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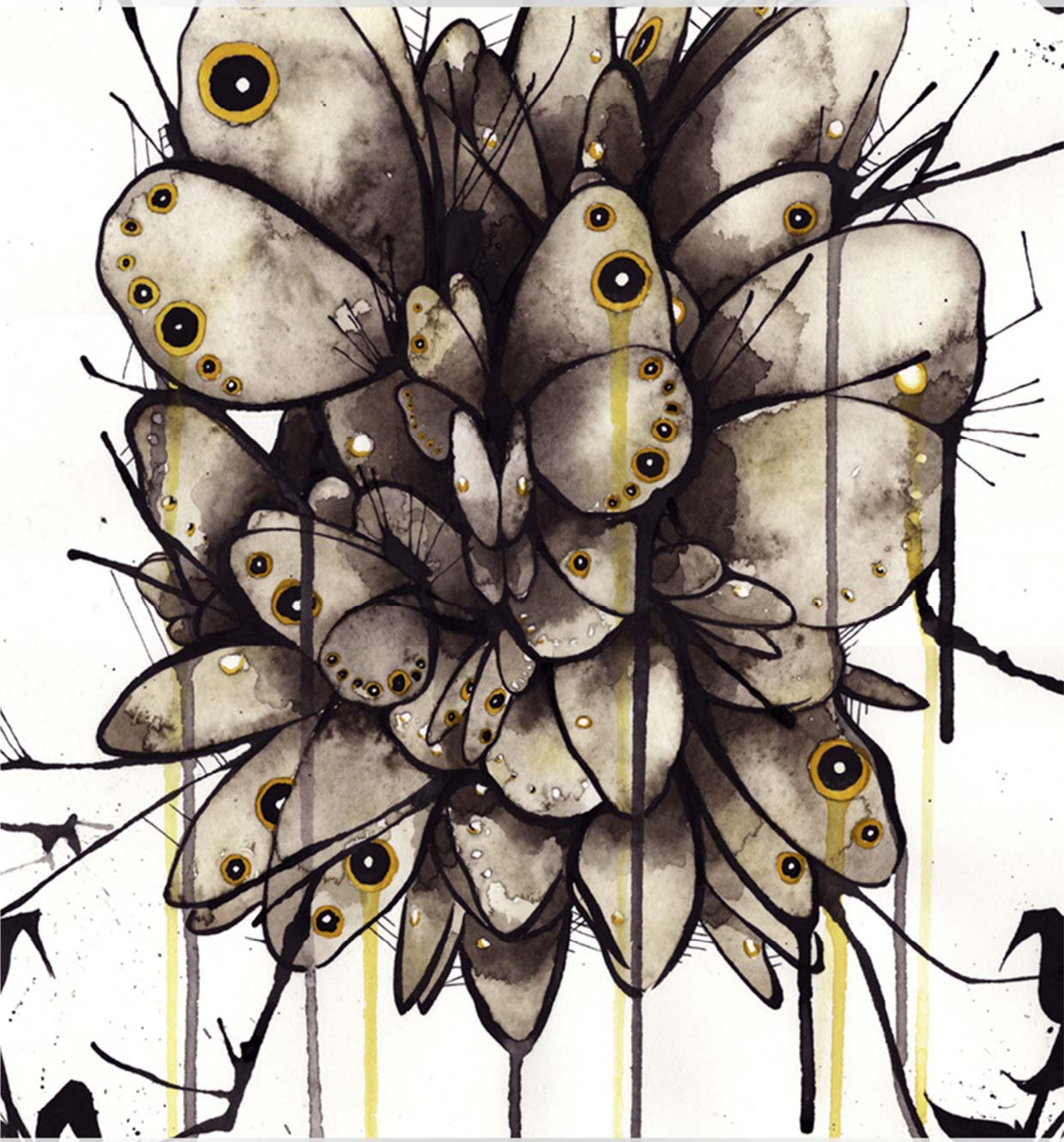

Ana Rita Amaro Mateus

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Temperature effects on genetic and physiological regulation of adaptive plasticity

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Aos meus pais, acima de tudo por acreditarem

"What you do makes a difference, and you have to decide what kind of difference you want to make."

Jane Goodall

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CHAPTER 1. EVOLUTION AND MOLECULAR MECHANISMS OF ADAPTIVE DEVELOPMENTAL PLASTICITY

ARA Mateus[1,2] & P Beldade[1,2]

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DEVELOPMENTAL PLASTICITY

It has become clear that more than a filter of phenotypic variation during the trans-generational process of natural selection, the environment also plays a key role in generating variation during organismal development. In fact, some degree of an effect of the external environment on phenotype seems pervasive in nature, and is accounted for in classical evolutionary genetics by the environment and the genetic-by-environment components of phenotypic variation. However, until recently environmentally-induced variation, or variation altogether, was seen more as a nuisance in developmental biology. Research in that field typically focused on single (often inbred) laboratory strains of one of a handful of model organisms kept in constant (often very unnatural) laboratory environments. This situation is rapidly changing as new disciplines are emerging and growing. Evolutionary developmental biology (evo-devo) brought the focus to intra- and inter-specific (morphological) variation and its genetic basis (see Stern 2000). More recently, ecological developmental biology (eco-devo, or eco-evo-devo) has started to bring the focus to how the external environment affects organismal development and how this impacts evolutionary change (see Gilbert & Epel 2009).

Phenotypic plasticity is the property whereby a single genotype produces distinct phenotypes in distinct environments. Organisms have different ways of adjusting to the environmental conditions they live in, including alterations in behavior and/or physiology and/or morphology leading to a better match between phenotype and selective environment (examples in Table 1.1). The term

developmental plasticity is used to refer to those cases where the environmentally-induced variation is the product of changes in pre-adult development (e.g. coat color variation in laboratory mice that depends on maternal diet, Waterland & Jirtle 2003). This thesis will focus on adaptive developmental plasticity linked to changes in development affecting morphological traits, with emphasis on the physiological and molecular mechanisms involved in the environmental-regulation of development and in the evolution of this phenomenon.

Traditionally, studies of developmental plasticity have focused on the phenotypic responses to environmental variation and on its ecological role and underlying physiological mechanisms. Researchers have also explicitly addressed the evolution of plasticity and its contribution to adaptive evolution. A detailed analysis of those topics has been covered in a number of insightful books and reviews (e.g. Callahan *et al.* 1997, Nijhout 2003, Pigliucci 2001, Schlichting & Pigliucci 1998, West-Eberhard 2003). New technological and conceptual advances are now being recruited to unravel the molecular mechanisms of developmental plasticity (e.g. Aubin-Horth & Renn 2009, Gilbert & Epel 2009, Minelli & Fusco 2010). This has precipitated a tremendous expansion of information on these mechanisms and their relationship to evolution justifying the pertinence of new synthetic efforts.

Some key concepts in developmental plasticity

Developmental plasticity refers to the property by which the same genotype can produce different phenotypes through environmental regulation of development (see main text). At the other end of the spectrum (Braendle & Felix 2009), *canalization* (or, *robustness*) is used to describe those situations where development produces the same phenotype despite environmental (and/or genetic) perturbation (e.g. blue solid line in Figure 1.1, Flatt 2005). Both plasticity and canalization are not absolute properties of a developmental program: the development of a particular trait might show environmental-sensitivity during a specific time window and be highly robust outside of that. Reversible changes in adult phenotypes, often in behavior or physiology, correspond to a form of phenotypic plasticity sometimes referred to as *acclimation* (e.g. Brakefield *et al.* 2007, Wilson & Franklin 2002) to distinguish from effects on development.

Table 1.1 - Examples of developmental plasticity for selected animal systems.

Biological system and plastic trait	Examples of inductive cues	Ecological relevance	References
Wings in female pea aphids	Crowding Nutrition Photoperiod Temperature	Dispersion	Braendle *et al.* 2006
Wing polyphenism in locusts	Crowding	Solitary versus gregarious and migratory morphs	Pener 1991, Simpson *et al.* 2001
Horns in dung beetles	Nutrition	Mating strategies	Moczek & Emlen 2000
Castes in social insects	Nutrition Pheromones	Division of labour	Korb & Hartfelder 2008
Teeth-like denticles in diplogastrid nematodes	Nutrition	Alternative diets	Bento *et al.* 2010
Seasonal polyphenism in butterflies	Temperature Photoperiod Nutrition	Anti-predator strategy Thermoregulation	Beldade & Brakefield 2002, Nijhout 1999
Gender determination in vertebrates (e.g. reptiles, fishes, amphibians)	Temperature	Optimal sex ratio	Janzen & Paukstis 1991, Ospina-Álvarez & Piferrer 2008, Nakamura 2008
Gender determination in invertebrates (e.g. Daphnia magna)	Photoperiod Crowding Temperature pH Nutrition Salinity	Optimal sex ratio	Hobaek & Larsson 1990; Cook 2002
Morphological defenses in planktonic crustaceans (Daphnia spp)	Density of predators (assessed via kairomones)	Defense	Dodson 1974, Stabell *et al.* 2003, Stibor & Lampert 2000
Head-size in spadefoot toad tadpoles	Density of conspecifics (assessed via food levels)	Food resources	Pfennig 1992, Pfennig *et al.* 2006

Reaction norms are graphical representations of the environmental dependence of the phenotype. Developmental plasticity can manifest itself in the form of graded variation in phenotype or in discrete switches between alternative developmental trajectories. A reaction norm displays phenotypic variation across an environmental gradient (see Schlichting & Pigliucci 1998). It is often used for situations where this environmental gradient corresponds to a more or less linear grading in phenotype (e.g. yellow line in Figure 1.1), but it can also describe situations of (nearly) discrete alternative phenotypes (e.g. non-linear relationship as in the orange line in Figure 1.1). Importantly, reaction norms can be obtained for different "end phenotypes" (morphology, life-history, behavior) but also for "intermediate phenotypes" such as hormone titers, methylation patterns and levels of gene expression during development (e.g. Aubin-Horth & Renn 2009). The reaction norms for such different phases do not necessarily need to have the same shape (dotted versus solid lines in Figure 1.1). In fact, even invariant phenotypes (i.e. flat reaction norm represented by the solid blue line in Figure 1.1) can result from cellular and molecular processes that are plastic (e.g. dotted blue line in Figure 1.1) (see Braendle & Felix 2008). Reaction norms drawn for different genetic backgrounds allow an assessment of genotype-by-environment interactions (e.g. Debat *et al.* 2009, Ostrowski *et al.* 2000, Sarkar & Fuller 2003). The genetic-by-environment component of phenotypic variation translates into reaction norms of different shapes for different genotypes, while the environment component corresponds to non-flat reaction norms.

Polyphenism describes a situation where inter-individual variation in phenotype does not result from differences in genotype, but rather from differences in the environment (e.g. wing development in pea aphid females influenced by different environmental cues, Braendle *et al.* 2006). The term polyphenism is used for situations where alternative phenotypes are discrete (e.g. orange line in Figure 1.1) – even if, in some cases, intermediate phenotypes can be produced (e.g. intercastes in ants). To contrast with polyphenism, the term *polymorphism* is used for those cases where inter-individual variation in phenotype is due to differences in genotype, often single or few alleles of large effect (e.g. wing development in pea aphid males influenced by allelic variation at the *aphicarus* locus, Braendle *et al.* 2006).

Genetic assimilation describes an evolutionary process by which an environmentally-induced phenotype becomes genetically fixed, so that the environmental cue is no longer necessary for the expression of that phenotype (see

Pigliucci *et al.* 2006). The term *genetic accommodation*, on the other hand, is a broad term referring to evolutionary mechanisms whereby selection acting on quantitative genetic variation moulds a novel phenotype, environmentally-induced (but also one arising by mutation), into an adaptive phenotype (e.g. Suzuki & Nijhout 2006). The concept of genetic accommodation describes trans-generational mechanisms of (quantitative) genetic change that can both fine tune developmental plasticity or canalize development. In contrast, the term *phenotypic accommodation* has been used to refer to intra-generational adjustment between developmental variables that does not depend on genetic change (see West-Eberhard 2003).

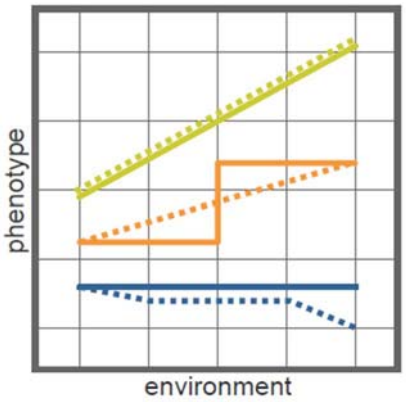

Figure 1.1 - Different shapes of reaction norms describing the environmental dependence of phenotypes produced from the same genotype. The lines can represent either end phenotype (solid) or some intermediate step such as gene expression (dotted), with different colors corresponding to different types of developmental-sensitivity to the environment. The blue example illustrates robust development, where even despite variation in underlying gene expression (non-flat dotted line), development always results in the same end phenotype across environments (flat solid line). Both the orange and yellow examples correspond to plastic development, where the same genotype will produce different phenotypes in different environments. The yellow is an example of a linear relationship between environmental and phenotypic gradient, and the orange to a non-linear relationship with discrete alternative phenotypes (i.e. polyphenism). Note that we intended to illustrate qualitatively different types of shapes of reaction norms; the heights and quantitative values being irrelevant here.

EVOLUTION OF AND VIA DEVELOPMENTAL PLASTICITY

Natural selection acting on genetic variation has led to differences between species (e.g. Scheiner 1993) and between populations of the same species (e.g. Crispo & Chapman 2010) in the degree and types of plastic responses. Analyses of those populations/species provide insights into the ecological conditions and biological properties that favor plastic versus non-plastic development, and into the mechanisms underlying evolutionary transitions between the two.

Evolutionary transitions to and from plastic development

Recent theoretical models have advanced our understanding of factors that favor the evolution of plasticity, including the predictability of environmental fluctuations (e.g. Leimar *et al.* 2006, Reed *et al.* 2010) and the costs of plasticity (see Snell-Rood *et al.* 2010). Transitions between plastic and robust development, as well as between environmentally and genetically determined alternative phenotypes (i.e. polyphenism and polymorphism, respectively) have been documented at different phylogenetic levels. For example, post-colonization erosion of plasticity of head-size was reported for snakes (Aubret & Shine 2009), the evolution of different degrees of genetic caste determination for ants (reviewed in Schwander *et al.* 2010), and back-and-forth transitions between genetic and environmental sex determination for vertebrates (see Stelkens & Wedekind 2010). Environmental sensitivity of developmental processes is probably the ancestral condition in most cases, with selection then working for the ability to buffer environmental effects (see Newman & Müller 2000, Nijhout 2003). This has been suggested, for example, for caste determination in ants (Anderson *et al.* 2006) and sex-determination in reptiles (Janzen & Paukstis 1991).

Beside studies of natural populations such as those mentioned above, there are also revealing studies where changes in plasticity resulted from artificial selection in laboratory populations. Temperature-dependent coloration in butterflies and moths offers some of the most compelling examples of these studies. Artificial selection on adult wing patterns in *Bicyclus anynana* butterflies and on larval coloration in *Manduca sexta* moths (Suzuki & Nijhout 2006) produced changes in the height and/or shape of the reaction norms that describe the relationship between environmental and phenotypic change. In both cases, these changes were associated with changes in hormone titer dynamics and were of polygenic nature. In contrast, the importance of single genes has also been documented; for instance, by analyses of mutants which loose or gain environmental sensitivity. Examples include loss of sensitivity to the hormone that mediates diet-associated mouth morphology in *daf-12* mutants of *Pristionchus pacificus* nematodes (Bento *et al.* 2010), and exposure of hidden temperature-sensitivity for larval coloration in *black* mutants of *Manduca sexta* (Suzuki & Nijhout 2006).

In recent years, sophisticated analyses have started to highlight specific developmental and genetic mechanisms that presumably confer robustness or

plasticity to development. Robustness may be enhanced by redundancy in cell precursors (e.g. Braendle & Felix 2008), in gene enhancers (e.g. Frankel *et al.* 2010), and in regulatory microRNAs (e.g. Brenner *et al.* 2010), as well as the action of particular gene families such as heat-shock proteins (e.g. Takahashi *et al.* 2010). Modularity in developmental genetic networks, in turn, has been proposed to have an important role in enabling phenotypic plasticity; decreased pleiotropy between networks may facilitate the induction of different modules under different environmental conditions (Snell-Rood *et al.* 2010). By acting on all those types of mechanisms, natural selection can presumably adaptively adjust the likelihood and/or the extent of plasticity in trait development. Through a process that has been referred to as genetic accommodation, natural selection can also fine-tune this plasticity, including its degree (e.g. Lind & Johansson 2007), which environmental cue triggers it (e.g. Edgell & Neufeld 2008), and the sensitivity thresholds for that cue (e.g. Moczek & Nijhout 2003).

Impact of developmental plasticity on adaptive evolution

The relevance of developmental plasticity to adaptive evolution is receiving increasing attention, despite the fact that developmental plasticity is characterized by phenotypic changes without changes in gene sequence, while adaptive evolution is specifically characterized by changes in allele frequencies. Phenotypic plasticity was often seen as being irrelevant or even a deterrent for adaptive evolution (see discussion in Pfenning *et al.* 2010): 1) irrelevant because the raw material for evolution by natural selection is heritable phenotypic variation, and not environmentally-induced phenotypes not transmitted from parents to progeny; and 2) deterrent because plasticity can shield genetic variation from natural selection, either because alternative genotypes can end up producing the same phenotype or because environment-specific genes (i.e. those expressed only in one environment) will be under relaxed selection in the non-inducing environment. However, this view has changed and increasing attention is now being given to the contribution of developmental plasticity to adaptive evolution and the mechanisms whereby this contribution can occur. Studies on different systems illustrate the impact of plasticity on phenotypic diversification (e.g. West-Eberhard 2003), including the origin of novel traits (e.g. Moczek 2010), and on speciation, including adaptive radiations (e.g. Wund

et al. 2008). The arguments and empirical evidence for these effects were reviewed recently by Pfennig *et al.* 2010.

Different types of non-mutually-exclusive mechanisms account for the potential positive impact of plasticity on adaptive evolution. Clearly, by providing the means by which organisms can cope with new environmental challenges (Yeh & Price 2004), plasticity can play an important role for the immediate survival of populations exposed to change in external environment. Then, exactly because phenotypic plasticity can shield genetic variation from natural selection, it can presumably promote the accumulation of cryptic variation (i.e. genetic variation which does not result in phenotypic variation). When released, this heritable variation can provide raw material for adaptive evolution and be important for phenotypic diversification (reviewed in Schlichting 2008). Under some circumstances, environmentally-induced phenotypes can become fixed through a process called genetic assimilation. It has been argued that plasticity can, in fact, accelerate adaptive evolution. For example, studies of melanogenesis in *Daphnia* have suggested that the developmental mechanism underlying ancestral plasticity was repeatedly co-opted to facilitate rapid adaptation (Scoville & Pfrender 2010).

Insights into the evolutionary transitions between environmentally-sensitive and environmentally-insensitive development, and into the contribution of plasticity to evolutionary diversification, require an understanding of both the ecological relevance of plasticity and the mechanisms by which the environment regulates development.

ECOLOGY AND DEVELOPMENT IN PHENOTYPIC PLASTICITY

Development translates genotypes into phenotypes in a process that is influenced by the external environment. Aside providing some basic building blocks, particular variables of the external environment, in some cases, function as cues that trigger switches in development and lead to the production of alternative phenotypes to face different types of ecological challenges (examples in Table 1.1). This section focuses on the ecological significance of developmental plasticity, and on the types of effects that external environmental cues can have on organismal development.

Ecological significance of environmentally-induced phenotypic variation

Developmental plasticity is adaptive when the environmentally-induced changes result in a better match between the adult phenotype and its selective environment. The induced alternative phenotypes typically correspond to different ecological tactics, such as alternative tactics to achieve copulation in horned (guarding of nest) versus hornless (sneaky copulations) males of *Onthophagus taurus* dung beetles (Moczek & Emlen 2000); alternative tactics to escape predation in cryptic versus conspicuous *Bicyclus anynana* butterflies; and presumably alternative foraging tactics in "toothless" (bacteriovorous) versus "toothed" (predatory) *Pristionchus pacificus* nematodes (Bento *et al.* 2010).

A good match between phenotype and ecological conditions is achieved when the environmental cue that triggers changes in development is a reliable predictor of the future selective environment (but not necessarily the same). Such external cues can be of different types, both abiotic (e.g. temperature and photoperiod) or biotic (e.g. presence of other species and density of conspecifics), and they typically reflect environmental heterogeneity in time and/or in space. For example, temperature fluctuations predict alternating seasons relating to many cases of seasonal polyphenims including coloration in butterflies; fish kairomone concentration reflects high predation environments that leads *Daphnia* crustaceans to develop morphological defenses; and leg rubbing in locusts reflects high population densities that result in the production of the winged migratory morph (see Table 1.1, also for references). The environment can also be manipulated by conspecific individuals. In most ants, for example, the high-nutrition diet that determines that a juvenile will develop into a queen is the result of feeding by adult workers. In this case, there is micro-environmental heterogeneity within which the different morphs co-occur and can carry out the division of labor within the colony.

Environmental cues and developmental sensitivity

The environmentally-induced phenotypic variation can be more or less continuous (e.g. larger or smaller wings in *Drosophila*, Powell *et al.* 2010) or discrete (e.g. presence or absence of wings in queens versus workers in some social insects). Both gradual or "switch-like" changes in development can be triggered by different types

of environmental cues, often in combination (e.g. Braendle *et al.* 2006), and result in simultaneous changes in different traits.

There is rarely, if ever, a "one cue to one trait" relationship. Plasticity often involves changes in multiple traits in the same organism. For example, environmentally-induced wing development in ants, locusts and pea aphids (references in Table 1.1) is associated with changes in other morphological traits (e.g. body mass and ovary development in ants, body pigmentation in locusts, antennae and eye development in aphids) and with changes in life-history traits (e.g. longevity and fertility in ants, gregarious versus solitary life-styles in locusts, mode of reproduction in aphids). On the other hand, there is also a substantial degree of cue specificity in determining how the development of particular traits is altered. For example, different species of predators induce different types of anti-predator morphologies in *Daphnia* (e.g. Beckerman *et al.* 2010, Laforsch & Tollrian 2004) as well as in frogs (Vonesh & Warkentin 2006). The same cue can affect different developmental switches at different developmental stages (e.g. low food availability determines formation of teeth and production of dauer larvae in some nematodes, Bento *et al.* 2010). Also, different cues can induce developmental switches at multiple stages. In ants with strong caste dimorphism, for example, queen-worker determination depends on hormones deposited by the queen during oogenesis (Passera & Suzzoni 1979), and the differentiation of subcastes (such as minor and major workers or soldiers) depends on nutrition during larval development (Wheeler & Nijhout 1983). These multiple environmentally-sensitive switch points along the developmental trajectory allow diversification of adult morphs specialized for different roles.

The effect of change in a particular environmental cue on phenotype, characteristically represented as a reaction norm, is highly dependent on developmental sensitivities. These sensitivities exist in relation to thresholds of the values of the inductive environmental cue beyond which there is change in development and in phenotype (Ostrowski *et al.* 2000). They also exist in relation to restricted time-windows of the development during which the external environment can influence the outcome (Ostrowski *et al.* 2002); development being quite robust outside these sensitive periods (Braendle & Felix 2008). Both sensitivity thresholds and sensitivity periods can evolve and might differ between populations.

Effects of the external environment on developmental timing and trajectories

The effects of the environment on developmental timing can be of different types; with the environmental cue more or less uniformly extending or reducing the total duration of development, affecting specifically particular developmental stages, or leading to arrested development altogether. For example, temperature (e.g. Bochdanovits *et al.* 2003), nutrition (e.g. Brian 1975), and presence of predators (e.g. Beckerman *et al.* 2010) often affect development time and lead to differences in body size and correlated life-history traits. In some arthropods, the duration but also the actual number of instars can vary across environments (e.g. Beckerman *et al.* 2010, Esperk *et al.* 2007). Furthermore, some organisms, typically in unfavorable environments, have environmentally-induced arrested development at different stages: embryonic diapause (Moriyama & Numata 2008), larval diapause (Golden & Riddle 1984), and pupal diapause (Belozerov *et al.* 2002). While it is clear that diapause represents an adaptive plastic response, the same is probably not true for many cases where developmental rates (and correlated body size) are affected by availability of energy resources (such as temperature or food) (see examples in Gotthard & Nylin 1995).

The environmental control of developmental rates can also affect body structure and result in the production of not just larger or smaller, but distinct adult morphologies. For example, if the rates of development of different traits are not affected in the same manner, environmental-sensitivity can modify the correlation between traits and generate novel trait combinations. A role for this type of heterochrony has been proposed in relation to differences between castes and body parts in ants (Miyazaki *et al.* 2010). Differential rates in association to different body structures have also been suggested to explain changes in allometry (i.e. characteristic patterns of relative organ size; see Stern & Emlen 1999) in environmentally-dependent omnivore versus carnivore morphs of spadefoot toad tadpoles (Storz & Travis 2007).

Aside from the global or local effects on developmental timing, the environmental cue can also trigger a switch between alternative developmental trajectories that result in drastically different morphologies. Studies of the actual process of development of different organisms are adding to a detailed characterization of the formation of alternative environmentally-induced

morphologies. These include some classic examples of adaptive developmental plasticity such as *Daphnia* anti-predator morphologies (Laforsch & Tollrian 2004, Miyakawa *et al.* 2010), beetle horns (Moczek 2007, Moczek & Nijhout 2002, Tomkins & Moczek 2009), pea aphid wings (Braendle *et al.* 2006, Brisson 2010, Legeai *et al.* 2010), and social insect castes (Abouheif & Wray 2002, Miura 2005). The way by which external environmental cues control patterns of gene expression that result in alternative phenotypes is now being elucidated for these and other examples of plastic development and is discussed in more detail below.

MOLECULAR MECHANISMS OF DEVELOPMENTAL PLASTICITY

Current research in adaptive developmental plasticity is characterizing the molecular mechanisms that link variation in external environmental cues to the changes in organismal development that result in the production of different phenotypes. For a long time, the external environment and plasticity were disregarded in studies of developmental biology. This is despite the fact that organismal development itself, with its characteristic tissue-by-stage specific gene expression, is perhaps the most compelling example of cellular plasticity. During organismal development, cell differentiation and pattern formation is the result of intrinsic signals that provide cells of developing organisms with information about their position. In developmental plasticity, the choice of alternative developmental trajectories is also fixed genetically, while the decision between those paths depends on different mechanisms that control gene expression.

Gene content and gene expression

Despite the fact that phenotypic plasticity is defined as environmentally-induced phenotypic variation produced from one single genotype (thus leaving out consideration of genetic variation), there are many revealing examples of a clear correlation between genetic composition and plasticity. This can be seen both in terms of allelic variation at specific loci and the extent of plasticity in different populations, as well as in the gene content on the genomes of species characterized by very plastic development.

Whatever the allelic or gene composition of an organism is, it is clear that environmentally-induced changes in development ultimately result from environmentally-induced changes in gene expression. The latter can have an effect on

which and to what level particular genes are expressed, and probably also particular alternative transcripts or alleles. An emblematic example of genes whose expression, and thus effect, depends on the environment is that of heat shock protein (Hsp) encoding genes. Their expression is characteristically influenced by temperature or other types of environmental stress to buffer perturbations to development and ensure the production of predictable phenotypes (e.g. Takahashi *et al.* 2010).

Analysis of plasticity in gene expression has also been carried out for groups of candidate genes or pathways involved in particular environmentally-sensitive developmental switches. Examples include analysis of wing development genes in queen versus worker ants (Abouheif & Wray 2002), of key body-plan and hormone-related genes in *Daphnia*'s induced defenses (Miyakawa *et al.* 2010), and of sex determining genes in species with environmental sex determination (Shoemaker *et al.* 2007). New analytical tools such as microarrays and RNA-Seq now make it possible to move from (necessarily biased) candidate gene approaches, to less biased (but of more challenging interpretation) whole transcriptome scans.

Environmental regulation of gene expression

Different mechanisms are known that act interactively to regulate gene expression, keeping it in tune with physiological adjustments to the environment. Among these, the role of endocrine hormones has received, and is receiving, special attention in the context of developmental plasticity (see Gilbert & Epel 2009).

The sensitivity of hormones to the environment, together with their widespread role as regulators of post-embryonic development, underscores their role as intermediaries in linking external environmental information with developmental switches (Nijhout 1998). In fact, a hormonal regulation has been characterized for most, if not all, well described examples of developmental plasticity (see Gilbert & Epel 2009, Nijhout 2003). Insect juvenile hormone and ecdysteroids, in particular, have been implicated in many cases of plastic development, including that of seasonal polyphenism in butterfly wing patterns and of castes in social hymenoptera. In many cases, the same hormone influences multiple developmental decisions and different traits during the development of one same organism; often associated to different sensitivity thresholds (Bento *et al.* 2010) and/or different sensitivity periods (Moczek & Nijhout 2002, Oostra *et al.* 2011). The environmental cues can induce changes in titers and/or dynamics of hormone production, and the hormones can then affect gene

expression. This can happen, for example, via their nuclear receptor proteins which, when activated by the hormone signal, have transcription regulator activity (Baniahmad & Tsai 1993) or possibly also via hormone-related changes in chromatin (Lu *et al.* 1998).

CHALLENGES AND TRENDS

In the section above we provided a broad overview of some of the best studied molecular mechanisms underlying developmental plasticity: changes in gene expression and its regulation by hormones. These mechanisms interact in complex ways whereby they regulate and are regulated reciprocally. For example, steroid hormones can influence gene expression by affecting chromatin states (Lu *et al.* 1998), and, conversely, their biosynthesis and action can itself be under epigenetic regulation (e.g. Martinez-Arguelles & Papadopoulos 2010).

A complete understanding of adaptive developmental plasticity will require knowing the different sensory and regulatory mechanisms, but also how these, in turn, affect development to produce changes in phenotype that result in differences in individual fitness in natural populations. In nature, the integration of all levels of information is complicated by the fact that the developmental environment is more complex than one single changing cue, the phenotype is more than one particular trait, and the selective environment presents more than one ecological challenge. Also, typically, there is extensive genetic variation in natural populations and different genotypes do not necessarily respond to environmental variation in the same manner. Current studies are starting to specifically address variation in nature also at the molecular level, including for gene expression (e.g. Scott *et al.* 2009), hormone dynamics (e.g. Zera 2007), and epigenetics (see Bossdorf *et al.* 2008 and Richards 2008). The integration of these different studies of the proximal mechanisms of the environmental-sensitivity of development will need to be done within an evolutionary framework, including the evolutionary history of the regulating mechanisms and their interactions (Johnson & Tricker 2010), as well as the origin and diversification of (plastic) developmental networks (Minelli & Fusco 2010). It is clear that environmentally-induced variation will need to continue to be studied in multiple systems (representing different types of cues, developmental and phenotypic changes, and ecological situations), at different levels of biological organization (changes in molecular processes, organismal development, and impact in natural populations) and

bringing together different disciplines (genetics, developmental biology, ecology and evolutionary biology).

Environmentally-induced variation is at the heart of new trends in biological and biomedical research. The new discipline of eco-(evo-)devo is perhaps the most emblematic example of this. It unites fields such as epigenetics and evo-devo (see Gilbert & Epel 2009) around the study of developmental plasticity. It takes explicit account of the environment in generating inter-individual variation in phenotype through changes in development, and in contributing to evolutionary diversification (see also West-Eberhard 2003). In fact, plasticity has been highlighted as one of the major themes for an extended evolutionary synthesis (Müller 2007, Pigliucci 2007). Aside its obvious place at the center of an effort to unite ecology and developmental biology and its contribution to evolutionary biology, the influence of the developmental environment on phenotype can also have important implications for biomedicine and biodiversity. First, both the *in utero* environment (including maternal stress and nutrition, e.g. Burdge & Lillycrop 2010), and trans-generational environmental effects carried in parental gamete epigenomes (including in the sperm; Puri *et al.* 2010) have been implicated in the developmental origin of adult disease (examples in Gilbert & Epel 2009, Gluckman *et al.* 2009). Second, the study of developmental plasticity can also be of relevance for appropriately assessing the biodiversity consequences of anthropogenic environmental change. Natural populations have different mechanisms for dealing with environmental change, including global change in climate (see Figure 1.2). While demographic and genetic mechanisms have received considerable attention in this context, the role of developmental mechanisms (Chevin *et al.* 2010, Reed *et al.* 2010) is lagging behind. Clearly, plasticity can help organisms exploit novel environments (e.g. Ghalambor *et al.* 2007, Yeh & Price 2004) and provides a means of rapidly adjusting to external change, but it might also pose problems. For example, in organisms with temperature-dependent sex determination, dramatic climate change can potentially lead to extremely biased sex ratios with serious demographic consequences (Janzen 1994, Miller *et al.* 2004).

It is clear that developmental plasticity will continue to be an active area of research, and will greatly profit from the availability of sophisticated methods of molecular analysis (which traditionally were a privilege of only a handful of classical laboratory models) for multiple systems with interesting ecology and/or unique

biological properties (see Abzhanov *et al.* 2008, Aubin-Horth & Renn 2009, Milinkovitch & Tzika 2007). It is also clear that a complete understanding of natural variation will gain from including the study of development, and it will continue to bring genetic models out of the laboratory, and ecological systems into the laboratory. These are certainly exciting times when different disciplines are joining efforts to understand what is arguably one of the most fascinating, and until recently largely ignored, properties of biological systems; that of variation.

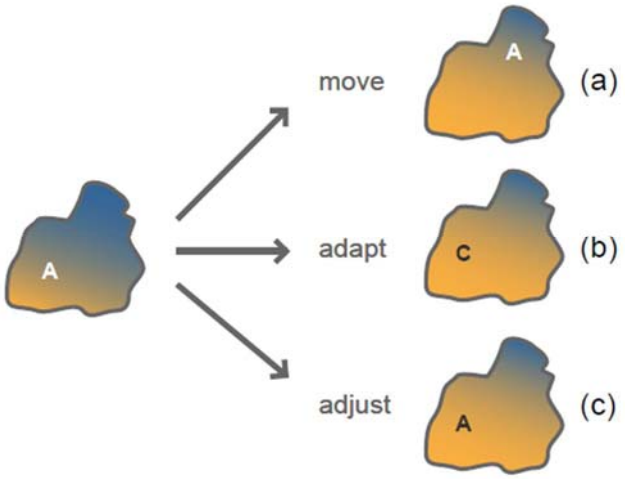

Figure 1.2 - Coping with changing environments. In nature, populations can deal with climate change in different ways: (a) through habitat tracking, individuals move to different places and this can result in changes in species distributions, (b) through natural selection on segregating genetic variation, allele frequencies change across generations as populations adapt to novel environmental situations, and (c) through phenotypic plasticity, individuals can adjust without changes in genetic composition. Background color shading represents an environmental gradient (e.g. temperature), and characters represent populations with letters (A or C) corresponding to different genotypes and colors (white or grey) to different phenotypes. Figure adapted from Beldade *et al.* 2011.

Here we will use an emerging model in evolutionary and ecological genomics, the tropical Nymphalid *Bicyclus anynana* butterflies, for which existing knowledge of the adaptive value of plasticity in natural populations (Brakefield *et al.* 2009) can be complemented with an understanding of its underlying mechanisms. *B. anynana* has been established as a laboratory model for research on the evolution and development of adaptive traits and it is an exceptional modem to address some of the current trends (e.g. Beldade & Brakefield 2002, Brakefield *et al.* 2009). It is small enough that large

laboratory populations can be maintained (essential for population-level analysis), but large enough that individuals can be easily manipulated (necessary for organismal-level analysis). More recently, genomic tools (Beldade *et al.* 2008) have been developed for this species allowing modern molecular-level approaches.

SEASONAL POLYPHENISM IN BICYCLUS ANYNANA BUTTERFLIES

Ecological and evolutionary context of B. anynana developmental plasticity

Like many butterflies from seasonal environments (examples in Beldade & Brakefield 2002), *B. anynana,* exhibits clear seasonal polyphenism in wing pattern and various life-history traits (Brakefield *et al.* 2007, Brakefield & Frankino 2009). In sub-Saharan Africa, where they occur naturally, larvae that develop during the wet season produce adults with conspicuous wing patterns that include large marginal eyespots, while those that develop during the dry season produce adults with dull brown colors and very small eyespots (Figure 1.3a). These alternative wing patterns correspond to alternative strategies to avoid predation. While the marginal large eyespots of the wet-season butterflies are thought to attract the predator's attention to the wing margin and away from the vulnerable body, the all-brown dry-season butterflies are thought to be cryptic against a background of dry leaves in the florest floor (Brakefield & Frankino 2009, Olofsson *et al.* 2010). Laboratory studies showed that the temperature during development, which predicts the natural seasonal fluctuations in precipitation, determines the production of the alternative wing pattern phenotypes (Brakefield & Frankino 2009). Curiously, only the pattern on the ventral side of the wings (the surface exposed at rest) shows plasticity in relation to developmental temperature (Brakefield *et al.* 1998) and has been associated to predator avoidance. Despite correlations between wing surfaces (e.g. Beldade & Brakefield 2003), the patterns on the dorsal side (exposed only during flight or courtship) are largely not plastic and have been implicated in mate choice (Robertson & Monteiro 2005). Examination of this contrast in a phylogenetic context suggested that ventral patterns, shaped by natural selection, evolved at a lower rate than dorsal patterns, shaped by sexual selection, during *Bicyclus* diversification (Oliver *et al.* 2009).

Phisiologycal underpininings

Like many polyphenisms, in *B. anynana* ecdysteroids are involved in the regulation of the differences in the wing pattern and life-history traits between the wet and the dry seasonal forms (Koch *et al.* 1996, Brakefield *et al.* 1998, Zijlstra *et al.* 2004, Oostra *et al.* 2011). Titers of ecdysone and 20-hydroxyecdysone peak relatively earlier at the higher temperature that typically leads to the production of large eyespots (Figure 1.3b). Furthermore, artificial manipulation of hormone titers can affect ventral eyespot size. Microinjections or infusions of 20-hydroxyecdysone into pupae resulted in the development of individuals reared at low temperatures into adults with wing patterns characteristic of the wet season form (Koch *et al.* 1996, Brakefield *et al.* 1998, Zijlstra *et al.* 2004). It is not yet known how precisely ecdysteroid dynamics regulates eyespot development, but, the ecdysone receptor, which has transcription factor function, possibly directly or indirectly regulates eyespot genes (Koch *et al.* 2003).

Correlated responses are regularly observed in artificial experiments which can be explained in part by the fact that ecdysone is involved in the regulation of multiple traits (Oostra *et al.* 2011). Lines that have been selected for short or long pupal development time show larger or small ventral eyespots, respectively (e.g. Zijlstra *et al.* 2004). Fast-developing butterflies have higher levels of ecdysone shortly after pupation in comparison with slow-developing individuals (Zijlstra *et al.* 2004). In addition, the slow-selected butterflies show a decreased response to ecdysone injections in the pupal stage relative to fast-selected butterflies.

The genetics of developmental plasticity in B. anynana

Previous studies of the genetic basis of developmental plasticity in *B. anynana* have used artificial selection to derive butterflies expressing wet or dry-like phenotypes across temperatures, changing the height of reaction norms but failing to significantly change their shape (Brakefield *et al.* 1996, Wijngaarden & Brakefield 2001, Wijngaarden *et al.* 2002). Butterflies from these lines, as well as from unselected laboratory populations reared at different temperatures, characterized the physiological and gene expression changes associated with the development of alternative wing patterns The eyespot gene *Distal-less*, proposed to contribute to variation in dorsal eyespot size (Beldade *et al.* 2002), has a larger area of expression in larval wings of individuals that develop into the wet-season-like phenotype with

larger eyespots (Brakefield *et al.* 1996). Further studies will be necessary to link hormone dynamics to the regulation of genes and processes involved in eyespot formation (Beldade & Brakefield 2002), as well as to investigate the involvement of other regulatory and sensory mechanisms in environmentally-sensitive wing pattern development. Genomic tools available today including e. g. expressed sequence tag (EST) data bases, microsatellite, linkage map and a custom designed microarray will be of extreme relevance in these studies (Beldade *et al.* 2006, Beldade *et al.* 2009a, Beldade *et al.* 2009b, Conceição *et al.* 2011).

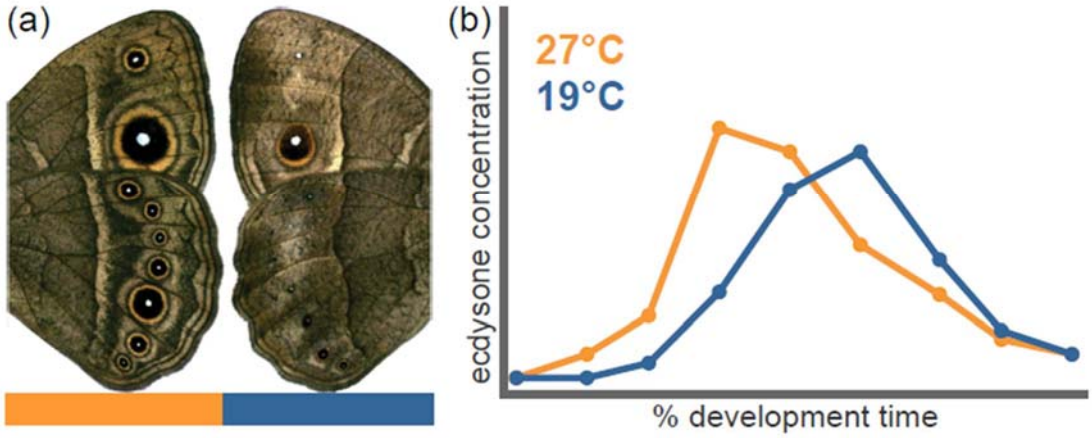

Figure 1.3 - Seasonal phenotypic plasticity in *B. anynana*. (a) B. anynana wet- (left) and dry-season-like (right) phenotypes obtained by rearing larvae at different temperatures. Note that the larger eyespot on the forewing is typically hidden behind the hindwing in resting butterflies (the posture relevant for the anti-predatory strategies described). Also, note that wing size (typically larger in dry-season phenotypes) was adjusted to emphasize comparison of color patterns. (b) Differences in hormone titer dynamics (adapted from Brakefield *et al.* 1998, Oostra *et al.* 2011) during pupal development, when patterning and pigment biosynthesis (cf. Wittkopp & Beldade 2009) genes are expressed. Figure adapted from Beldade *et al.* 2011.

OUTLINE OF THIS THESIS

Developmental plasticity is an important strategy for adaptation to fluctuating environments (reviewed in this CHAPTER 1). Such plasticity has one of its most compelling examples in seasonal polyphenism in butterflies; individuals can have different wing patterns and life-histories in alternating seasons. Previous studies have shown that the mechanism that mediates seasonal polyphenism involve ecdysteroids

hormones; with alternative seasonal forms being characterized by differences in the timing of hormone increase after pupation. This thesis will contribute to a broader understanding of the genetic, developmental and physiological mechanisms that regulate developmental plasticity represented by temperature-regulated variation in butterfly wing color patterns. We will focus on a lab model for the study of adaptive phenotypic plasticity: color patterns on the wings of *Bicyclus anynana* butterflies. The adaptive value of the alternative seasonal phenotypes in this species is well documented, and their underlying physiological underpinnings have started to be explored, however how animals perceive and assess temperature and how that influences development is still a black box.

In CHAPTERS 2 AND 3 we explored the coordination of responses of different plastic traits to temperature and hormone manipulations. Both in CHAPTER 2 AND 3 we studied the integration of response of different traits by combining the analysis of changes induced by temperature in hormone physiology and traits development that lead to changes in phenotype. For that purpose, we explored the effects of manipulating external temperature, and internal levels of the active form of ecdysone and analyze phenotypic effects on different wing pattern (CHAPTER 2) and life-history traits (CHAPTER 3). In CHAPTER 2 we also explored the mechanism for local sensitivities to systemic levels of ecdysone by testing the hypothesis that groups of cells that responded differently to ecdysone manipulations would differ in expression of ecdysone receptor. In CHAPTER 3 we additionally tested the ecological consequences of any hormone-induced changes in morphology and physiology observed by manipulating ecdysteroid at a single temperature and injection time point, and monitoring the effects on multiple aspects of adult fitness.

Genotypes can differ in many properties of reaction norms such as height, slope, or shape. In CHAPTERS 4 AND 5 we explored the genetic basis of variation in developmental plasticity. In CHAPTER 4, we explored the effect of alleles of large effect on wing pattern on plasticity therein. To achieve this goal, we characterized thermal reaction norms for the size of eyespot color rings for *B. anynana* mutants with altered eyespot size and/or color composition. In CHAPTER 5 we explored standing genetic variation for alternative plastic phenotypes. To explore genotype (G), temperature (T), and GxT effects on *B. anynana* development, we derived artificial selection lines expressing extreme wet season-like or dry-season-like phenotypes at intermediary temperatures and, we characterized thermal reaction norms for several

traits for a wider range of temperatures than is usually explored in this species to characterize the shape of reaction norms.

In CHAPTER 6, I summarized the conclusions from the previous chapters and provide ideas for future research to deeper our understanding of developmental plasticity.

REFERENCES

Abouheif E, Wray GA (2002) Evolution of the gene network underlying wing polyphenism in ants. *Science* **297**, 249–252.

Abzhanov A, Extavour CG, Groover A, Scott HA, Hopi HE, Elena KM, Monteiro A (2008) Are we there yet? Tracking the development of new model systems. *Trends in Genetics* **24**, 353–360.

Anderson KE, Gadau J, Mott BM, Brendon MM, Robert JA, Annette A, Christoph S, Jennifer FH (2006) Distribution and evolution of genetic caste determination in Pogonomyrmex seed-harvester ants. *Ecology* **87**, 2171–2184.

Aubin-Horth N, Renn SCP (2009) Genomic reaction norms: using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Molecular Ecology* **18**, 3763–3780.

Aubret F, Shine R (2009) Genetic assimilation and the postcolonization erosion of phenotypic plasticity in island tiger snakes. *Current Biology* **19**, 1932–1936.

Baniahmad A, Tsai MJ (1993) Mechanisms of transcriptional activation by steroid hormone receptors. *Journal of Cellular Biochemistry* **51**, 151–156.

Beckerman AP, Rodgers GM, Dennis SR (2010) The reaction norm of size and age at maturity under multiple predator risk. *Journal of Animal Ecology* **79**, 1069–1076.

Beldade P, Brakefield PM (2002) The genetics and evo-devo of butterfly wing patterns. *Nature Reviews Genetics* **3**, 442–452.

Beldade P, Brakefield PM (2003) Concerted evolution and developmental integration in modular butterfly wing patterns. *Evolution & Development* **5**, 169–179.

Beldade P, Brakefield PM, Long AD (2002) Contribution of Distal-less to quantitative variation in butterfly eyespots. *Nature* **415**, 315–318.

Beldade P, Rudd S, Gruber JD, Long AD (2006) An Expression Sequence Tag resource for Bicyclus anynana butterflies, an evo-devo model. *BMC Genomics* **7**, 130.

Beldade P, Saenko SV, Pul N, Long AD (2009a) A gene-based linkage map for Bicyclus anynana butterflies allows for a comprehensive analysis of synteny with the lepidopteran reference genome. *PLoS Genetics* **5**, e1000366.

Beldade P, van't Hof A, Jerónimo MA, Long AD (2009b) Microsatellite markers associated with genes expressed in developing wings of Bicyclus anynana butterflies. *Molecular Ecology Resources* **9**, 1487-1492.

Beldade P, McMillan WO, Papanicolaou A (2008) Butterfly genomics eclosing. *Heredity* **100**, 150-157.

Belozerov VN, Fourie LJ, Kok DJ (2002) Photoperiodic control of developmental diapause in nymphs of prostriate ixodid ticks (Acari: Ixodidae). *Experimental & Applied Acarology* **28**, 163–168.

Bento G, Ogawa A, Sommer RJ (2010) Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. *Nature* **466**, 494–497.

Bochdanovits Z, van der Klis H, de Jong G (2003) Covariation of larval gene expression and adult body size in natural populations of Drosophila melanogaster. *Molecular Biology & Evolution* **20**, 1760–1766.

Bossdorf O, Richards CL, Pigliucci M (2008) Epigenetics for ecologists. *Ecology Letters* **11**, 106–115.

Braendle C, Davis GK, Brisson JA, Stern DL (2006) Wing dimorphism in aphids. *Heredity* **97**, 192–199.

Braendle C, Felix MA (2008) Plasticity and errors of a robust developmental system in different environments. *Developmental Cell* **15**, 714–724.

Braendle C, Felix MA (2009) The other side of phenotypic plasticity: a developmental system that generates an invariant phenotype despite environmental variation. *Journal of Biosciences* **34**, 543–551.

Brakefield PM, Beldade P, Zwaan BJ (2009) The African butterfly Bicyclus anynana: a model for evolutionary genetics and evolutionary developmental biology. *in* Emerging Model Organisms: A Laboratory Manual, pp 291-329. Edited by Behringer RR, Johnson AD, Krumlauf RE. New York: Cold Spring Harbor Laboratory Press.

Brakefield PM, Frankino WA (2009) Polyphenisms in Lepidoptera: multidisciplinary approaches to studies of evolution. In: Phenotypic Plasticity in

Insects. Mechanisms and Consequences, pp. 281–312. Science Publishers, Inc., Plymouth.

Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden PJ, Monteiro A, French V, Carroll SB (1996) Development, plasticity and evolution of butterfly eyespot patterns. *Nature* **384**, 236–242.

Brakefield PM, Kesbeke F, Koch PB (1998) The regulation of phenotypic plasticity of eyespots in the butterfly Bicyclus anynana. *American Naturalist* **152**, 853–860.

Brakefield PM, Pijpe J, Zwaan BJ (2007) Developmental plasticity and acclimation both contribute to adaptive responses to alternating seasons of plenty and of stress in Bicyclus butterflies. *Journal of Biosciences* **32**, 465–475.

Brenner JL, Jasiewicz KL, Fahley AF, Kemp BJ, Abbott AL (2010) Loss of individual microRNAs causes mutant phenotypes in sensitized genetic backgrounds in C. elegans. *Current Biology* **20**, 1321–1325.

Brian MV (1975) Caste determination through a queen influence on diapause in larvae of ant Myrmica rubra. *Entomologia Experimentalis et Applicata* **18**, 429–442.

Brisson JA (2010) Aphid wing dimorphisms: linking environmental and genetic control of trait variation. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**, 605–616.

Burdge GC, Lillycrop KA (2010) Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. *Annual Review of Nutrition* **30**, 315-339.

Callahan HS, Pigliucci M, Schlichting CD (1997) Developmental phenotypic plasticity: where ecology and evolution meet molecular biology. *Bioessays* **19**, 519–525.

Chevin LM, Lande R, Mace GM (2010) Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLoS Biology* **8**, e1000357.

Conceição IC, Long AD, Gruber JD, Beldade P (2011) Genomic sequence around butterfly wing development genes: annotation and comparative analysis. *PLoS ONE* **6**, e23778.

Crispo E, Chapman LJ (2010) Geographic variation in phenotypic plasticity in response to dissolved oxygen in an African cichlid fish. *Journal of Evolutionary Biology* **23**, 2091–2103.

Debat V, Debelle A, Dworkin I (2009) Plasticity, canalization, and developmental stability of the Drosophila wing: joint effects of mutations and developmental temperature. *Evolution* **63**, 2864–2876.

Edgell TC, Neufeld CJ (2008) Experimental evidence for latent developmental plasticity: intertidal whelks respond to a native but not an introduced predator. *Biology Letters* **4**, 385–387.

Esperk T, Tammaru T, Nylin S (2007) Intraspecific variability in number of larval instars in insects. *Journal of Economic Entomology* **100**, 627–645.

Flatt T (2005) The evolutionary genetics of canalization. *The Quarterly Review of Biology* **80**, 287–316.

Frankel N, Davis GK, Vargas D, Wang S, Payre F, David SL (2010) Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature* **466**, 490–493.

Ghalambor CK, McKay JK, Carroll SP, Reznick DN (2007) Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology* **21**, 394–407.

Gilbert SF, Epel D (2009) Ecological developmental biology: integrating epigenetics, medicine, and evolution Sinauer Associates, Inc., Massachusetts.

Gluckman PD, Hanson MA, Bateson P, Beedle AS, Law MC, Bhutta AZ, Vanokhin K, Bougneres P, Ratan CG, Dasgupta P, Smith GD, Ellison PT, Forrester TE, Gilbert SF, Jablonka E, Kaplan H, Prentice AM, Simpson SJ, Uauy R, West-Eberhard MJ (2009) Towards a new developmental synthesis: adaptive developmental plasticity and human disease. *Lancet* **373**, 1654-1657.

Golden JW, Riddle DL (1984) The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. *Developmental Biology* **102**, 368–378.

Gotthard K, Nylin S (1995) Adaptive plasticity and plasticity as an adaptation: A selective review of plasticity in animal morphology and life history. *Oikos* **74**, 3–17.

Janzen FJ (1994) Climate change and temperature-dependent sex determination in reptiles. *Proceedings of the National Academy of Sciences USA* **91**, 7487–7490.

Janzen FJ, Paukstis GL (1991) Environmental sex determination in reptiles: ecology, evolution, and experimental design. *The Quarterly Review of Biology* **66**, 149–179.

Johnson LJ, Tricker PJ (2010) Epigenomic plasticity within populations: its evolutionary significance and potential. *Heredity* **105**, 113–121.

Koch PB, Brakefield PM, Kesbeke F (1996) Ecdysteroids control eyespot size and wing color pattern in the polyphenic butterfly Bicyclus anynana (Lepidoptera: Satyridae). *Journal of Insect Physiology* **42**, 223–230.

Koch PB, Merk R, Reinhardt R, Weber P (2003) Localization of ecdysone receptor protein during colour pattern formation in wings of the butterfly Precis coenia (Lepidoptera : Nymphalidae) and co-expression with Distal-less protein. *Development Genes and Evolution* **212**, 571–584.

Laforsch C, Tollrian R (2004) Embryological aspects of inducible morphological defenses in Daphnia. *Journal of Morphology* **262**, 701–707.

Legeai F, Rizk G, Walsh T, Edwards O, Gordon K, Lavenier D, Leterme N, Méreau A, Nicolas J, Tagu D, Jaubert-Possamai S (2010) Bioinformatic prediction, deep sequencing of microRNAs and expression analysis during phenotypic plasticity in the pea aphid, Acyrthosiphon pisum. *BMC Genomics* **11**, 281.

Leimar O, Hammerstein P, Van Dooren TJ (2006) A new perspective on developmental plasticity and the principles of adaptive morph determination. *American Naturalist* **167**, 367–376.

Lind MI, Johansson F (2007) The degree of adaptive phenotypic plasticity is correlated with the spatial environmental heterogeneity experienced by island populations of Rana temporaria. *Journal of Evolutionary Biology* **20**, 1288–1297.

Lu BY, Ma J, Eissenberg JC (1998) Developmental regulation of heterochromatin-mediated gene silencing in Drosophila. *Development* **125**, 2223–2234.

Martinez-Arguelles DB, Papadopoulos V (2010) Epigenetic regulation of the expression of genes involved in steroid hormone biosynthesis and action. *Steroids* **75**, 467–476.

Milinkovitch MC, Tzika A (2007) Escaping the mouse trap: the selection of new Evo-Devo model species. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* **308B**, 337–346.

Miller D, Summers J, Silber S (2004) Environmental versus genetic sex determination: a possible factor in dinosaur extinction? *Fertil Steril* **81**, 954-964.

Minelli A, Fusco G (2010) Developmental plasticity and the evolution of animal complex life cycles. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**, 631–640.

Miura T (2005) Developmental regulation of caste-specific characters in social-insect polyphenism. *Evolution & Development* **7**, 122–129.

Miyakawa H, Imai M, Sugimoto N, Ishikawa Y, Ishikawa A, Ishigaki H, Okada Y, Miyazaki S, Koshikawa S, Cornette R, Miura T (2010) Gene up-regulation in response to predator kairomones in the water flea, Daphnia pulex. *BMC Developmental Biology* **10**, 45.

Miyazaki S, Murakami T, Kubo T, Azuma N, Higashi S, Miura T (2010) Ergatoid queen development in the ant Myrmecina nipponica: modular and heterochronic regulation of caste differentiation. *Proceedings of the Royal Society B: Biological Sciences* **277**, 1953–1961.

Moczek AP (2007) Pupal remodeling and the evolution and development of alternative male morphologies in horned beetles. *BMC Evolutionary Biology* **7**, 151.

Moczek AP (2010) Phenotypic plasticity and diversity in insects. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**, 593–603.

Moczek AP, Emlen DJ (2000) Male horn dimorphism in the scarab beetle, Onthophagus taurus: do alternative reproductive tactics favour alternative phenotypes? *Animal Behaviour* **59**, 1273–1273.

Moczek AP, Nijhout HF (2002) Developmental mechanisms of threshold evolution in a polyphenic beetle. *Evolution & Development* **4**, 252–264.

Moczek AP, Nijhout HF (2003) Rapid evolution of a polyphenic threshold. *Evolution & Development* **5**, 259–268.

Moczek AP, Snell-Rood EC (2008) The basis of bee-ing different: the role of gene silencing in plasticity. *Evolution & Development* **10**, 511–513.

Moriyama M, Numata H (2008) Diapause and prolonged development in the embryo and their ecological significance in two cicadas, Cryptotympana facialis and Graptopsaltria nigrofuscata. *Journal of Insect Physiology* **54**, 1487–1494.

Müller GB (2007) Evo-devo: extending the evolutionary synthesis. *Nature Reviews Genetics* **8**, 943–949.

Nijhout HF (1998) Insect Hormones Princeton University Press, Princeton, New Jersey.

Nijhout HF (2003) Development and evolution of adaptive polyphenisms. *Evolution & Development* **5**, 9–18.

Newman SA, Müller GB (2000) Epigenetic mechanisms of character origination. *Journal of Experimental Zoology* **288**, 304–317.

Oliver JC, Robertson KA, Monteiro A (2009) Accommodating natural and sexual selection in butterfly wing pattern evolution. *Proceedings of the Royal Society B: Biological Sciences* **276**, 2369–2375.

Olofsson M, Vallin A, Jakobsson S, Wiklund C (2010) Marginal eyespots on butterfly wings deflect bird attacks under low light intensities with UV wavelengths. *PLoS One* **5**, e10798.

Oostra V, de Jong MA, Invergo BM, Kesbeke F, Wende F, Brakefield PM , Zwaan BJ (2011) Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly. *Proceedings of the Royal Society B: Biological Sciences* **278**, 789–797.

Ostrowski MF, Jarne P, Berticat O, David P (2002) Ontogenetic reaction norm for binary traits: the timing of phallus development in the snail Bulinus truncatus. *Heredity* **88**, 342–348.

Ostrowski MF, Jarne P, David P (2000) Quantitative genetics of sexual plasticity: the environmental threshold model and genotype-by-environment interaction for phallus development in the snail Bulinus truncatus. *Evolution* **54**, 1614–1625.

Passera L, Suzzoni JP (1979) Le role de la reine de Pheidole pallidula (Nyl.) (Hymenoptera, Formicidae) dans la sexualisation du couvain après traitement par l'hormone juvénile. *Insectes Sociaux* **24**, 343–353.

Pfennig DW, Wund MA, Snell-Rood EC, Cruickshank T, Schlichting CD, Moczek AP (2010) Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology & Evolution* **25**, 459–467.

Pigliucci M (2001) Phenotypic plasticity: beyond nature and nurture The John Hopkins University Press, Baltimore.

Pigliucci M (2007) Do we need an extended evolutionary synthesis? *Evolution* **61**, 2743–2749.

Pigliucci M, Murren CJ, Schlichting CD (2006) Phenotypic plasticity and evolution by genetic assimilation. *Journal of Experimental Biology* **209**, 2362-2367.

Powell AM, Davis M, Powell JR (2010) Phenotypic plasticity across 50MY of evolution: drosophila wing size and temperature. *Journal of Insect Physiology* **56**, 380–382.

Puri D, Dhawan J, Mishra RK (2010) The paternal hidden agenda: epigenetic inheritance through sperm chromatin. *Epigenetics* **5**, 386–391.

Reed TE, Waples RS, Schindler DE, Hard JJ, Kinnison MT (2010) Phenotypic plasticity and population viability: the importance of environmental predictability. *Proceedings of the Royal Society B: Biological Sciences* **277**, 3391–3400.

Richards EJ (2008) Population epigenetics. *Current Opinion in Genetics & Development* **18**, 221–226.

Robertson KA, Monteiro A (2005) Female Bicyclus anynana butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. *Proceedings of the Royal Society B: Biological Sciences* **272**, 1541–1546.

Sarkar S, Fuller T (2003) Generalized norms of reaction for ecological developmental biology. *Evolution & Development* **5**, 106–115.

Scheiner SM (1993) Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics* **24**, 35–68.

Schlichting CD (2008) Hidden reaction norms, cryptic genetic variation, and evolvability.

Annals of the New York Academy of Sciences **1133**, 187–203.

Schlichting CD, Pigliucci M (1998) Phenotypic evolution: a reaction norm perspective inauer Associates Inc, Massachusetts.

Schwander T, Lo N, Beekman M, Oldroyd BP, Keller L (2010) Nature versus nurture in social insect caste differentiation. *Trends in Ecology & Evolution* **25**, 275–282.

Scott CP, Williams DA, Crawford DL (2009) The effect of genetic and environmental variation on metabolic gene expression. *Molecular Ecology* **18**, 2832–2843.

Scoville AG, Pfrender ME (2010) Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. *Proceedings of the National Academy of Sciences* **107**, 4260–4263.

Shoemaker CM, Queen J, Crews D (2007) Response of candidate sex-determining genes to changes in temperature reveals their involvement in the molecular network underlying temperature-dependent sex determination. *Molecular Endocrinology* **21**, 2750–2763.

Snell-Rood EC, Van Dyken JD, Cruickshank T, Wade MJ, Moczek AP (2010) Toward a population genetic framework of developmental evolution: the costs, limits, and consequences of phenotypic plasticity. *Bioessays* **32**, 71–81.

Stelkens RB, Wedekind C (2010) Environmental sex reversal, Trojan sex genes, and sex ratio adjustment: conditions and population consequences. *Molecular Ecology* **19**, 627–646.

Stern DL (2000) Evolutionary developmental biology and the problem of variation. *Evolution* **54**, 1079–1091.

Stern DL, Emlen DJ (1999) The developmental basis for allometry in insects. *Development* **126**, 1091–1101.

Storz BL, Travis J (2007) Temporally dissociated, trait-specific modifications underlie phenotypic polyphenism in Spea multiplicata tadpoles, which suggests modularity. *Development & Embryology* **2**, 60–71.

Suzuki Y, Nijhout HF (2006) Evolution of a polyphenism by genetic accommodation. *Science* **311**, 650–652.

Takahashi K, Rako L, Takano-Shimizu T, Hoffmann A, Lee S (2010) Effects of small Hsp genes on developmental stability and microenvironmental canalization. *BMC Evolutionary Biology* **10**, 284.

Tomkins JL, Moczek AP (2009) Patterns of threshold evolution in polyphenic insects under different developmental models. *Evolution* **63**, 459–468.

Vonesh JR, Warkentin KM (2006) Opposite shifts in size at metamorphosis in response to larval and metamorph predators. *Ecology* **87**, 556–562.

Waterland RA, Jirtle RL (2003) Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Molecular and Cellular Biology* **23**, 5293–5300.

West-Eberhard MJ (2003) Developmental plasticity and evolution Oxford University Press, New York.

Wheeler DE, Nijhout HF (1983) Soldier determination in Pheidole bicarinata: effect of methoprene on caste and size within caste. *Journal of Insect Physiology* **29**, 847–854.

Wijngaarden PJ, Brakefield PM (2001) Lack of response to artificial selection on the slope of reaction norms for seasonal polyphenism in the butterfly Bicyclus anynana. *Heredity* **87**, 410–420.

Wijngaarden PJ, Koch PB, Brakefield PM (2002) Artificial selection on the shape of reaction norms for eyespot size in the butterfly Bicyclus anynana: direct and correlated responses. *Journal of Evolutionary Biology* **15**, 290–300.

Wilson RS, Franklin CE (2002) Testing the beneficial acclimation hypothesis. *Trends in Ecology & Evolution* **17**, 66–70.

Wittkopp PJ, Beldade P (2009) Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. *Seminars in Cell and Developmental Biology* **20**, 65–67.

Wund MA, Baker JA, Clancy B, Golub JL, Fosterk SA (2008) A test of the "Flexible stem" model of evolution: ancestral plasticity, genetic accommodation, and morphological divergence in the threespine stickleback radiation. *American Naturalist* **172**, 449–462.

Yeh PJ, Price TD (2004) Adaptive phenotypic plasticity and the successful colonization of a novel environment. *American Naturalist* **164**, 531–542.

Zera AJ (2007) Endocrine analysis in evolutionary-developmental studies of insect polymorphism: hormone manipulation versus direct measurement of hormonal regulators. *Evolution & Development* **9**, 499–513.

Zijlstra WG, Steigenga MJ, Koch PB, Zwaan BJ, Brakefield PM (2004) Butterfly selected lines explore the hormonal basis of interactions between life histories and morphology. *American Naturalist* **163**, 76–87.

CHAPTER 2. ADAPTIVE DEVELOPMENTAL PLASTICITY: COMPARTMENTALIZED RESPONSES TO ENVIRONMENTAL CUES AND CORRESPONDING INTERNAL SIGNALS

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ABSTRACT

The environmental regulation of development can result in the production of distinct phenotypes from the same genotype, and provide the means for organisms to cope with environmental heterogeneity. The effect of the environment on developmental outcomes is typically mediated by hormonal signals which convey information about external cues to the developing tissues. While such plasticity is a wide-spread property of development, not all developing tissues are equally plastic. To understand how organisms integrate environmental input into coherent adult phenotypes, we must know how different body parts respond, independently or in concert, to external cues and to the corresponding internal signals. We quantified the effect of temperature and ecdysone hormone manipulations on post-growth tissue patterning in an experimental model of adaptive developmental plasticity. Following a suite of traits evolving by natural or sexual selection, we found that different groups of cells

within the same tissue have sensitivities and patterns of response that are surprisingly distinct for the external environmental cue and for the internal hormonal signal. All but those wing traits presumably involved in mate choice responded to developmental temperature and, of those, all but the wing traits not exposed to predators responded to hormone manipulations. On the other hand, while patterns of significant response to temperature contrasted traits on autonomously-developing wings, significant response to hormone manipulations contrasted neighboring groups of cells with distinct color fates. We also showed that the spatial compartmentalization of these responses cannot be explained by the spatial or temporal compartmentalization of the hormone receptor protein. Our results unravel the integration of different aspects of the adult phenotype into developmental and functional units which both reflect and impact evolutionary change. Importantly, our findings underscore the complexity of the interactions between environment and physiology in shaping the development of different body parts.

KEYWORDS

Bicyclus anynana, Developmental recombination, Ecdysone, Environmental input, Modularity, Phenotypic flexibility, Physiology, Seasonal polyphenism, Thermal plasticity, Trait-specific sensitivities

INTRODUCTION

In numerous species, the external environment can affect development and lead to the production of distinct phenotypes from the same genotype (Beldade *et al.* 2011). This phenomenon is called developmental plasticity. The resulting alternative phenotypes can be as dramatically different as the nutrition-induced differences between workers and queens in social insects (e.g. Miura 2005, Schwander *et al.* 2010, Keller *et al.* 2014) and the seasonal forms of many insects (e.g. Simpson *et al.* 2011, Brakefield & French 1999, Nijhout 2003). All organisms have traits that are plastic. However, not all body parts of plastic organisms are equally flexible (e.g. Guthrie & Brown 1968, David *et al.* 1998, Shingleton *et al.* 2009). The ability of tissue development to both resist and integrate environmental input is crucial for organismal fitness in heterogeneous environments. An important step towards understanding how organisms can adaptively respond to the environment by expressing alternative phenotypes, and organize this response across body parts and traits, is to determine to

which degree and by what mechanism body parts are integrated into coordinated modules that correspond to functional, evolutionary, and/or developmental units (Cheverud 1996, Wagner 1996). This will include understanding how different body parts respond to external environmental cues, as well as to the internal signals that convey information about those cues to the developing tissues.

In insects, ecdysteroid hormones work as internal signals that mediate key developmental transitions, such as molting and metamorphosis, and can also mediate developmental plasticity (Nijhout 2003). The external environment typically affects systemic hormone titers which, in turn, affect developing tissues. So that different traits which respond to the same hormone signal can develop and evolve independently, hormone effects need to be compartmentalized in time and space (Nijhout 2003, Ketterson *et al.* 2009). This type of compartmentalization has been characterized in relation to the environmental regulation, mostly by nutrition, of the growth of different organs during insect larval development (Shingleton *et al.* 2009, Tang *et al.* 2011, Koyama *et al.* 2013). Much less is known about the compartmentalization of hormone effects for different groups of cells within the same tissue, and during post-growth tissue patterning. We investigate this process here for an evolutionary ecology model of developmental plasticity.

The butterfly *Bicyclus anynana* has become a textbook example of adaptive developmental plasticity (Beldade *et al.* 2011, Brakefield *et al.* 1996, Schlichting & Pigliucci 1998, Gilbert & Epel 2009, Beldade & Brakefield 2002). Its study combines knowledge about the ecological and evolutionary significance of plasticity with the analysis of its genetic and physiological underpinnings (Beldade *et al.* 2011, Brakefield *et al.* 2009). In natural populations, butterflies developing in the dry versus the wet season have cryptic versus conspicuous ventral wing patterns, each associated with different seasonal strategies to avoid predation (Beldade *et al.* 2011). The wing phenotypes encompass a whole suite of pattern elements which differ between the seasons. In the laboratory, the development of wet- versus dry-like phenotypes can be induced by the temperature experienced during pre-adult stages (Beldade *et al.* 2011): warmer temperatures induce wet-like wing patterns, while cooler temperatures induce dry-like phenotypes. Previous studies showed differences between warm- versus cool-reared pupae in the dynamics of ecdysone levels (Oostra *et al.* 2011) (Figure 2.1A) and established these as a cause for changes in wing pattern (*Oostra et al.* 2011). Various studies of *B. anynana* wing pattern plasticity characterized the

effects of the temperature and/or ecdysteroid levels on a few indicative pattern traits (Brakefield *et al.* 1998, Koch *et al.* 1996, Zijlstra *et al.* 2004, Prudic *et al.* 2011). Limiting these analyses to only a few traits has precluded an assessment of how the effects of external and internal signals are compartmentalized in the developing wings. A systematic analysis of both types of cues on multiple aspects of wing patterns is lacking.

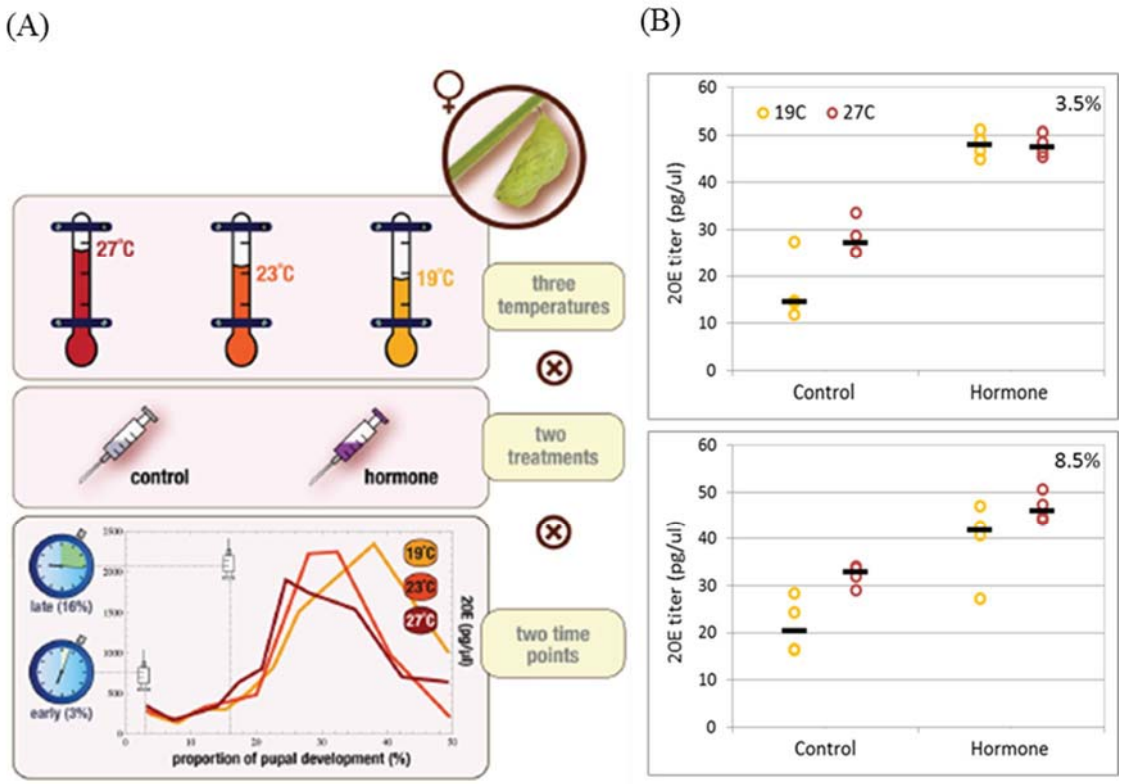

Figure 2.1 - Dynamics and manipulation of internal levels of ecdysone. (A) Experimental design for hormone manipulations. Hydroxyecdysone (20E) and control injections were done on female pupae reared at 19°C, 23°C, or 27°C at two developmental stages corresponding to different phases of the natural 20E dynamics (cf. Oostra *et al.* 2011): "early", before ecdysone concentration starts to increase (at 3% of the total time it takes to complete pupal development at each of the temperatures), and "late", corresponding to the ascending phase of the ecdysone level (at 16% of the total pupal development time). (B) Effect of early hormone injections on hormone titers. Internal levels of 20E at 3.5% (top panel) and 8.5% (bottom) of total pupal development time after "early" injection of hormone and control solutions at 19°C and 27°C.The bar represents the median value of four individuals per treatment, temperature, and time point (see Material and Methods). We tested for the effect of temperature and injection treatment on the levels of 20E at two time points using the model *20E ~ time point + temperature * injection*, for which the residuals showed no significant departure from

normality (Shapiro-Wilk W test: W =0.950, P =0.146) or from homogeneity of variances (Fligner-Killeen test: *Median Chi Square* =1.176, *df* =1, P =0.185). The analysis of variance revealed a statistically significant effect of *temperature* ($F_{(1,32)}$ = 13.848, P =0.0009) and *injection* ($F_{(1,32)}$ =114.501, P =3.25e-11), but not of *time point* ($F_{(1,32)}$ = 0.026, P =0.874) or *temperature*injection* ($F_{(1,32)}$ =3.670, P =0.066), (see Annex 2.4 for more on this analysis).

To characterize the effects of external cues and internal signals on tissue patterning, we manipulated temperature during pre-adult development and manipulated the levels of active ecdysone in the pupal haemolymph (Figure 2.1). We then compared the suite of adult wing traits that constitute the seasonal wing phenotype. The traits we chose (Figure 2.2) reflect increasing levels of spatial resolution in the analysis of the compartmentalization of plasticity. They allow comparisons between: 1) different wings derived from autonomously- developing imaginal discs (fore- and hindwing), 2) different surfaces of the same wing that correspond to distinct cell sheets (dorsal and ventral surfaces) and evolve under different selection regimes (Oliver *et al.* 2009), 3) different types of pattern elements (eyespots and band) displaying weak genetic correlations between them, 4) different repeats of the same type of pattern element (anterior and posterior eyespots on the same wing surface) with stronger correlations between them (Beldade & Brakefield 2002, 2003), and 5) different rings of the same eyespot (central white focus, middle black disc, and external golden ring) that correspond to groups of neighboring cells responding to a morphogen signal originated at each presumptive eyespot center (Beldade & Brakefield 2002, Beldade *et al.* 2002a, Beldade *et al.* 2002b, Allen *et al.* 2008, Saenko *et al.* 2010). Our data on this extensive set of traits allows us to investigate the coordination of responses to external cues and internal signals across groups of wing epidermal cells, and the mechanism for the spatial compartmentalization of the sensitivities to those signals. We discuss our results in terms of whether tighter or looser integration between traits might be adaptive and/or might represent (constrained) properties of the development in response to environmental variation.

MATERIAL AND METHODS

Experimental animals

We used a large outbred laboratory colony of *Bicyclus anynana* butterflies (Brakefield *et al.* 2009). Hundreds of eggs collected from this stock were distributed over three climate-controlled rooms (70% relative humidity, 12:12 hr light/dark cycle) differing in ambient temperature (±0.5°C). We chose temperatures that simulate the conditions of the natural dry (19°C) and wet (27°C) seasons, and an intermediate temperature (23°C). Larvae were fed ad libitum with young maize plants. Pre-pupae were collected daily and pupation times determined (±15 min) by time-lapse digital photography (Canon EOD 100 camera, GT Time-lapse remote control). Female pupae from each temperature were split into three experimental groups: non-injected, injected with control solution, and injected with hormone solution (see below). We started with 28–70 per temperature per treatment but final sample sizes were smaller for some groups (e.g. due to mortality associated to early hormone injections (see below). For non- injected butterflies, we obtained 33 females reared at 19°C, 31 from 23°C, and 38 from 27°C.

Image analysis of target traits

The ventral surface of the right forewing and hindwing, and the dorsal surface of the forewing of the eclosed females with undamaged wings were photographed (Leica DC200 digital camera) under a binocular microscope (Leica MZ12) with controlled light and 10x magnification. We included a ruler for conversion from pixels to millimeters and a color reference card (QPcard 201) for background correction. The resulting images were analyzed with a custom macro image processing system using ImageJ-based open-source Fiji software package (Schindelin *et al.* 2012). For each trait, areas were calculated by a threshold method in which the image was first converted to black and white and values of intensity under or above user-established threshold values were chosen. The measurements of the white central areas of the smaller more anterior eyespots on the forewing (dorsal and ventral, traits 1 and 3, respectively) and hindwing (trait 5) were excluded because of high measurement error. In total, we measured 19 traits characterizing the area of wings and of various color pattern components (Figure 2.2). We also counted the number of white eyespot centers on the dorsal surface of the hindwing of the non-injected butterflies

(Westerman *et al.* 2014). Note that the number of females obtained for each treatment is not necessarily equal to the number of measurements available for the 19 traits. This is because not all traits could be measured in all females (e.g. in cases of some damaged wings). Final sample sizes for all traits in all experimental groups are given in Annex 2.1 for the non-injected individuals, and Annex 2.2 for early and late injections, respectively.

Hormone injections

For each temperature, we had two injection treatments: "hormone" for injection of a solution of 20-hydroxyecdysone (20E), the biologically-active form of ecdysone (Richards 1981), and "control" for injection of the same volume of just solvent. Because the duration of pupal stage varies with temperature, as does the dynamics of ecdysone titers (Oostra *et al.* 2011), we used % of the duration of the pupal stage when choosing the injection time points. Injections were done on pupae at two stages corresponding to different phases of the natural ecdysone dynamics titers (Oostra *et al.* 2011): "early" (at 3% of the total pupal development time) before ecdysone levels start to increase, and "late" (at 16% of the total pupal development time) corresponding to the ascending phase of the ecdysone levels (Figure 2.1A). Pupae were injected (10 µL Hamilton syringe with a 0.3 mm gauge needle) on the left side in the region of the fifth abdominal segment with 3 µL of 0.25 µg 20-hydroxyecdysone (Sigma; hormone stock solution 1 mg/ml in 100% ethanol) in insect Ringer's buffer (Merck) with vital red artificial coloring (Fluka). This hormone concentration was chosen to obtain an optimal balance between hormonal effects and pupal survival (cf. Koch *et al.* 1996). After injection, pupae were placed back at their respective rearing temperature until emergence, and adults were frozen (−20°C) until wing analysis. The numbers of females phenotyped for early injections of control:hormone were 32:19 for 19°C, 29:8 for 23°C, and 35:7 for 27°C. For late injections, these numbers were 32:32 for 19°C, 23:30 for 23°C, and 34:32 for 27°C. Because not all traits could be measured for each female, final number of measurements for each trait can be different and are shown in Annex 2.2 for early and late injections. Smaller sample sizes for early hormone injections are due to higher mortality associated to that treatment.

Hormone titers

We injected female pupae reared at 19°C and 27°C with hormone and control solutions at 3% of the duration of pupal stage, and measured internal 20E at 3.5% or at 8.5% of total pupal development time. For that, we extracted 50 µl of haemolymph from each of four pupae per treatment and time point, and measured 20E levels using the ACE enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI) following manufacturer's instructions. Briefly, samples were extracted from individual pupae by homogenization followed by addition of 200 µl of 70% methanol. The homogenates were dried using a rotary evaporator at room temperature and dissolved in assay buffer. Calibration curves were generated using commercially available 20E (Sigma; 0.5 µg/µl in 100% ethanol). Absorbance for controls, standards, and haemolymph samples was measured by spectrophotometry at a wavelength of 405 nm (VICTOR Multilabel Plate Reader). Note that this hormone quantification method can detect concentrations down to a minimum concentration of 7.8 pg/µl, which is below the detection level of the method used previously to characterize the titer dynamics displayed in Figure 2.1A (Beldade *et al.* 2011).

Immunohistochemistry

Antibody stainings of pupal wings were performed as described in (Brakefield *et al.* 2009) using a custom antibody against *B. anynana* EcR (Conceição *et al.* 2011) obtained from Proteintech (peptide within region common to all isoforms; CWDVADVNSAQPPPVFDHASDL) at a final dilution of 1:50 (after testing a range of concentrations). The antibody was tested together with other antibodies to assess: 1) specificity by comparing its localization with the *Manduca* anti-EcR (we observed similar patterns but with less background for the *B. anynana*-specific antibody), 2) detection of the active form of EcR by comparing its localization with that of known downstream EcR target Broad, 3) association to the eyespot field and intra-cellular localization by comparing with localization of DAPI. We also detected EcR in younger "clearer" tissues (larval wings) in order to confirm the intra-cellular localization of this antibody. We performed stainings of wings dissected from multiple pupae and covering 6-30% of pupal duration for each of the two extreme rearing temperatures 27°C and 19°C. The primary anti-*EcR* antibody was detected with Alexa Fluor 594 anti-rabbit (Molecular Probes) and images were

collected on a Leica DMIRE2, Leica SP5 confocal laser scanning and Nikon Eclipse TE2000-S Screening microscopes.

Statistical analysis of effects of developmental temperature on wing traits

All data analyses were done in *R* statistics package (R-Core-Team 2012) and *Mathematica* software package (Wolfram 1996). We tested for the effect of temperature on wing traits of non-injected individuals (Figure 2.3) using ANOVA with *temperature* as a factor (three levels: 19°C, 23°C, 27°C) and, for wing pattern traits 1–8, using the respective *wing area* as covariate with the model *trait ~ wing area + temperature*. Trait areas were used untransformed or log10 transformed to meet Shapiro-Wilk normality test ($P \geq 0.05$). When *temperature* was found to have a significant effect on trait values ($P < 0.01$), we did post-hoc comparisons between pairs of temperatures using lsmeans (see Annex 2.1). To test for the effect of temperature on the number of white pupils on the dorsal surface of the hindwing we used an ANOVA with a Chi-square test and a quasi-Poisson distribution. We tested the model *pupil nr ~ temperature*, using *temperature* as a factor (three levels: 19°C, 23°C, 27°C).

Statistical analysis of differences in hormone titers

We tested for the effect of temperature and injection treatment on the levels of 20E at two developmental time points (Figure 2.1B) using the model *20E ~ time point + temperature * injection*. We first confirmed that the residuals showed no significant departure from normality (Shapiro-Wilk test) or from homogeneity of variances (Fligner-Killeen test). We then used ANOVA to test for the effect on levels of 20E of *time point* (factor with two levels: 3.5% and 8.5%), *temperature* (factor with levels 19°C and 27°C), *injection* (factor with two levels: hormone and control) and the interaction *temperature*injection*. Because there was no significant effect of *time point*, we did pairwise comparisons between *temperature* and *injection* groups using Tukey's honest significance tests (see Annex 2.4).

Statistical analyses of the effects of hormone manipulations on wing traits

We tested for the effect of hormone injections, done at different temperatures and at different developmental time points, on wing traits (Figure 2.4) using core and confirmatory tests in a series of steps. Details of the analyses are shown in Annex 2.2.

To facilitate between- trait comparisons, we rescaled raw trait measurements to an identical [0–1] range. This was done for each of 114 groups (3 temperatures x 2 injection treatments x 2 time points x 19 traits) by setting the minimum trait value to 0 and the maximum value to 1, and rescaling intermediate values proportionally. We then checked Normal distribution of the rescaled trait values in each group (Jarque-Bera test, alpha =0.01). For normally distributed values, we used a two-tailed T test to compare control and hormone treatment means for each trait, temperature and time point. For the one non-normally distributed group values (hindwing area, trait 10, after early injection at 27°C), we used a two-tailed Mann–Whitney U test to compare control and hormone medians. We used the False Discovery Rate procedure (Benjamini & Hochberg 1995) with alpha =0.05 to determine the contextual significance of each of the 57 p-values obtained per injection time point.

To take into account differences across treatments in sample size and, particularly, the reduced sample sizes in the early hormone injection groups (Koch *et al.* 1996), we carried out an extra validation statistical analysis. We combined two types of resampling techniques (Benjamini & Hochberg 1995): (1) bootstrap (a good method to estimate population parameter differences from small samples) and (2) permutation tests to determine the significance (p-values) of the parameter differences (or displacements) obtained via the bootstrap distributions. We performed a bootstrap-based estimation of the displacement of mean/median for each group by resampling 1000 times from the original distributions of trait values (keeping sample size with replacement). Because the bootstrap distributions did not depart significantly from normality (Jarque-Bera test, alpha =0.01), we used the mean of that distribution as the estimator of mean displacement (difference) between control and hormone-injected groups. We then used permutation tests to compare differences between control and hormone injections (for each trait, temperature, and time point) assessed from the original dataset with those from the resampled dataset. For each of the 57 pairs (19 traits x 3 temperatures x 2 time points) of control and hormone groups, we computed the difference between their original means, and then estimated mean difference 1000 times from resampled data as follows (note that only means were used on the basis that no bootstrap distribution for the previous goal departs significantly from normality): 1) we merged the two distributions (control with hormone values) into a single distribution, 2) 1000 times, we divided the values in this distribution into two groups of the same sizes as the original control and hormone

groups, 3) we calculated the mean difference between these groups, 4) we thus produced a list of 1000 mean differences (in absolute value), 5) we calculated a p-value for our original comparison of control versus hormone means as the proportion of those 1000 values that is different from the original mean difference divided by 1000 (two-tailed test). The p-values obtained were also subjected to the False Discovery Rate procedure (Benjamini & Hochberg 1995) with alpha =0.05 to determine the contextual significance of each of the 57 p-values obtained per injection time point. We compared both sets of results obtained from the core test (k-sample t-test or Mann–Whitney as appropriate and from permutation tests and found them to be not in conflict (see Annex 2.2).

RESULTS AND DISCUSSION

Our results show that different groups of cells on the developing wing epidermis, which correspond to different aspects of the color pattern on adult female wings, have characteristic sensitivities to changes in temperature during pre-adult development (Figure 2.3), as well as to changes in ecdysone levels during the pupal stage (Figure 2.4). We could identify not only which traits are and are not responsive to manipulations of the external cue and internal signal, but also identify groups of sensitive traits that display distinct patterns of coordinated responses (Figure 2.5). Finally, we show that the spatial compartmentalization of hormone sensitivities is not due to the spatial or temporal compartmentalization of the hormone receptor protein (Figure 2.6).

Response of wing traits to developmental temperature

To assess how different groups of cells on the developing wings respond to external environmental cues, we measured wing patterns of butterflies reared at three temperatures, representing typical wet- and dry-inducing extremes (27°C and 19°C, respectively) and an intermediate temperature (23°C). We then compared phenotypes between temperatures. Figure 2.3 shows the thermal reaction norms for the 19 target traits in adult females. For the first time, this involved considering separately and simultaneously the distinct color rings (white, black, and gold) of multiple eyespots on different parts (anterior and posterior) of the same wing surface and on different wing surfaces (ventral and dorsal) (Figure 2.2).

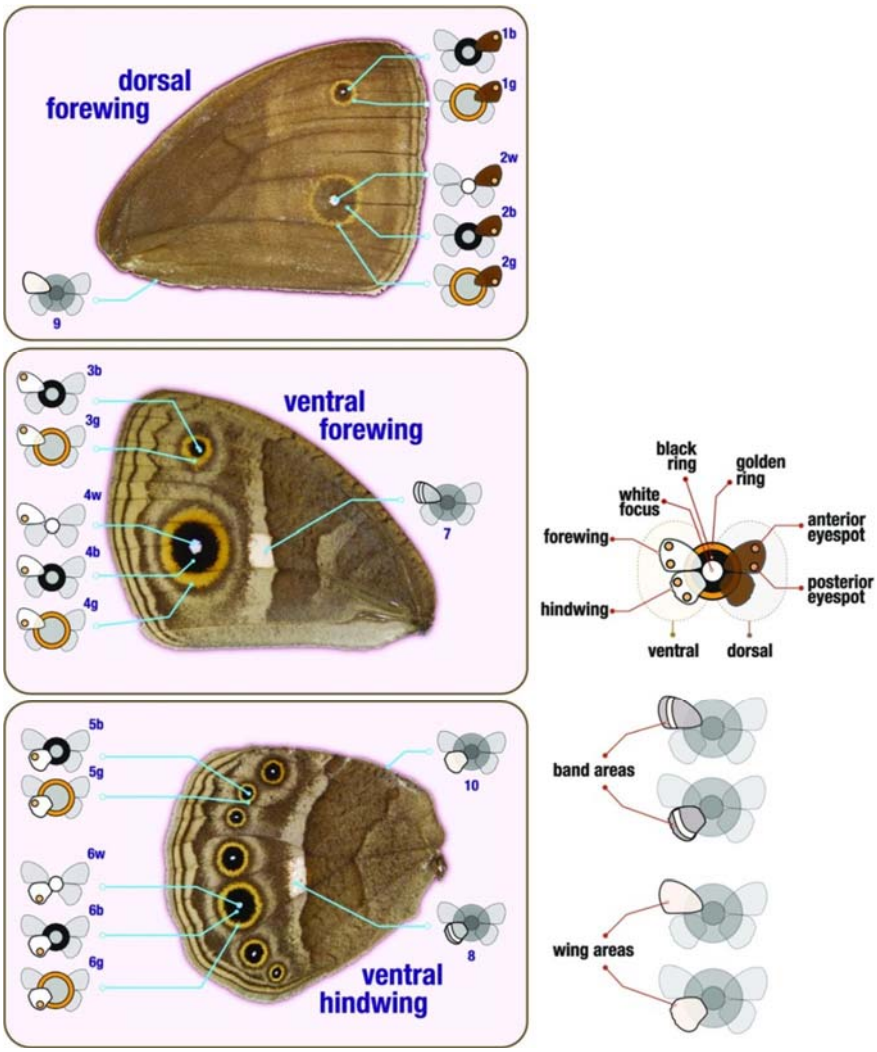

Figure 2.2 - Wing traits measured in adult females. The photos represent the typical phenotype of female *Bicyclus anynana* reared at 27°C. Note that the dorsal surface of the hindwing does not always have color patterns beyond occasional extra eyespots or just their white pupils which are generally too small for accurate size measurements. For each individual, we obtained 19 wing measurements corresponding to four categories of traits: dorsal eyespots, ventral eyespots, ventral band, and wing areas. Note that each eyespot corresponds to a different trait number and we use different letter codes to refer to the corresponding white centers (w), black discs (b), and golden rings (g). The diagram on the right panel displays the symbols used to refer to each of the traits in the other figures. On each wing surface (ventral represented in white and dorsal in brown), we measured two eyespots (one more anterior represented by a circle on the top and one more posterior by a circle on the bottom). The color of the circles at the center of the image corresponds to each of the color rings that make up each eyespot.

This extensive analysis of wing pattern traits revealed that, in contrast to what had been described, some aspects of the dorsal wing pattern are plastic in relation to developmental temperature (Figure 2.3A). Previous studies of plasticity on dorsal forewing color pattern had investigated the most posterior eyespot (our trait 2) and found it to be largely non-plastic across seasonal environments (Brakefield *et al.* 1998, Prudic *et al.* 2011). Our results confirm this but, by also analyzing other pattern elements on the same wing surface, show that the lack of temperature-sensitivity is not a property of the whole dorsal wing surface. The more anterior eyespot on the dorsal forewing (trait 1) did increase significantly with temperature (Figure 2.3A). As expected from previous studies, wing pattern components on the ventral surface of the wings showed clear thermal plasticity (Figure 2.3B, C, E, F, G; see Annex 2.1).

Only the wing pattern element implicated in mate choice does not respond to temperature

Previous work largely focused on ventral wing patterns because this is the surface exposed to predators in butterflies at rest, and thus the surface under predator-driven natural selection for plasticity (Brakefield *et al.* 2009). Seasonal variation in ventral wing patterns is associated with seasonal variation in the resting background and to alternative strategies for butterflies to avoid predation. In the cooler dry season, duller brown wing patterns with no striking color elements are cryptic in relation to the resting background of dry brown leaves. In the warmer wet season, more conspicuous color elements along wing margins can function as targets for predator attacks away from the more fragile body (Beldade *et al.* 2011, Brakefield & French 1995).

The dorsal patterns, on the other hand, are typically not exposed in the butterfly at rest and presumably not under selection by predators. Instead, those patterns are exposed during courtship and thought to evolve under sexual selection (Prudic *et al.* 2011, Oliver *et al.* 2009, Breuker & Brakefield 2002). In particular, some of the UV-reflecting white pupils of dorsal eyespots have been shown to influence mate choice (Prudic *et al.* 2011, Westerman *et al.* 2014). In our study of female butterflies, the only eyespot that showed no significant response to temperature (Figure 2.3D; trait 2) was the one that is sexually selected in males (Prudic *et al.* 2011). The white center of this eyespot had been found to be plastic in males; being larger and more UV-reflecting in wet season courting individuals

(Prudic *et al.* 2011). Even though it has been proposed that dry season females do courtship (Prudic *et al.* 2011), in a case of seasonally-plastic sexual selection, we found that the corresponding trait is not plastic in females (Figure 2.3D; trait 2w). Instead, a recent study proposed that male choice among potential dry-season mating partners depends on the number of white pupils found on the dorsal surface of the female hindwing (Westerman *et al.* 2014). The number of such pupils was shown to vary between females reared at 17°C versus 27°C (Westerman *et al.* 2014). In our study, we found that the mean (but not the median) number of white pupils on the ventral surface of the hindwing of non-injected females decreases with increasing temperature, but not significantly so (Figure 2.3I).

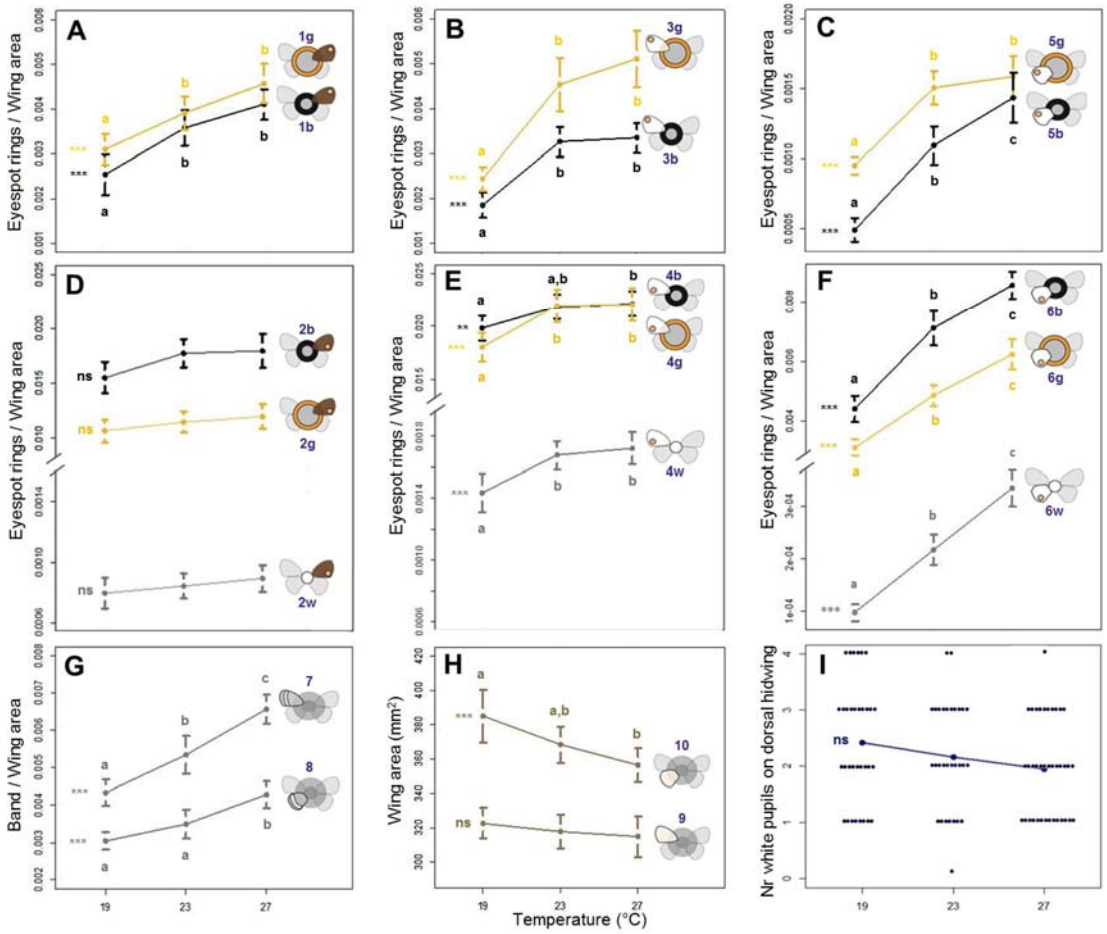

Figure 2.3 - Effect of temperature experienced during development on wing traits. For each trait, we plot the mean value as a function of temperature and use bars to represent the standard deviation for 24–38 measurements per temperature. These representations, called reaction norms, are the standard way of displaying plasticity. Trait icons cf. Figure 2.2 are given on the right of the respective reaction norm line: (A-B) dorsal eyespots, (C-F) ventral eyespots on forewing and hindwing, (G) ventral bands, and (H) wing areas. We tested for the

effect of temperature on wing pattern trait using the model trait ~ temperature + wing (where the area of the corresponding wing is a covariate), and on wing area using wing ~ temperature (see Material and Methods). Trait values were used untransformed or log10 transformed to meet Shapiro-Wilk normality test (alpha =0.05). Statistical significance for effects of temperature on wing traits (see Material and Methods) is indicated to the left of each reaction norm: ns (non-significant) P > 0.01, ** P <0.01, *** P <0.001. When ANCOVA/ANOVA showed significant effects of temperature on trait value, we compared across temperatures. For each reaction norm, different letters indicate pairwise comparisons that revealed statistically significant differences (lsmeans P <0.01) (see Annex 2.1 for more details on these statistical analyses). For the number of white pupils (n = 30–38 individuals, Annex 2.1) on the dorsal surface of the hindwing in panel (I), we found no significant effect of temperature using the model pupil nr ~ temperature with a quasi-Poisson distribution (Deviance =1.894, df =2, P =0.1172).

Response of wing traits to hormone manipulations

To examine how different groups of cells on the wings respond to changes in hormone levels, we measured the effect of hormone manipulations during the early pupal stage when the signaling from eyespot organizers and the response of the surrounding cells to the ring- determining morphogen are known to take place (Beldade & Brakefield 2002). We manipulated the levels of active ecdysone in the haemolymph by injecting female pupae with 20-hydroxyecdysone (20E) (Brakefield *et al.* 1998, Koch *et al.* 1996, Zijlstra *et al.* 2004) at two developmental time points (Figure 2.1). For each temperature and injection time point, we then compared adult wings between control-injected and hormone-injected individuals. Figure 2.4 shows the magnitude and statistical significance of the difference between control and hormone treatments for each of the target traits, injection time points, and rearing temperatures (see also Annex 2.2).

Only traits that responded to changes in temperature during development responded to changes in hormone titers during early pupal life. That is, all traits for which differences between control-injected and hormone-injected individuals were significant (i.e., any red circles in Figure 2.4) are traits for which the differences between temperatures for non-injected individuals were also significant (i.e. reactions norms marked with stars in Figure 2.3). However, not all wing pattern traits that responded to temperature were affected by the hormone treatment. We found no significant effect of hormone manipulations for any of the traits in the dorsal wing

surface (Figure 2.4A). In contrast, many traits on the temperature- plastic ventral wing surfaces significantly increased in area in response to hormone injections. In some cases, lack of effect of our hormone injections on temperature-responsive traits can be explained by the fact that trait determination occurred before the hormone treatment. This is the case for the white eyespot centers (traits 4w, 6w in Figure 2.2) and for hindwing area (trait 10). The establishment of the eyespot organizing centers (Saenko *et al.* 2010) and most of wing growth (Nijhout *et al.* 2014) are known to take place during larval life, before our hormonal injections were done. However, for other non-responsive traits, notably eyespot color rings, that is not the case (see below).

Only pattern elements on the wing surface exposed to predators respond to changes in pupal ecdysone levels. For all dorsal (traits 1 and 2) and some ventral thermally-responsive color pattern elements (traits 4 and 7) that did not respond to hormone treatment, it seems unlikely that our treatment missed the relevant windows of trait determination. Certainly for eyespot rings, we know that it is during early pupal development that signaling from eyespot centers establishes concentric rings of cells fated to produce different color pigments (Allen *et al.* 2008, Saenko *et al.* 2010). The lack of response of those traits to our hormone manipulations could be due to lower sensitivities to hormone titers and, i.e., due to them requiring hormone concentrations higher than those we produced artificially. This too, at least alone, seems unlikely because our post-injection hormone levels at 19°C surpassed the control levels at 27°C, a temperature difference for which the traits did change (see below and Figure 2.1B). The lack of response to hormonal manipulations suggests that thermal plasticity for these traits is not mediated (exclusively) by ecdysone.

It is curious to note that the color traits established in early pupae which we found to be thermally-sensitive but ecdysone-resistant are presumably under no, or weaker selection by predators. As discussed before, this is the case for color patterns on the dorsal surface of the wing which is not exposed in the butterflies resting against the seasonally color-variable background foliage. Also, unlike other ventral pattern elements, the wing region containing the hormone-unresponsive traits 4 and 7 is typically covered by the hindwing in the resting butterfly. Therefore, these traits too are presumably less exposed to the predators that drive selection for seasonally plastic ventral wing patterns. A weaker selection pressure by natural enemies could explain why these particular traits evolved different levels of plasticity.

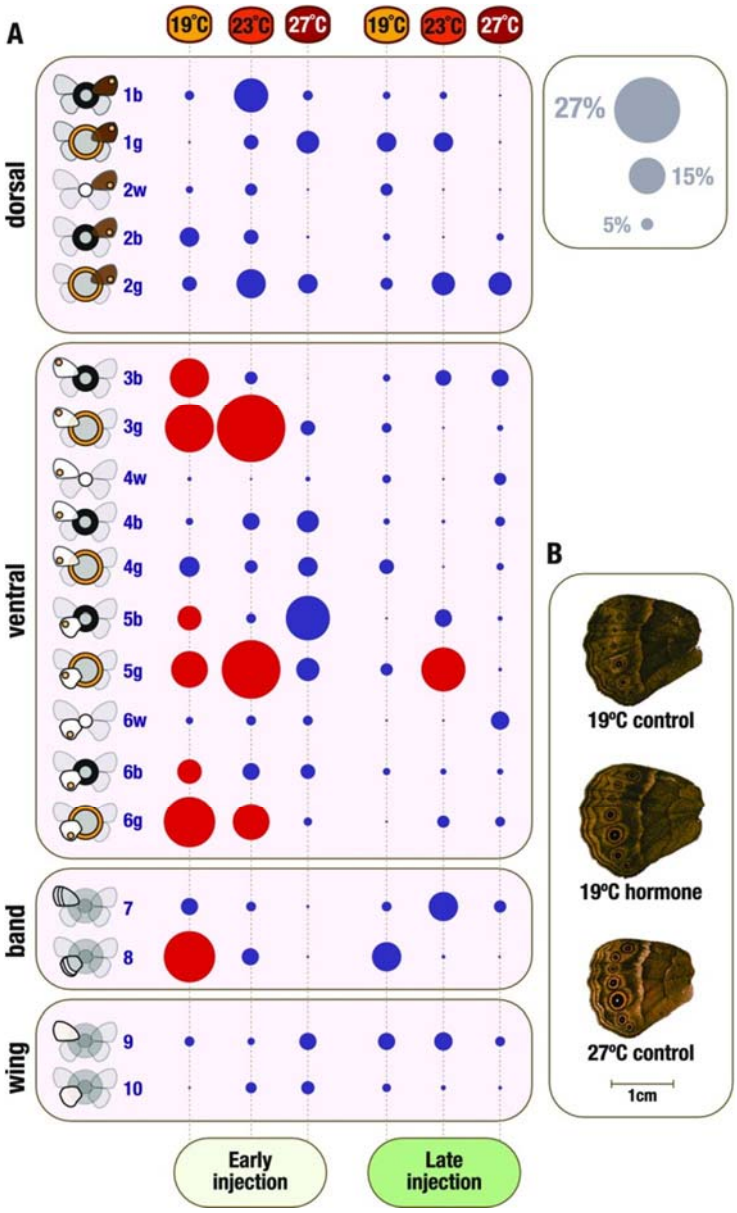

Figure 2.4 - Effect of pupal hormone manipulations on different wing traits. (A) For each trait, temperature and time point combination, the circles represent the magnitude (circle size; scale on top right corner) and statistical significance (circle color, with red for significant differences; cf. permutation test explained in the Material and Methods) of the difference between hormone- versus control-injected individuals (details in Annex 2.2). As for Figures 2.2 and 2.3, the traits are organized per type: dorsal eyespot traits, ventral eyespot traits, ventral bands, and wing areas. Final number of measurements for each trait in each experimental group can be found in Annex 2.2. The difference between control and hormone treatments was tested using a series of core and confirmatory statistical tests, all giving largely the same results (details in Materials and Methods and Annex 2.2). (B) Photos of the ventral surface of adult hindwings representing the phenotypes of different temperature and

injection treatments: control-injected individual at 27°C, hormone-injected individual at 19°C, and control-injected individual at 19°C. Scale bar corresponds to 1 cm. All images are from butterflies injected as pupae at 3% of their development time. These wings illustrate how early hormone manipulations at lower temperature increase the area of different color pattern components, bringing the phenotypes closer to those of individuals reared at higher temperature.

Levels and time windows of sensitivity to hormone manipulations

All traits that responded to hormone injection treatment (Figure 2.4) were larger in hormone- treated relative to control-treated butterflies. The hormone-induced increase in size is consistent with the temperature plasticity: development at warmer temperatures associated with an earlier increase in natural hydroxyecdysone titers (Oostra *et al.* 2011, Brakefield *et al.* 1998, Koch *et al.* 1996, Zijlstra *et al.* 2004); see Figure 2.1A), leads to the production of more conspicuous wing patterns with larger areas of non-background color (Figure 2.3). By artificially increasing hormone levels at the lower temperatures, we induced the production of the same type of phenotypic effect that higher temperatures have on wing patterns (Figure 2.4B, see also Annex 2.3). The fact that the artificial increase in hormone levels phenocopied the temperature effect confirms a role for ecdysteroids at this early-pupal developmental stage in mediating thermal plasticity in wing patterns.

Strikingly, we detected the strongest responses to hormone manipulations for injections done at the early developmental time point, when the natural levels of pupal ecdysone are very low and differences between temperatures were previously undetectable (Oostra *et al.* 2011), and not for the later time point when hormone titer differences between temperatures are clear (Figure 2.1). This suggests a window of sensitivity to the hormone between our two injection time points, i.e. between 3% and 16% of pupal life. For only one of the target traits (trait 5 g), did we see an effect of later hormone manipulation. This indicates some level of heterochrony in the development of this trait, which appears to have a later window of sensitivity to the hormone. Heterochrony, differences in the developmental times and/or rates, is an important contributor to phenotypic diversification, including for butterfly wing patterns (Koch *et al.* 2000, ffrench-Constant 2012). We have shown previously that hormone manipulations at later time points do affect a number of life-history traits (Oostra *et al.* 2014).

We did not observe significant effects of hormone manipulations at higher temperatures (Figure 2.4), even if our manipulations did significantly change hormone titers. We measured hydroxyecdysone concentration in the haemolymph of pupae at 3.5% and 8.5% of pupal development time for the two extreme experimental temperatures after early injection of hormone and of control solutions (Figure 2.1B). Hormone levels are significantly higher for hormone-injected versus control-injected pupae at both rearing temperatures (see Annex 2.4). Control pupae show higher 20E levels when reared at 27°C relative to 19°C, consistent with the relatively faster increase in natural hormone titers that occurs at higher temperatures (Figure 2.1A). After hormone injection we can no longer detect differences in internal levels between temperatures (Figure 2.1B).

Differences in trait associations in response to external and internal cues

Focusing on the eyespot traits that are plastic in relation to temperature and/or to hormone titers, we can identify different categories of response (Annex 2.5 summarized in Figure 2.5). Note that a Principal Component Analyses (Annex 2.6), a standard approach for analysis of multidimensional datasets such as ours, identified traits with similar and contrasted responses but not with the same resolution we could do with the analyses of individual traits (cf. Figures 2.3 and 2.4).

The groups identified based on the response to temperature largely contrast eyespots on the forewing versus hindwing (Figures 2.3 and 2.5A). All forewing eyespot traits are significantly smaller at 19°C and do not differ between 23°C and 27°C, while all hindwing eyespot traits significantly increase in size with temperature. In summary, for the effects of temperature on wing patterning, we observed looser integration across autonomously-developing wings, and tighter coordination of traits on the same wing. The single hindwing trait (trait 5 g) that responds to temperature in the same manner as all forewing traits (Figure 2.3 and Annex 2.5: Figure 2.S2A) is also the only trait significantly affected by late hormone manipulations (Figure 2.4). It is unclear what, developmentally or ecologically, might be the uniqueness of this trait.

For the traits that we found to be sensitive to early manipulations of pupal hormone levels, we found a different pattern of coordinated responses. Because 1) color rings of each eyespot are specified by the same morphogen gradient established from each eyespot's center (Beldade & Brakefield 2002, Brakefield & French 1995),

2) each eyespot center produces morphogen independently of other eyespots (Brakefield & French 1995), and 3) eyespot centers have been shown to have higher levels of ecdysone receptor protein (Koch *et al.* 2003), we had hypothesized that all rings of a single eyespot would respond to hormone manipulations in concert and relatively independently from those of other eyespots (Beldade & Brakefield 2002, Beldade *et al.* 2002a). However, rings of the same color, and not rings of the same eyespot, responded in a similar manner (Figures 2.4 and 2.5B). All plastic black rings showed hormone-related changes only at 19°C while all golden rings showed hormone-related changes both at 19°C and 23°C (Figure 2.4). Among the golden rings, we can further distinguish between those from the anterior versus the posterior-half of the wings. They differ in relation to how much hormone-related change we saw at 19°C versus 23°C (Figure 2.4, Annex 2.5: Figure 2.S2B). This is consistent with studies showing coupling of anterior (and of posterior) portions across wing surfaces (Beldade & Brakefield 2003) and uncoupling of anterior versus posterior eyespots within the same wing surface (Beldade *et al.* 2002a, Oostra *et al.* 2014, Beldade *et al.* 2002c).

Compartmentalization of hormone effects is not explained by hormone receptor localization

As a mechanism for local sensitivities to systemic levels of 20E, we hypothesized that groups of cells that responded differently to 20E manipulations would differ in expression of ecdysone receptor (EcR). To test this hypothesis, we investigated the localization of EcR protein in wings from pupae reared at different temperatures using an antibody against *B. anynana*'s EcR (Conceição *et al.* 2011). We found EcR in cells on the entire pupal wing epidermis at all temperatures and throughout the whole early pupal life, extending well after the 16% of developmental pupal time used as our last injection time point (Figure 2.6). The density of EcR-positive cells was higher in circular regions corresponding to the eyespot organizing centers (Koch *et al.* 2003). These regions were smaller for pupae reared at 19°C relative to 27°C (Figure 2.6B, C versus 2.6F, G; Oliver *et al.* 2013), and for smaller versus larger eyespots (Figure 2.6B, 2.6F versus 2.6C, 2.6G).

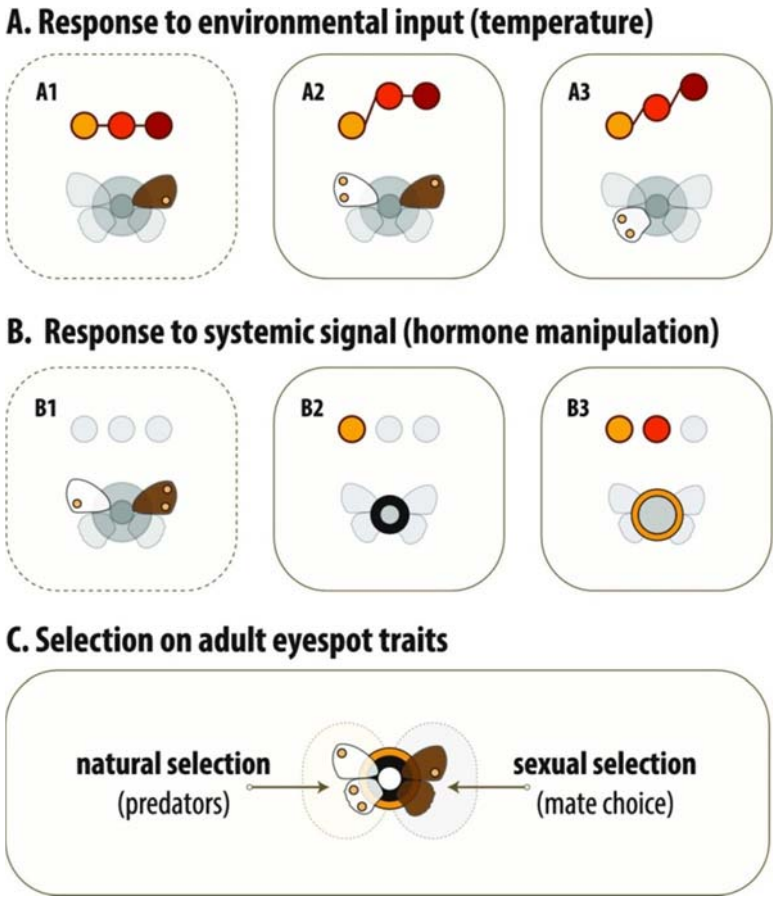

Figure 2.5 - Patterns of coordinated response to external and internal signals. Each box includes eyespot traits that responded in a similar manner to differences in developmental temperature (A) and to hormone injections (B). Boxes in dashed lines represent traits (symbols cf. Figure 2.2) that do not respond to temperature (A1) or to hormone injections (B1). The other boxes represent distinct patterns of response to temperature (A2-A3) or to ecdysone (B2-B3) (see details in Annex 2.5). The three circles at the top of each box represent each of the three experimental temperatures: from right to left, 19°C, 23°C, and27°C. In panel (A), lines between those circles illustrate the shapes of the corresponding thermal reaction norms (cf. Figure 2.3): flat for A1, 19°C <23°C ~27°C for A2, and 19°C<23°C <27°C for A3. In panel (B), the circles not in grey represent temperatures for which phenotypes were significantly different between control- and hormone-injected individuals (cf. Figure 2.4): no effect of hormone manipulations for whichever temperature in B1, effect only for 19°C in B2, and effect both at 19°C and 23°C in B3. The only traits that do not respond to temperature (A1) correspond to the eyespot shown to be under sexual selection, while those that do not respond to hormone manipulations (B1) are those not exposed to predators in resting butterflies (C). The patterns of response to temperature contrast fore- and hindwing while those for hormone manipulations contrast black and golden

color rings. A detailed scheme of the patterns of response showing all traits can be found in Annex 2.5.

Surprisingly, however, this pattern of EcR expression was detected both for the highly plastic ventral and the hormone-unresponsive dorsal eyespots. This shows that the non- responsiveness of the dorsal color traits to hormone manipulations cannot be due to the corresponding cells not having the receptor for the systemic signal, as had been previously proposed (Brakefield *et al.* 1998). Our data also did not reveal visible differences in EcR levels between the regions of the presumptive black versus golden eyespot rings (Figure 2.6B-D and 2.6F-G) that showed different sensitivities to the hormone injections (Figure 2.5). This indicates that differences in the way they respond to hormone manipulations (Figure 2.5B) must be determined either upstream of the binding of 20E to its receptor in the cell nucleus (e.g. cell permeability to hormone) or downstream of that (e.g. factors interacting with the activated EcR (cf. Tang *et al.* 2011).

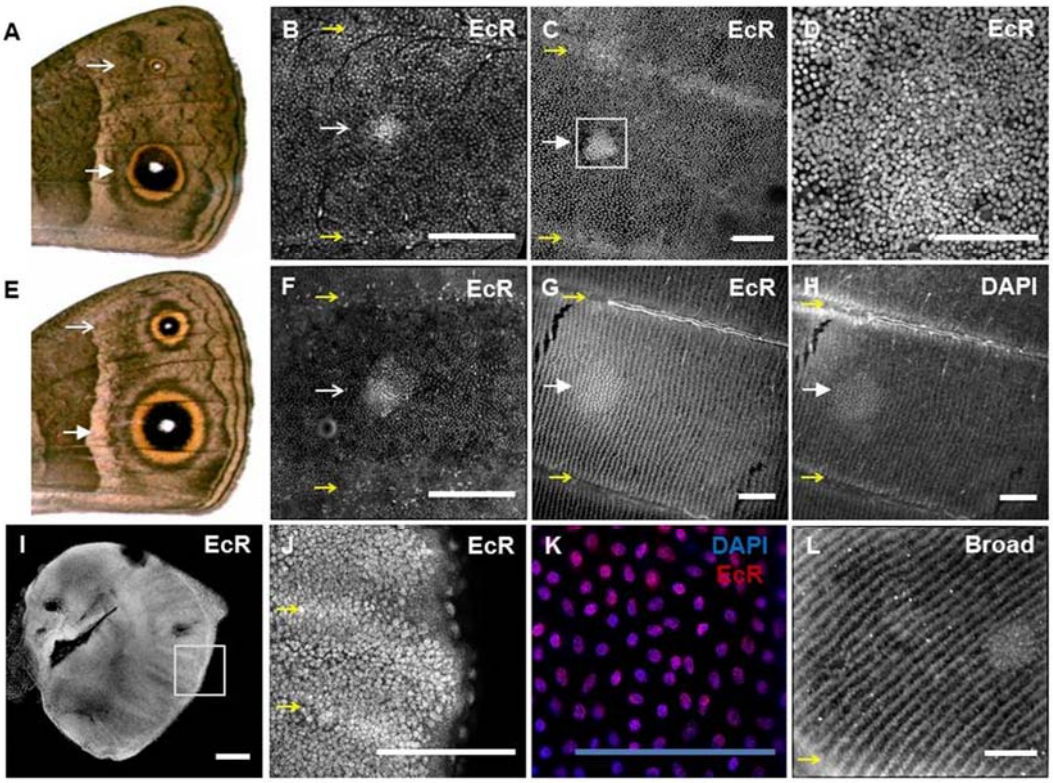

Figure 2.6 - Localization of Ecdysone Receptor (EcR) protein in pupal wings. (A) Ventral surface of forewing (distal section shown) of non-injected individual reared at 19°C with different arrow heads pointing at anterior (trait 3) versus posterior (trait 4) eyespots. Corresponding region of anterior (B) and posterior (C) eyespot fields of developing pupal

forewing at 19°C around 6% and 23% of pupal time, respectively. Panel (D) is a detail of the presumptive eyespot center in panel (C). (E) Ventral surface of forewing (distal section) of non-injected individual reared at 27°C with arrow heads pointing at anterior and posterior eyespots. Corresponding region of anterior (F) and posterior (G) eyespot fields of developing pupal forewing at 27°C around 6% and 23% of pupal time, respectively. Panel (H) corresponds to the DAPI (nuclear) stain in panel (G) showing higher density and lack of row-like organization of the cells at the center of the presumptive eyespot. Panel (I) corresponds to EcR expression in larval hindwing and (J) is a detail of (I). (K) Detail of overlap in EcR protein and DAPI from developing forewing at 27°C (around 6% of pupal duration), showing nuclear localization of EcR. (L) Presumptive eyespot center (around 23% of pupal duration at 27°C) expressing EcR's target gene Broad (core isoform) shows that EcR is active. Yellow arrows indicate veins for reference. All in all, we see EcR-positive cells over the entire wing since larval to late pupal stages, and in higher cell density in the presumptive eyespot centers. These centers are larger for larger eyespots. Scalebar=100μm.

CONCLUSIONS

Environmental cues can have systemic effects but also localized effects in developing organisms. These are typically mediated by hormone signals in the circulating haemolymph which carry the information about the external environment to the developing tissues. However, not all organs and groups of cells within organs have equal sensitivities to the external cues and internal signals. The compartmentalization of these effects reflects what has been called phenotypic integration to imply tight connections between traits, or phenotypic independence to refer to connections that are readily uncoupled (cf. Ketterson *et al.* 2009).

The present study identified such differing modes of connections for different aspects of butterfly wing patterns in relation to external temperature and to internal levels of ecdysone. With our systematic analysis of multiple traits in different temperature and hormone contexts (Figures 2.1 and 2.2), we have: 1) identified which traits are and which are not responsive to temperature during development (Figure 2.3), and to changes in ecdysone levels in early pupal life (Figure 2.4), 2) identified which of the sensitive traits respond in concert to each of the cues, and shown that these groupings are not the same for both types of cues (Figure 2.5), and finally 3) revealed that the mechanism for spatial compartmentalization of the responses does not reflect the spatial or temporal compartmentalization of the receptor for the internal signal (Figure 2.6).

Overview of the effects of developmental temperature and ecdysone manipulations on plastic wing patterning

We found unexpected differences between sensitivity to temperature and to hormone, both in terms of traits that are responsive versus those that are unresponsive, and also in terms of the traits that respond in a coordinated manner (Figure 2.5). In relation to the effects of external temperature on wing patterning, we showed that all color traits increase in size with increasing temperature (Figure 2.3) with the exception of the rings of a single eyespot (Figure 2.3D, Figure 2.5A1 and C) previously shown to be under sexual selection in males (Prudic *et al.* 2011). Among the temperature-sensitive eyespot traits, we found that all color elements on the forewing respond in the same fashion and differently from all but one color element on the hindwing (Annex 2.5: Figure 2.S2A, summarized in Figure 2.5A). The contrast between fore- and hindwing is consistent with the hypothesis that traits on autonomously-developing organs are more loosely integrated than traits on the same organ.

In relation to the effect of increasing hormone levels in early pupal life, we showed that only ventral color patterns, known to be associated to seasonally-plastic strategies for avoiding predators, responded (Figure 2.4, Figure 2.5B1 and C). Among the hormone-responsive eyespot traits, we found that rings of the same color respond in concert and in a pattern distinct from rings of other color (Annex 2.5: Figure 2.S2B, summarized in Figure 2.5B). This contrast is not consistent with the hypothesis that all rings of the same eyespot show similar sensitivity to hormone levels because they are all specified by a morphogen gradient originating from the eyespot center expressing hormone receptor (Koch *et al.* 2003). We further show that the spatial compartmentalization of hormone effects is not due to the spatial compartmentalization of the levels of hormone receptor protein (Figure 2.6), as had been suggested (Brakefield *et al.* 1998). Overall, our results point at complex interactions between the environmental cues that induce developmental plasticity and the internal signals that carry information about those cues to the developing tissues.

Sensitivities to external cues and internal signals are shaped by and impact phenotypic evolution

The coordinated trait sensitivities are properties of development that may have been favored by selection; for example, because it is important for fitness that traits change in concert. However, they may also be properties of development that are selectively

neutral (i.e. it is irrelevant whether or not traits develop in concerted fashion) or even evolutionarily constrained (i.e. it could be advantageous for traits to change independently but the way they develop makes that difficult (Maynard-Smith *et al.* 1985). The integration between traits can be a factor constraining future responses to selection if integrated traits are selected to change in opposite ways (evolutionary constraint hypothesis (Ketterson *et al.* 2009, Hau 2007). On the other hand, having traits responding independently to systemic hormone or external input can allow more rapid evolution of new arrangements of traits (evolutionary potential hypothesis, Hau 2007). It has been proposed that trait "reorganization" produced by exposure to novel environmental conditions can lead to the production of new phenotypic variants and differences between species, through a process that has been called developmental recombination (West-Eberhard 2005).

To fully understand this type of phenomenon it will be necessary to expand on studies such as ours. It is fundamental to combine the analysis of how different traits are integrated in their response to internal and external cues with an analysis of the mechanisms of differences in response to those cues and the ecological implications of changes in individual traits. In nature, the integration of all levels of information is further complicated by the fact that the developmental environment is more complex than one single changing cue, the phenotype is more than one particular trait, and the selective environment presents more than one ecological challenge.

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ANNEXES

All six annexes can be found at http://www.biomedcentral.com/1741-7007/12/97/additional.

ANNEX 2.1 - Summary of ANOVA and lsmeans pairwise comparison **r**esults to test the effect of temperature on wing traits of un-injected individuals (cf. Figure 2.3). http://www.biomedcentral.com/content/supplementary/s12915-014-0097-x-s1.pdf

ANNEX 2.2 - Summary of statistical analyses for wing trait values upon early and late control and hormone injections. This file supports results in Figure 2.4 and contains Tables 2.S1 (for early injections) and 2.S2 (for late injections) displaying sample sizes, mean and standard error of the re-scaled trait values, difference between hormone and control values (before and after bootstrap), as well as the p-values for the statistical significance of those differences.
http://www.biomedcentral.com/content/supplementary/s12915-014-0097-x-s2.pdf

ANNEX 2.3 - Hormone injection phenocopies effects of higher developmental temperature. This figure shows the extent to which hormone manipulations at lower temperatures increase trait areas to levels characteristic of higher temperatures. http://www.biomedcentral.com/content/supplementary/s12915-014-0097-x-s3.pdf

ANNEX 2.4 - Summary of ANOVA results to test the effect of temperature and injection treatment on the levels of 20E at two developmental time point (compare with Figure 2.1B).
http://www.biomedcentral.com/content/supplementary/s12915-014-0097-x-s4.pdf

ANNEX 2.5 - Patterns of coordinated response to external and internal signals. This figure illustrates which traits responded in concert and in contrast to either the temperature treatment (compare with Figure 2.3) or the hormone manipulations (compare with Figure 2.4) and shows in detail the findings summarized in Figure 2.5. http://www.biomedcentral.com/content/supplementary/s12915-014-0097-x-s5.pdf

ANNEX 2.6 - Principal components analysis (PCA) for variation in eyespot traits, separately for non-injected individuals (compare with Figure 2.3) and for hormone manipulations (compare with Figure 2.4). This file contains the material and methods,

figures 2.S3 and 2.S4, as well as results and discussion of the PCA. http://www.biomedcentral.com/content/supplementary/s12915-014-0097-x-s6.pdf

REFERENCES

Allen CE, Beldade P, Zwaan BJ, Brakefield PM (2008) Differences in the selection response of serially repeated color pattern characters: Standing variation, development, and evolution. *BMC Evolutionary Biology* **8**, 94.

Beldade P, Brakefield PM (2002) The genetics and evo–devo of butterfly wing patterns. *Nature Review Genetics* **3**, 442–452.

Beldade P, Koops K, Brakefield PM (2002a) Developmental constraints versus flexibility in morphological evolution. *Nature* **416**, 844–847.

Beldade P, Koops K, Brakefield PM (2002b) Modularity, individuality, and evo-devo in butterfly wings. *Proceedings of the National Academy of Sciences USA* **99**, 14262–14267.

Beldade P, Brakefield PM, Long AD (2002c) Contribution of Distal-less to quantitative variation in butterfly eyespots. *Nature* **415**, 315–318.

Beldade P, Brakefield PM (2003) Concerted evolution and developmental integration in modular butterfly wing patterns. *Evolution & Development* **5**, 169–179.

Beldade P, Mateus ARA, Keller RA (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology* **20**, 1347–1363.

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* **57**, 289–300.

Brakefield PM, French V (1995) Eyespot Development on Butterfly Wings: The Epidermal Response to Damage. *Developmental Biology* **168**, 98–111.

Brakefield PM, French V (1995) Eyespot Development on Butterfly Wings: The Focal Signal. *Developmental Biology* **168**, 112–123.

Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden P, Monteiro A, French V, Carroll S (1996) Development, plasticity and evolution of butterfly eyespot patterns. *Nature* **384**, 236–242.

Brakefield PM, Kesbeke F, Koch PB (1998) The regulation of phenotypic plasticity of eyespots in the butterfly Bicyclus anynana. *American Naturalist* **152**, 853–860.

Brakefield PM, French V (1999) Butterfly wings: the evolution of development of color patterns. *Bioessays* **21**, 391–401.

Brakefield PM, Beldade P, Zwaan BJ (2009) The African butterfly Bicyclus anynana: a model for evolutionary genetics and evolutionary developmental biology. In Emerging Model Organisms: A Laboratory Manual. Edited by Behringer RR, Johnson AD, Krumlauf RE, pp. 281-312. New York: Cold Spring Harbor Laboratory Press.

Breuker CJ, Brakefield PM (2002) Female choice depends on size but not symmetry of dorsal eyespots in the butterfly Bicyclus anynana. *Proceedings of the Royal Society B: Biological Sciences* **269**, 1233–1239.

Cheverud JM (1996) Developmental Integration and the Evolution of Pleiotropy. *American Zoologist* **36**, 44–50.

Conceição IC, Long AD, Gruber JD, Beldade P (2011) Genomic sequence around butterfly wing development genes: annotation and comparative analysis. *PLoS One* **6**, e23778.

David JR, Gilbert P, Pla E, Petavy P, Karan D, Moreteau B (1998) Cold stress tolerance in Drosophila: analysis of chill coma recovery in D. melanogaster. *Journal of Thermal Biology* **23**, 291–299.

Efron B, Tibshirani RJ (1993) An Introduction to the Bootstrap. New York: Chapman & Hall; 1993.

ffrench-Constant RH (2012) Butterfly wing colors are driven by the evolution of developmental heterochrony. Butterfly wing colors and patterning by numbers. *Heredity* **108**, 592–593.

Gilbert SF, Epel D (2009) Ecological Developmental Biology: Integrating Epigenetics, Medicine, and Evolution. Massachusetts: Sinauer Associates, Inc.

Guthrie HA, Brown ML (1968) Effect of severe undernutrition in early life on growth, brain size and composition in adult rats. *Journal of Nutrition* **94**, 419–426.

Hau M (2007) Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. *Bioessays* **29**, 133–144.

Keller RA, Peeters C, Beldade P (2014) Evolution of thorax architecture in ant

castes highlights trade-off between flight and ground behaviors. *eLife* **3**, e01539.

Ketterson ED, Atwell JW, McGlothlin JW (2009) Phenotypic integration and independence: Hormones, performance, and response to environmental change. *Integrative & Comparative Biology* **49**, 365–379.

Koyama T, Mendes CC, Mirth CK (2013) Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects. *Frontiers in Physiology* **4**, 263.

Koch PB, Brakefield PM, Kesbeke F (1996) Ecdysteroids control eyespot size and wing color pattern in the polyphenic butterfly Bicyclus anynana (Lepidoptera: Satyridae). *Journal of Insect Physiology* **42**, 223–230.

Koch PB, Lorenz U, Brakefield PM, ffrench-Constant RH (2000) Butterfly wing pattern mutants: developmental heterochrony and co-ordinately regulated phenotypes. *Development Genes & Evolution* **210**, 536–544.

Koch PB, Merk R, Reinhardt R, Weber P (2003) Localization of ecdysone receptor protein during color pattern formation in wings of the butterfly Precis coenia (Lepidoptera: Nymphalidae) and co-expression with Distal-less protein. *Development Genes & Evolution* **212**, 571–584.

Miura T (2005) Developmental regulation of caste-specific characters in social-insect polyphenism. *Evolution & Development* **7**, 122–129.

Maynard Smith J, Burian R, Kauffman S, Alberch J, Campbell B, Goodwin R, Lande R, Raup D, Wolpert L (1985) Developmental constraints and evolution. *The Quarterly Review of Biology* **60**, 265–287.

Nijhout HF (2003) Development and evolution of adaptive polyphenisms. *Evolution & Development* **5**, 9–18.

Nijhout HF, Cinderella M, Grunert LW (2014) The development of wing shape in Lepidoptera: mitotic density, not orientation, is the primary determinant of shape. *Evolution & Development* **16**, 68–77.

Oliver JM, Robertson KA, Monteiro A (2009) Accommodating natural and sexual selection in butterfly wing pattern evolution. *Proceedings of the Royal Society B: Biological Sciences* **276**, 2369–2375.

Oliver JC, Ramos D, Prudic KL, Monteiro A (2013) Temporal Gene Expression Variation Associated with Eyespot Size Plasticity in Bicyclus anynana. *PLoS One* **8**, e65830.

Oostra V, de Jong MA, Invergo B, Kesbeke F, Wende F, Brakefield PM, Zwaan BJ (2011) Translating environmental gradients into discontinuous reaction norms via hormone signaling in a polyphenic butterfly. *Proceedings of the Royal Society B: Biological Sciences* **278**, 789–797.

Oostra V, Mateus ARA, van der Burg KR, Piessens T, van Eijk M, Brakefield PM, Beldade P, Zwaan BJ (2014) Ecdysteroid hormones link the juvenile environment to alternative adult life histories in a seasonal insect. *American Naturalist* **184**, E79-E92.

Prudic KL, Jeon C, Cao H, Monteiro A (2011) Developmental plasticity in sexual roles of butterfly species drives mutual sexual ornamentation. *Science* **331**, 73–75.

R-Core-Team (2012) R - A Language and Environment for Statistical Computing.

Richards GP (1981) Insect hormones in development. *Biological Reviews* **56**, 501–549.

Saenko SV, Brakefield PM, Beldade P (2010) Single locus affects embryonic segment polarity and multiple aspects of an adult evolutionary novelty. *BMC Biology* **8**, 111.

Saenko SV, Marialva MS, Beldade P (2011) Involvement of the conserved Hox gene Antennapedia in the development and evolution of a novel trait. *EvoDevo* **2**, 9.

Schlichting CD, Pigliucci M (1998) Phenotypic Evolution: A Reaction Norm Perspective. Massachusetts: Sinauer Associates, Inc.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji - an open source platform for biological image analysis. *Nature Methods* **9**, 676–682.

Schwander T, Lo N, Beekman M, Oldroyd BP, Keller L (2010) Nature versus nurture in social insect caste differentiation. Trends in *Ecology & Evolution* **25**, 275–282.

Shingleton AW, Estep CM, Driscoll MV, Dworkin I (2009) Many ways to be small: different environmental regulators of size generate distinct scaling relationships in Drosophila melanogaster. *Proceedings of the Royal Society B: Biological Sciences* **276**, 2625–2633.

Simpson SJ, Sword GA, Lo N (2011) Polyphenism in insects. *Current Biology* **21**, 738–749.

Tang HY, Smith-Caldas MH, Driscoll MV, Salhadar S, Shingleton AW (2011) FOXO regulates organ-specific phenotypic plasticity in Drosophila. *PLoS Genetics* **7**, e1002373.

Wagner GP (1996) Homologues, Natural Kinds and the Evolution of Modularity. *American Zoology* **36**, 36–43.

Westerman EL, Chirathivat N, Schyling E, Monteiro A (2014) Mate preference for a phenotypically plastic trait is learned, and may facilitate preference-phenotype matching. *Evolution* **68**, 1661–1670.

West-Eberhard MJ (2005) Developmental plasticity and the origin of species differences. *Proceedings of the National Academy of Sciences USA* **102**, 6543–6549.

Wolfram S (1996) Mathematica 3.0 User's Guide. New York: Cambridge University Press.

Zijlstra WG, Steigenga MJ, Koch PB, Zwaan BJ, Brakefield PM (2004) Butterfly selected lines explore the hormonal basis of interactions between life histories and morphology. *American Naturalist* **163**, 76–87.

CHAPTER 3. ECDYSTEROID HORMONES LINK THE JUVENILE ENVIRONMENT TO ALTERNATIVE ADULT LIFE HISTORIES IN A SEASONAL INSECT

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This chapter has been published as Oostra V[1,3], Mateus ARA[1,2], Van der Burg K[1], Piessens T[1], Van Eijk M[1], Brakefield PM[1,3], Beldade P[1,2] & Zwaan B[4] (2014) Ecdysteroid hormones link the juvenile environment to alternative adult life histories in a seasonal insect. *American Naturalist* **184**, E79-92. In this paper I was involved designing and performing the first part of the experiment and in the supervision of Thomas Piessens´s bachelor thesis where he analyzed the effects of ecdysteroids in development time and resting metabolic rate. I was also involved in the general writing and submission of the paper.

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ABSTRACT

The conditional expression of alternative life strategies is a widespread feature of animal life, and a pivotal adaptation to life in seasonal environments. To optimally match suites of traits to seasonally changing ecological opportunities, animals living in seasonal environments need mechanisms linking information on environmental quality to resource allocation decisions. The butterfly *Bicyclus anynana* expresses alternative adult life histories in the alternating wet and dry seasons of its habitat, as end points of divergent developmental pathways triggered by seasonal variation in pre-adult temperature. Pupal ecdysteroid hormone titers are correlated with the seasonal environment, but whether they play a functional role in coordinating the coupling of adult traits in the alternative life histories is unknown. Here, we show that manipulating pupal ecdysteroid levels is sufficient to mimic in direction and magnitude the shifts in adult reproductive resource allocation normally induced by

seasonal temperature. Crucially, this allocation shift is accompanied by changes in ecologically relevant traits, including timing of reproduction, lifespan and starvation resistance. Together, our results support a functional role for ecdysteroids during development in mediating strategic reproductive investment decisions in response to predictive indicators of environmental quality. This study provides a physiological mechanism for adaptive developmental plasticity, allowing organisms to cope with variable environments.

KEYWORDS

Bicyclus anynana, Developmental plasticity, Diapause, Hormonal regulation, Life history, Polyphenism, Resource allocation, Seasonal adaptation, 20-hydroxyecdysone

INTRODUCTION

Understanding how animals cope with the seasonal fluctuations in environmental quality that characterize many temperate and tropical habitats is a key challenge in evolutionary ecology, and an important requirement if we want to predict ecological responses to climate change (Hofmann & Todgham 2010, Visser *et al.* 2010, Meylan *et al.* 2012). To optimally match suites of traits - i.e. the alternative life histories - to seasonally changing ecological opportunities, animals living in seasonal environments need mechanisms linking information on environmental quality to resource allocation decisions. In many animals, hormones provide such mechanisms (Nijhout 2003, Beldade *et al.* 2011, Simpson *et al.* 2011). They play crucial regulatory roles in transducing indicators of seasonal progression, for instance temperature or photoperiod, into adaptive alterations of the phenotype, such as timing of reproduction or preparation for diapause (e.g. Denlinger 2002, Dawson 2008, Brakefield & Zwaan 2011). These same hormonal mechanisms are also involved in the regulation of phenotypic plasticity when the environmental stimulus is not (directly) related to seasonality, such as crowding (e.g. in crickets and locusts; Simpson & Sword 2009, Zera 2009), nutrition (e.g. in nematodes, social insects and beetles; Smith *et al.* 2008, Sommer & Ogawa 2011, Emlen *et al.* 2012), or a combination of stimuli (e.g. in aphids; Brisson 2010). Understanding seasonal adaptations from an evolutionary perspective will require combining a detailed dissection of hormonal mechanisms of plasticity with ecological experiments aimed at establishing the relationships between these mechanisms and fitness in the field (Zera *et al.* 2007, Visser *et al.* 2010, Beldade

et al. 2011, Braendle *et al.* 2011, Gilbert 2012). However, addressing seasonal plasticity in an integrative way, from the environmental sensitivity, the hormonal changes, the sensitivity of the target phenotype to the hormone, through to the ecological relevance of the altered phenotype, is not possible in many systems. Here, we take such an approach and study seasonal adaptation in the butterfly *Bicyclus anynana* from the developmental and hormonal mechanism through to the alternative life history strategies relevant for natural populations.

The East African butterfly *B. anynana* expresses distinct life strategies in each season. During the warm wet season, larval and adult food is plentiful, larvae develop fast and adults live active lives with rapid reproduction and relatively short lifespans. In contrast, during the cool dry season characterized by no larval resources and adult food scarcity, adults display a higher investment in body reserves, have longer lifespans and postpone reproduction (Brakefield & Reitsma 1991, Brakefield & Zwaan 2011). These phenotypic differences are determined by the seasonal temperatures that the larvae and pupae experience during development, with a high temperature signaling the wet season and a decline to lower temperatures predicting the approaching dry season (Brakefield & Reitsma 1991). In the laboratory several aspects of these alternate life histories can be induced by development at different temperatures (Fischer *et al.* 2003, Pijpe *et al.* 2007, Steigenga & Fischer 2007, de Jong *et al.* 2010). Recently, we showed that females reared at high temperatures (wet season conditions) develop a relatively larger abdomen compared to those reared at low temperatures (dry season conditions). This response is discontinuous, with a threshold at an intermediate temperature (Oostra *et al.* 2011). Resting metabolic rate (RMR) in young adults is also affected by developmental temperature: butterflies developed at low temperatures have a higher RMR as adults, irrespective of adult temperatures (Pijpe *et al.* 2007, Oostra *et al.* 2011). The proximate mechanisms linking pre-adult temperatures to adult phenotypes are unknown, but previous observations suggest an involvement of ecdysteroid hormones during the pupal stage. Seasonal temperatures experienced during larval development drive dynamics of pupal ecdysteroids, with an earlier peak in hormone concentration in pupae reared at high versus low temperatures (Koch *et al.* 1996, Brakefield *et al.* 1998). A detailed characterization of hormonal reaction norms showed that the shift in hormone dynamics is discontinuous, with a similar shape and identical threshold temperature as the phenotypic reaction norm for female abdomen size (Oostra *et al.* 2011). Together,

these correlative studies suggested that ecdysteroid signaling is a regulator of the developmental plasticity in life history.

The first aim of the present study was to establish the extent to which pupal ecdysteroids play a functional role in fully inducing the alternative life histories in response to developmental temperature. We approached this question by manipulating ecdysteroids in pupae reared at three different temperatures spanning the range of natural seasonal environments (Brakefield & Reitsma 1991), and then monitoring the phenotypic effects for a suite of seasonally plastic traits: 1) pupal development time, 2) adult RMR, 3) allocation of adult body mass to abdomen, and 4) adult fat content.

The second aim of this study was to assess windows of hormone sensitivity during the pupal stage. In our previous experiments, we observed differences in thermal responses among traits putatively regulated by the same hormone, and suggested that these could arise as a result of differences among traits in their windows of sensitivity to that hormone (Oostra *et al.* 2011). To assess hormone sensitivity across time, a pupa was injected at one of four separate time points, representing different stages of the natural dynamics in ecdysteroid concentrations during the pupal stage (Brakefield *et al.* 1998, Zijlstra *et al.* 2004, Oostra *et al.* 2011).

Our third goal was to test, in an independent follow-up experiment, the ecological consequences of any hormone-induced changes in morphology and physiology observed in the initial experiment. We again manipulated ecdysteroids, focusing on a single temperature and injection time point, and monitored effects on multiple aspects of adult fitness: 1) onset of oviposition, 2) early life fecundity, 3) egg size, 4) lifespan, and 5) starvation resistance.

In this study, we show that ecdysteroids are responsible for the temperature-induced seasonal developmental plasticity of allocation of body resources to the abdomen in *B. anynana* females. In addition, we demonstrate that the ecdysteroid-induced allocation changes between thorax and abdomen have consequences for fitness: pupal hormone injections accelerate onset of oviposition and increase egg size, but reduce fecundity later in life as well as lifespan. These results support a functional role for ecdysteroids in reproductive investment decisions during development in response to variation in environmental quality, and provide insight into mechanisms enabling organisms to persist in fluctuating environments.

MATERIAL AND METHODS

Experimental design

We first performed a full factorial experiment with three developmental temperatures and four injection time points. Immediately after hatching, larvae were divided over three temperature treatments: 19, 23 and 27°C. We recorded pupations to the nearest 15 minutes using time-lapse photography, excluded male pupae and assigned female pupae to one of four injection time points: 3, 16, 29 or 34 % of total pupal development time (DT). Total pupal development time in absence of hormone manipulation was strongly affected by temperature, with pupae reared at 19, 23 and 27°C developing on average in 356, 193, and 158 hours, respectively. For pupae reared at 19°C, the four injection time points thus correspond to 10h41', 56h58', 103h14' and 121h02' after pupation, for pupae at 23°C to 5h47', 30h53', 55h58' and 65h37', and for pupae at 27°C these time points corresponded to 4h44', 25h17', 45h49' and 53h43' after pupation. Previous data on natural ecdysone titers in absence of manipulations for the three temperatures allowed us to identify four time points representing relevant stages of the pupal ecdysteroid pulse: (i) overall low titers (3% DT), (ii) titers ascending for wet season but not for dry season (16%), (iii) titers descending for wet season but not for dry season (29%), and, (iv) titers descending for dry season and low for wet season (34%). At the latter three time points the natural titers differ between wet and dry season pupae (Oostra *et al.* 2011). Pupae were injected with either 20-hydroxyecdysone (20E) or control solutions, after which they were allowed to continue development and eclose individually at their respective larval temperatures. After eclosion, we measured resting metabolic rate (RMR) and abdominal dry weight and fat content in N = 15-45 per temperature per injection time point per injection treatment.

In the follow-up experiment, we reared larvae at 23°C, injected the pupae at 16% DT, and measured fecundity, lifespan and starvation resistance in the adult females (N = 50-80 per injection treatment). In both experiments, all larvae were derived from the same outbred *B. anynana* captive population and reared on young maize plants sprayed with an antifungal agent (see Brakefield *et al.* 2009 for rearing protocols).

Hormone injections

Fresh injection solutions were prepared daily by combining 107 µ 1x Ringer's physiological solution with 3 µ Vital Red dye (Fluka) and either 10 µ 100% ethanol (control treatments) or 10 µ 1 mg / ml 20E (Sigma) in 100% EtOH (hormone treatments). Using a 10 µ Hamilton micro syringe with a 0.3 mm needle, we injected pupae laterally between the 4th and 5th abdominal segments, with 3 µ injection solution (0 or 0.25 µg 20E for the control and hormone treatments, respectively), injecting each female only once. To avoid easily induced pharmacological effects of exogenous hormone applications it is critical that titers of injected hormones are well within physiological ranges, and this can only be established by knowledge on natural hormone concentrations (Zera 2007). Therefore, we based the amount of hormone to inject on previous studies on pupal ecdysteroids in *B. anynana*, which yielded detailed knowledge on natural 20E concentrations throughout the pupal stage as well as dose-response curves for mortality (Koch *et al.* 1996, Brakefield *et al.* 1998, *Zijlstra et al.* 2004, Oostra *et al.* 2011). These data also allowed us to inject at biologically relevant time points, when ecdysteroids are active and their titers differ between seasonal morphs (see above). In addition, we quantified how 20E hormone injections affect internal 20E titers and found that these levels are similar to the natural 20E concentrations during the early pupal stage, and much lower than peak concentrations (Mateus *et al.* 2014). Thus, our hormone manipulations did not raise 20E titers to unnatural levels.

Measurements of phenotypic responses

a. First experiment: pupal development time, RMR, abdominal dry weight and fat content

All pupae were weighed to the nearest 0.1 mg within 36 hours of pupation. In the first experiment, a subset of pupae (ca. 20%) was kept separately to measure pupal development time with 15 minutes precision. We monitored these pupae towards the end of the pupal period and recorded new eclosions every 15 minutes by time-lapse photography. One day after eclosion, we measured RMR for each female as the individual rate of CO_2 respiration (ml per hour) over a period of 20 min, following Pijpe *et al.* (2007). All RMR measurements were done at 27°C during the dark phase of the diurnal cycle. Next, abdomens were cut off to measure their dry weight, extract

total fat (triglyceride and free fatty acids) and measure fat-free dry weight following Oostra *et al.* (2011). Fat content was calculated by subtracting the fat-free dry weight from the initial dry mass.

b. Second experiment: fecundity, lifespan and starvation resistance

One day after eclosion, we weighed each adult female to the nearest 0.1 mg and introduced her into a mating cage with 10-30 virgin males (3-10 days old), keeping the ratio of females to males in these cages below one. We inspected the cages every 15 minutes and separated mating pairs into cylindrical oviposition pots. After each mating had finished, we removed the male and provided the female with *ad libitum* food and a fresh cutting of *Oplismenus sp.* grass for oviposition. After 72 hours we moved the female to a new pot. This was repeated three times, yielding a total of four consecutive egg measurement periods with age classes of: 2-4, 5-7, 8-10, and 11-13 days. After each period, we counted the total number of eggs in the oviposition pot. To estimate egg size, we photographed the spherical eggs against a black background using a Leica DC200 digital still camera connected to a Leica MZ12 stereo microscope (3.2X magnification). On every image, we measured egg area as a measure of egg size (following Fischer *et al.* 2003), using an automated macro in ImageJ software. After four egg measurement periods covering the 12 days after mating, we transferred females to larger cages, with a maximum of 10 females per cage, provided oviposition plants and food *ad libitum*, and monitored survival daily. Females that laid only unfertilized eggs were excluded from the analysis.

Each day, we separated a fraction of newly eclosed females and excluded them from the fecundity assay. Instead, we kept them virgin, introduced them into larger cages with a maximum of 15 females per cage, and provided them with *ad libitum* access to water (wet cotton) but not food to record starvation resistance (SR). We scored and removed dead females twice a day.

Statistical analyses

In the first experiment we initially analyzed data using a three-way analysis of variance (ANOVA) for each phenotypic trait, with rearing temperature, injection time point and hormone treatment as fixed variables (see Annex 3.3). To identify time point-specific treatment effects, we subsequently analyzed, in cases where injection

time point interacted significantly with hormone treatment, each time point separately using two-way ANOVAs, with rearing temperature and hormone treatment as fixed effects (see Annex 3.5). Prior to the ANOVAs, pupal development time was natural log transformed. We analyzed RMR, abdomen dry weight, abdomen fat content and abdomen fat-free dry weight first in separate linear regressions models with pupal mass as the only predictor variable (see Annex 3.4), and subsequently used the residuals of these regressions as dependent variables in the ANOVAs. Post hoc comparisons between 20E and control treated females at specific temperatures were performed with Tukey's honest significant differences (HSD) tests.

In the second experiment, fecundity was strongly non-normally distributed during the first egg measurement period (age 2-4 days), as a large fraction of females had not yet laid any eggs in this period. Therefore, we chose to analyze this first period separately, treating fecundity as a categorical variable: females either had or had not started to lay eggs in this period. Numbers of females in each category were compared between injection treatments using a χ^2 test. For the three subsequent egg-laying periods (ages 5-13 days), we analyzed fecundity using a repeated measures general linear model (GLM) with injection treatment and age as fixed variables, and individual as random variable. In order to obtain p-values for each main effect, we constructed a model without the main effect and compared it to the full model with a likelihood-ratio test. For specific comparisons at each age class between 20E and control treated females, we obtained p-values using a Markov Chain Monte Carlo method (Baayen 2011). We also analyzed egg size using repeated measures GLM with injection treatment and age as fixed variables, and individual as a random variable. We analyzed lifespan and starvation resistance using a Cox proportional hazard model with adult mass as covariate and injection treatment as fixed variable; age at death was used as the dependent variable. All analyses were performed in R (R Development Core Team 2012) with packages *survival* (Therneau 2012), *lme4* (Bates *et al.* 2011) and *languageR* (Baayen 2011).

RESULTS

Ecdysteroids accelerate pupal development and increase adult mass allocation to abdomen

20E treatment affected pupal development time differently depending on the time of injection, as indicated by a significant interaction between injection time point and treatment in the three-way ANOVA (Annex 3.3). When pupae were injected at 3 and 16% development time (DT), 20E treatment induced a substantial acceleration of pupal development, while this was not the case at 29 nor at 34 % DT (Figure 3.1, Annex 3.5). Pupae reared at 27°C showed the weakest response to early 20E treatment compared to pupae reared at the other temperatures. At this same temperature, 20E treatment at 34% DT had the reverse effect on these pupae: development was slowed rather than accelerated (Tukey's HSD $p < 0.0005$). No such effect was observed at the other temperatures. The overall acceleration in development upon injections earlier in development was due to a higher proportion of butterflies eclosing a full day or more earlier, and was not accompanied by a change in time of day at which they eclosed.

Relative abdomen mass was substantially increased after pupal 20E injection at 3 or 16%, but not at 29 or 34% DT (Figure 3.2, Annexes 3.3 and 3.5). This reveals a period of ecdysteroid sensitivity during development of the abdomen. The effect of 20E treatment on relative abdomen mass was similar in magnitude and direction to the effect of developmental temperature. In particular, 20E injected pupae reared at 19°C have a similarly sized abdomen as control injected pupae reared at 23°C, and 20E injected pupae reared at 23°C have a similarly sized abdomen as control injected pupae reared at 27°C (Figure 3.2, Annex 3.5). Thus, exogenous ecdysteroids phenocopy the temperature-induced seasonal differences in abdomen size.

We then asked whether this hormone-induced increase in abdomen mass was due to an increase in fat content, fat-free dry weight, or both. As was the case for total abdomen mass, the effect of 20E treatment on abdominal fat content depended on the timing of injections: fat content was higher in females injected as pupae with 20E compared to controls for manipulations at 3 and 16% DT, but not at 29 or 34% DT (Annexes 3.1, 3.3 and 3.5).

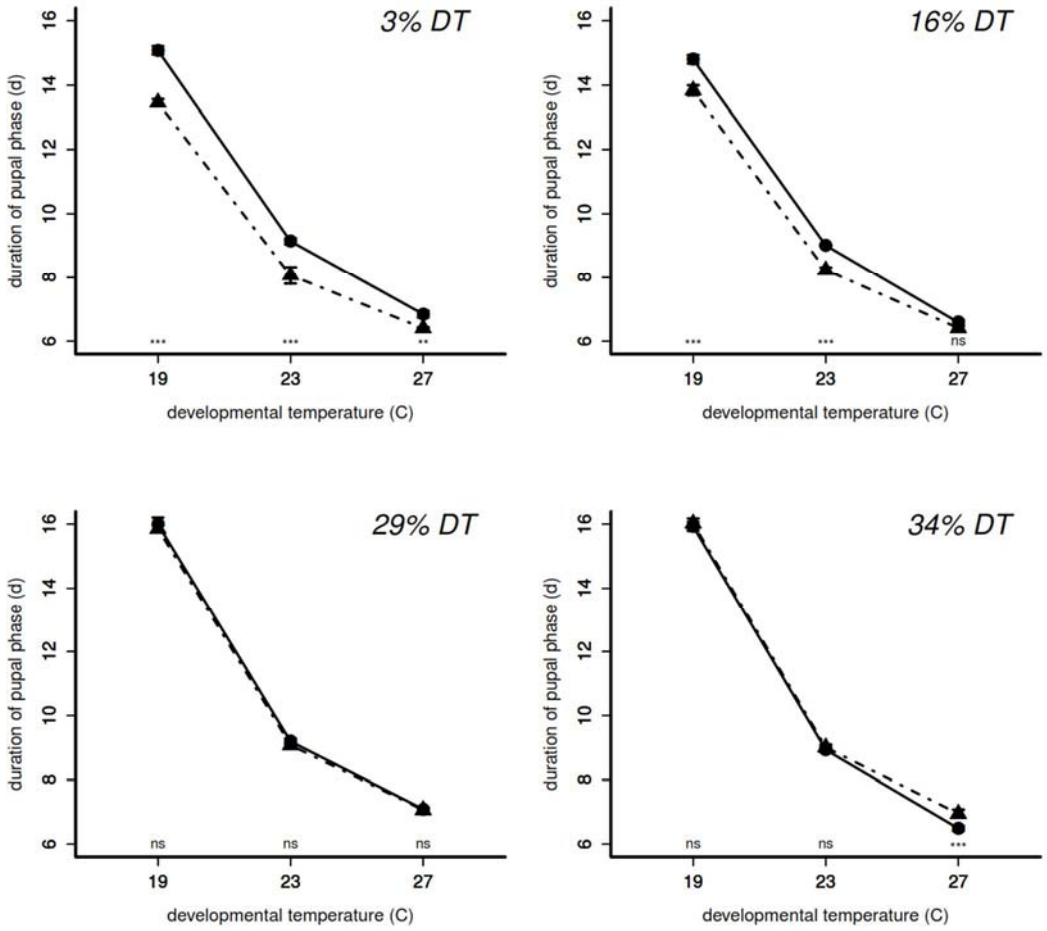

Figure 3.1 - Early but not late 20-hydroxyecdysone (20E) treatment accelerates pupal development. Duration of pupal stage (days, ±SEM) is strongly affected by developmental temperature, as indicated by the shape of reaction norms and large differences between extreme temperatures. In addition, pupae injected with 20E (triangles and dashed line) at 3 or 16% of pupal development time (DT) show significant acceleration of development in comparison with controls (circles and solid line; two-way ANOVA $p < 0.00001$), while those injected at 29 or 34 % DT show no such effect. Late injections (34% DT) decelerate development, but only at 27°C (Tukey's HSD $p < 0.001$). See Annexes 3.3 and 3.5. Asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate significant differences between control and 20E treated animals; in the case of significant temperature by treatment interaction in two-way ANOVAs, p values from post-hoc Tukey's HSD are reported; when this interaction was not significant, the overall treatment effect of the two-way ANOVA is given. For pupae reared at 19°C, the four injection time points correspond to 10h41', 56h58', 103h14' and 121h02' after pupation, for pupae at 23°C to 5h47', 30h53', 55h58' and 65h37', and for pupae at 27°C these time points corresponded to 4h44', 25h17', 45h49' and 53h43' after pupation.

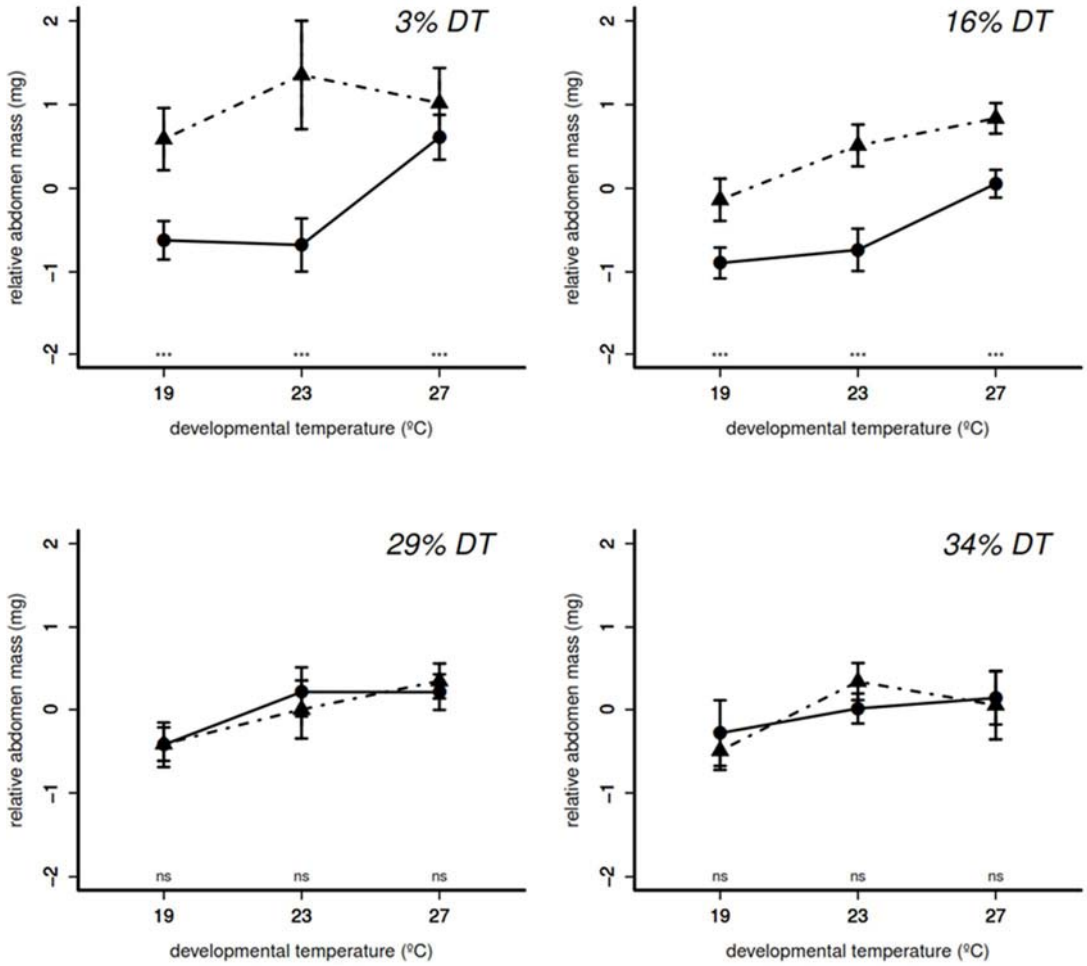

Figure 3.2 - Pupal ecdysteroids induce high, wet-season like allocation to abdomen mass. Mass-corrected abdomen dry weight (mg, see Annex 3.4) is significantly affected by developmental temperature with females reared at high temperatures (wet season conditions) having a larger abdomen. In addition, pupae injected with 20E (dashed line) at 3 or 16, but not at 29 or 34% DT, show a substantial increase in abdomen mass compared to controls (solid line), similar in magnitude and direction to the temperature effect (two-way ANOVA $p < 0.0005$). See Annexes 3.1, 3.2, 3.3 and 3.5.

In addition, at 3% DT we observed a significant interaction between treatment and temperature (Annex 3.5); pupae reared at 19 and 23°C showed a response to 20E (Tukey's HSD $p < 0.001$), whereas those at 27°C did not. Likewise, abdominal fat-free dry weight increased in response to pupal 20E injections, but again only when injected at 3 and 16% and not at 29 or 34% (Annexes 3.2, 3.3and 3.5). Considered together, we conclude that the increase in abdomen mass in the females injected with 20E as pupae in the earlier time points was due to an increase in both fat and non-fat

mass, with both traits showing an identical window of sensitivity to the 20E injections.

Developmental signature on adult RMR is not mediated by ecdysteroids

We found no evidence for a role for ecdysteroids in mediating the pre-adult temperature effect on adult RMR. As observed previously (Pijpe *et al.* 2007, Oostra *et al.* 2011), RMR corrected for body size (see Annex 3.4) was higher in females developed at lower (dry season) temperatures. However, we observed no significant effect of 20E treatment on size-corrected RMR for any of the four injection time points at any of the three temperatures (Figure 3.3, Annexes and 3.5).

Pupae show a limited window of sensitivity to ecdysteroid manipulation

Sensitivity of pupal development rate, abdomen dry weight and fat content to 20E treatment was not constant in time, as indicated by a significant effect on all of these traits of the interaction between treatment and injection time point (Annex 3.3). In particular, the traits were most strongly affected by injections at the two earlier time points (3 and 16% DT, Figures 3.1, 3.3, Annexes 3.1, 3.2 and 3.5), when natural Ecdysone titers are rising (Oostra *et al.* 2011, see also Material and Methods). In contrast, later in the pupal stage (29 and 34% DT), when natural Ecdysone titers are decreasing (Oostra *et al.* 2011), these traits showed little if any response to injections. Furthermore, this window of hormone sensitivity was affected by the temperature at which the pupae had developed. Pupae from 19°C or 23°C developed an enlarged abdomen with increased fat content and accelerated pupal development rate in response to 20E injections at both 3 and 16% DT. However, those reared at the wet season temperature of 27°C only showed increased abdominal fat content when injected at 16, not 3 % DT, and accelerated development when injected at 3, not 16% DT. In the same 27°C cohort (and not at 19 or 23°C), late injections at 34% DT had the reverse effect on rate of development compared to injections at 3 and 16% DT: development was slowed rather than accelerated.

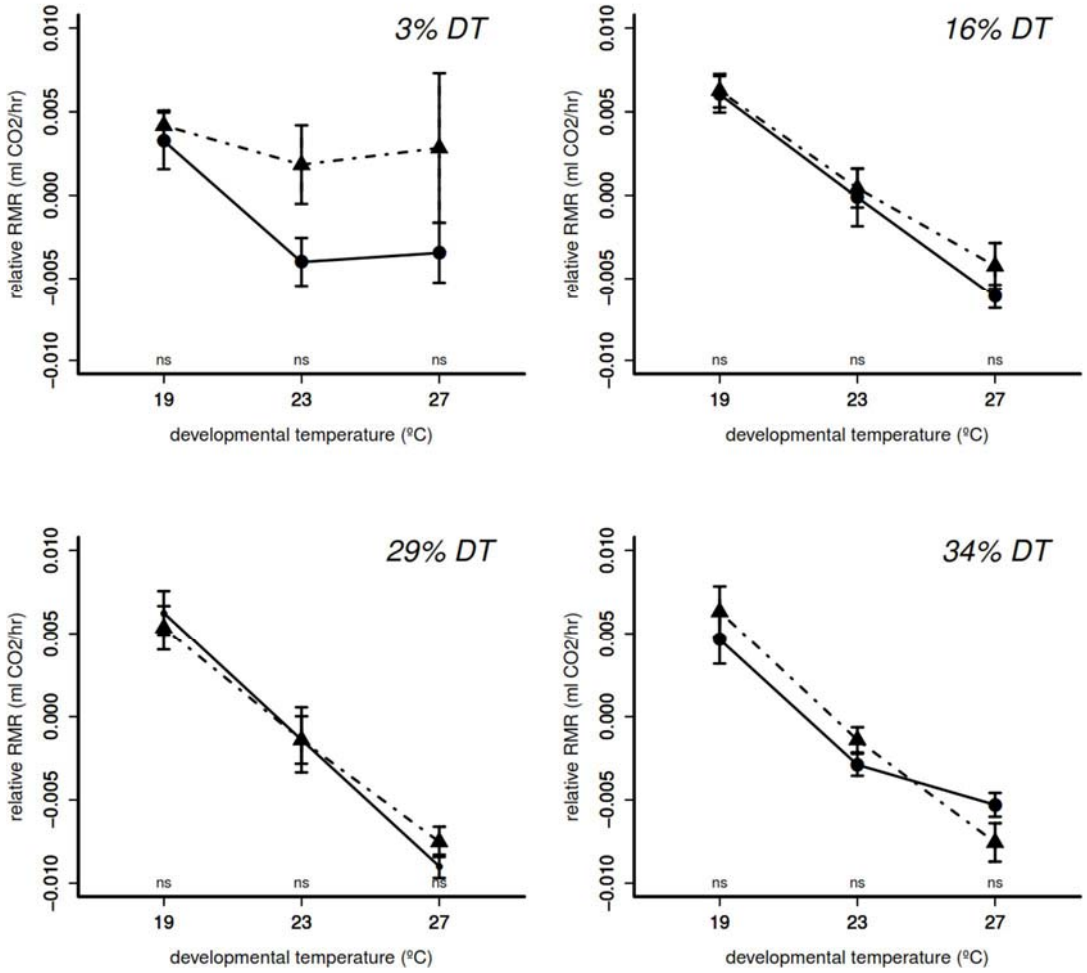

Figure 3.3 - Developmental temperature signature on adult resting metabolic rate (RMR) is not mediated by pupal ecdysteroids. Mass-corrected RMR (ml CO_2 hr^{-1}, see Annex 3.3) is significantly affected by developmental temperature with individuals reared at lower temperature having higher RMR (two-way ANOVA $p < 0.005$). However, 20E treatment in the pupal stage has no significant effect on RMR at any of the four injection time points (compare solid and dashed line reaction norms). See Annexes 3.1 and 3.5.

Pupal ecdysteroids affect reproductive schedule, lifespan and starvation resistance

To assess whether the observed induction of relatively larger, wet season-like abdomens by pupal ecdysteroid levels has fitness consequences for the adult life history, we reared an independent cohort of larvae at 23°C, injected females at 16% of pupal development time, and measured effects on adult performance. We focused on this temperature and time point because they revealed the largest effects of ecdysteroids on abdomen size in the first set of experiments (Figure 3.2).

After eclosion, females were mated and allowed to oviposit. In the first period of oviposition (age 2-4 days), not all females had started laying eggs. Among the control treated females, 35% had not laid their first egg during this period, while this percentage was less than half (17%) among the 20E treated individuals (Figure 3.4B). Thus, 20E treatment during the early pupal stage significantly accelerated the onset of first egg laying (χ^2 $p < 0.05$; Annex 3.6), resulting in a ca. 31% increase in mean number of eggs produced in this period (Figure 3.4A). Among those females that laid eggs in this period, there was no significant difference in mean number of eggs between the 20E and control treated group (Annex 3.6). This indicates that ecdysteroids probably do not increase the rate of egg production once it has started, but instead bring forward the onset of oviposition.

Later in life, after the peak in egg laying, the 20E treated females laid fewer eggs compared to control females (Figure 3.4A, Annex 3.6); at age 8-10 days the reduction was 9 % (MCMC $p = 0.19$, see Material and Methods), but in the final oviposition period that was monitored (age 11-13 days) the difference was more substantial (23%, MCMC $p < 0.005$). Although the total number of eggs produced in all four oviposition periods combined was 7% lower in the 20E treated females compared to controls, this effect was not significant (Annex 3.6). Thus, it appears that pupal 20E treatment, while accelerating the onset of oviposition, inflicts a fecundity cost later in life by accelerating the normal age-related decline in fecundity.

Since females can alter their egg size and number (Fischer *et al.* 2003), we wanted to know whether the decrease in later-life fecundity was offset by an increase in egg size. This was indeed the case: eggs of the 20E treated females were larger compared to control treated females (Figure 3.4C, Annex 3.6). However, this was only observed at age 8-10 days (MCMC $p < 0.05$) and to a lesser extent at age 11-13 days (MCMC $p = 0.07$).

After the final fecundity measurements, we monitored individual daily survival. Females treated with 20E as pupae lived, on average, 12% fewer days than control females (Figure 3.4D, Cox proportional hazard $p=0.06$; hazard ratio = 1.38, Annex 3.6). Splitting the females into two groups according to early reproductive status revealed that the negative effect of 20E treatment on lifespan was only significant for those females that had reproduced before the age of 4 days; the females that showed accelerated egg laying in response to 20E showed reduced lifespan (Cox proportional hazard $p < 0.05$, hazard ratio = 1.58, Annex 3.6), while those that did not

lay eggs in that period showed the same lifespan as control females. It appears that, in addition to reducing fecundity later in life (Figure 3.4A), ecdysteroid-induced acceleration in onset of oviposition (Figure 3.4B) inflicts a fitness cost on lifespan (Figure 3.4D).

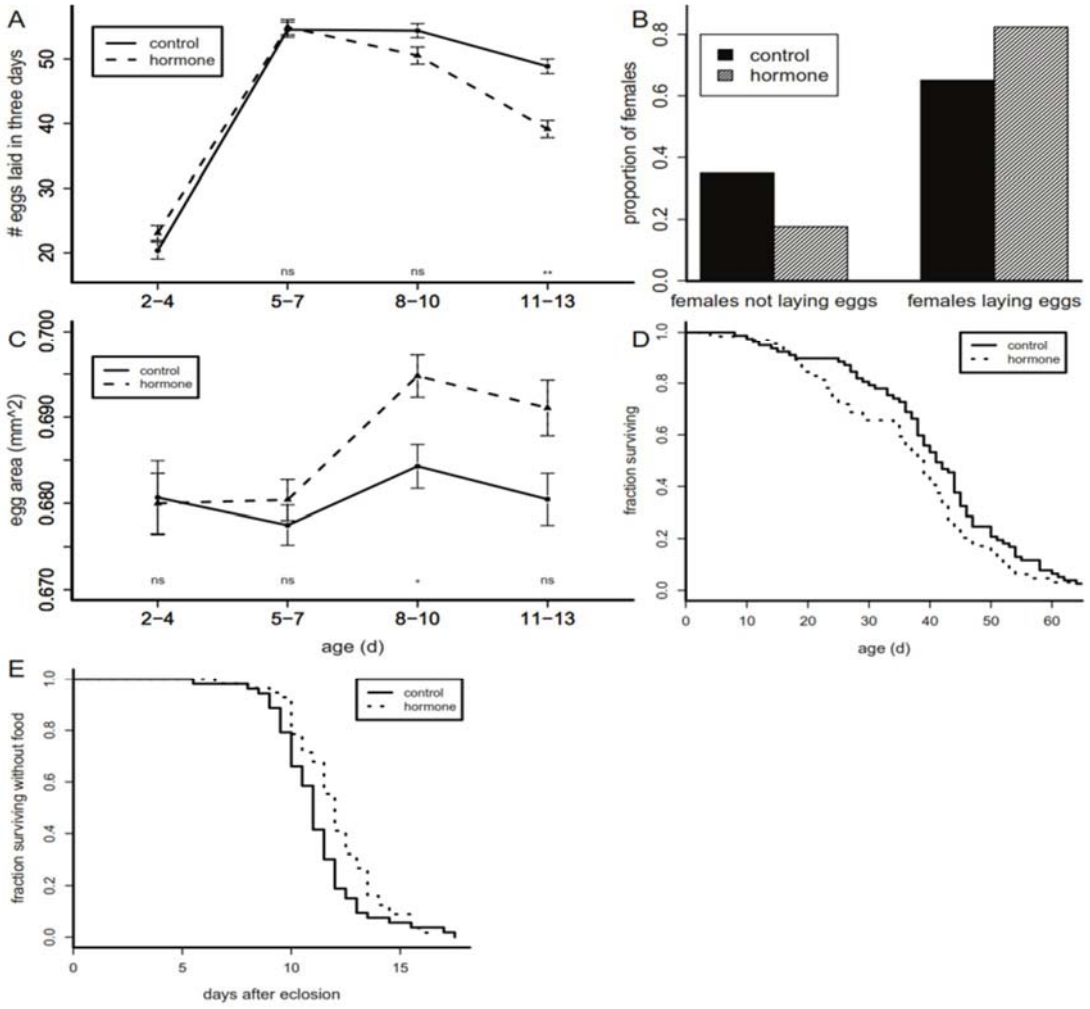

Figure 3.4 - Pupal ecdysteroids affect reproductive schedule, lifespan and starvation resistance. A) Female fecundity (number of eggs laid) is highly affected by female age ($p < 0.00001$ for likelihood ratio test (LRT) between model with and without age). In addition, adult females injected as pupa with 20E (dashed line) had lower fecundity compared to controls (solid line), but only later in life ($p < 0.001$ for LRT with and without treatment x age interaction). B) Pupal ecdysteroids accelerate onset of oviposition. Proportion of females that have already started laying eggs at age 4 days is significantly higher when injected as pupa with 20E (shaded bars) than when injected with control solution (solid bars; χ^2 $p < 0.05$). All females not laying eggs at age 4 days did lay eggs later in life. C) Pupal ecdysteroids induce increased egg size. Egg area (mm^2) is significantly affected by female age ($p < 0.00001$ for

likelihood ratio test (LRT) between model with and without age), and females injected as pupa with 20E (dashed line) lay larger eggs than control females (solid line), but only at age 8-10 days ($p < 0.05$ for LRT with and without treatment x age interaction). D) Pupal ecdysteroids reduce adult lifespan of mated females. Daily adult survival under *ad libitum* food is reduced in mated females injected as pupa with 20E (dotted line) compared to controls (solid line; Cox proportional hazard $p = 0.06$; hazard ratio = 1.38). Lifespan reduction was stronger for females that had started laying eggs before age 4 d (Cox proportional hazard $p < 0.05$; hazard ratio = 1.58) than for those that did not lay eggs before age 4 d (see Annex 3.6: Table A4). E) Pupal ecdysteroids enhance adult starvation resistance in virgin females. Daily adult survival without food is increased in virgin females injected as pupa with 20E (dotted line) compared to controls (solid line; Cox proportional hazard $p < 0.01$; hazard ratio = 0.68). See also Annex 3.6.

The increased allocation to abdomen mass in the ecdysteroid-injected females observed in the first experiment (Figure 3.2) could also have been related to aspects of adult performance other than fecundity. In particular, both non-fat and fat mass were increased in these females (Annexes 3.1 and 3.2) which could contribute to survival under starvation (Zwaan *et al.* 1991). To test this hypothesis, we measured starvation resistance (SR) in adult females from the cohort of larvae reared at 23°C and injected at 16% pupal DT. We found that 20E treated females survived, on average, *ca.* 1 day (8%) longer without food compared to the control treated females (Figure 3.4E, Cox proportional hazard $p < 0.01$, hazard ratio = 0.68). In addition, smaller females showed the largest increase in adult SR when injected with 20E (Cox proportional hazard $p < 0.05$ for mass-by-treatment interaction, Annex 3.6). This suggests that virgin females with an ecdysteroid-induced increased abdomen mass are able to use the increased abdominal resources to live longer when confronted with food stress.

DISCUSSION

Seasonal developmental plasticity in *B. anynana* involves a suite of morphological, physiological and life history traits co-varying across the seasons in response to developmental temperature. Previously, we observed a correlation between expression of some of these adult traits and ecdysteroid dynamics during the pupal stage. Here, we functionally test the involvement of these hormones in the developmental regulation of the alternative adult life histories. We manipulate ecdysteroids during pupal development, and observe significant shifts in adult reproductive resource

allocation, mimicking in direction and magnitude the seasonal phenotypic changes normally induced by temperature experienced during development. This reveals that pupal ecdysteroid hormone titers provide the causal link between the seasonal environment during development and allocation of adult mass to reproductive function. Crucially, these allocation changes are accompanied by changes in ecologically relevant adult performance traits, including timing of reproduction, egg size, lifespan and survival under starvation. Thus, ecdysteroids after pupation mediate strategic adult reproductive investment decisions in response to variation in the quality of the environment.

As reported previously for *B. anynana* (Koch *et al.* 1996, Zijlstra *et al.* 2004), exogenous ecdysteroids applied early in the pupal stage accelerate pupal development. In the present study, we included two additional, later injection time points and found no such hormone-induced acceleration later in the pupal stage (Figure 3.1, Annexes 3.3 and 3.5). Thus, as was the case for abdomen size (Figure 3.2), we observed a restricted window of sensitivity to hormone manipulations. In both cases, sensitivity was limited to the earliest 16% of the pupal stage. We have thus identified a critical period during which ecdysteroids are able to alter the developmental trajectory and ultimately the adult phenotype. This critical hormone-sensitive period is transient and occurs early in the pupal stage, when wet season pupae already have increasing natural ecdysteroid titers, while those of dry season pupae are still lower (Oostra *et al.* 2011). We chose our injection time points precisely because they represent the main stages in natural ecdysteroid dynamics (low, ascending, or descending titers), and at the three latest time points the seasonal morphs differ most in their ecdysone titers. Thus, in the wet season increasing natural ecdysteroid titers coincide with the hormone-sensitive period, whereas in the dry season the hormone-sensitive period passes with low ecdysteroid titers. In *B. anynana*, ecdysteroids can thus be considered to act as a developmental switch *sensu* Nijhout (2003). Such developmental switches have been identified for numerous other animals displaying alternative phenotypes (discussed in Hartfelder & Emlen 2011). For example, in *Araschnia levana* pupae destined to develop directly, an ecdysteroid-sensitive period coincides with a pulse of high ecdysteroid titers during early development. This same sensitive period occurs in pupae destined to go into diapause, but the ecdysteroid pulse occurs much later, after the critical period (Koch and Bückmann 1987). A similar temporal match or mismatch between ecdysteroid titers and ecdysteroid sensitivity determines development of

Junonia (*Precis*) *coenia* pupae into summer and autumn adult forms, respectively. With 25-56 hours after pupation, the hormone-senstive period in *B. anynana* is similar to that of *J. coenia* (28 - 48 hours; Rountree & Nijhout 1995), but shorter than that of *A. levana* (3 - 9 days after pupation; Koch & Bückmann 1987). As *B. anynana* belongs to a group of Lepidoptera in which oocytes mature after eclosion (Ramaswamy *et al.* 1997), and no vitellogenins are yet detectable in pupae or freshly eclosed females (Geister *et al.* 2008), the much earlier occurring ecdysteroid signaling is unlikely to directly affect adult reproductive function. Instead, the early developmental switch in *B. anynana* probably acts as a cascade switch (West-Eberhard 2003), in which the initial ecdysteroid-mediated decision sets in motion downstream alternative developmental pathways which ultimately produce the seasonal morphs. Such a scenario explains the lack of phenotypic response to our late injections (Figure 3.2). After the hormone-sensitive period, the downstream developmental pathways have already been initiated, and can no longer be modified by ecdysteroids.

It is likely that these downstream pathways involve other hormones, as studies in other insects show myriad interactions at a variety of life stages between ecdysteroids and other hormones (e.g. Shingleton *et al.* 2007). In particular, insulin-like peptides are expected to play an important role in the developmental pathways that regulate abdomen size. Thus, early ecdysteroid manipulations likely assert their ultimate phenotypic effects indirectly, by initiating alternative developmental pathways whose downstream mechanisms are unknown but likely involve other hormones.

Another mechanism by which ecdysteroids induce the alternate seasonal morphs in *B. anynana* may be changes in timing of developmental events (see Annex 3A). Both pupal development time and abdomen size showed the same window of hormone sensitivity. Furthermore, pupal development time and timing of ecdysteroid pulses in the pupal stage are genetically correlated (Zijlstra *et al.* 2004), and discrete variation in timing of ecdysteroid pulses in the pupal stage is phenotypically correlated with adult reproductive allocation (Oostra *et al.* 2011). In the wet season, an early ecdysteroid pulse coinciding with the sensitive period would accelerate development, resulting in an increased abdomen size and accelerated onset of oviposition. This is consistent with the well-known function of ecdysteroids as a developmental timer during the larval stage (Klowden 2007). In our experiment, pupal

development time was more strongly affected by the seasonal environment than by the hormonal manipulations (Figure 3.1), i.e. ecdysteroids did not fully phenocopy the temperature response. Temperature is known to have a major impact on rates of growth and development in ectotherms, independent of any adaptive plasticity and likely as a result of the direct effect of temperature on metabolic rate (Nylin & Gotthard 1998).

Developmental plasticity in *B. anynana* might also share components of its regulatory mechanisms with larval and pupal diapause expression in other insects, which has been linked to ecdysteroids (Denlinger 2002). In some cases, ecdysteroid titers are lower in diapausing larvae or pupae (e.g. Koch 1996, Munyiri & Ishikawa 2004), and in other cases exogenous ecdysteroid applications terminate diapause and induce the continuation of normal development (Arpagaus *et al.* 1986, Singtripop *et al.* 1999). In adult insects, ecdysteroids interplay with other hormones (in particular juvenile hormones) to regulate several aspects of female reproduction (Klowden 2007). For example, ovarian growth in young *Gryllus firmus* adults is positively correlated with ecdysteroid titers (Zera 2009). Mutant *Drosophila melanogaster* females with reduced ecdysteroid signaling show reduced rates of oocyte maturation or oviposition, as well as increased lifespan (reviewed in Schwedes & Carney 2012). Adult reproductive diapause in *D. melanogaster* females, characterized by arrested reproductive development and increased lifespan (see Schmidt 2011), can be terminated by ecdysteroid injection (Richard *et al.* 2001). Such a reproductive function of ecdysteroids in adult females is consistent with the increased abdomen size and accelerated onset of oviposition we observed in ecdysteroid-injected *B. anynana* females, suggesting some overlap in function between ecdysteroid signaling in the pupal and adult stages.

The environmental induction of alternative phenotypes consists not only of the developmental switch and subsequent cascade, but is preceded by a period of environmental sensitivity. During this period the developing organism senses and processes environmental cues which then yield the hormone-mediated decision between alternative pathways, as discussed above. The environmental sensitive period generally occurs much earlier in development, and it is well known that in seasonally plastic insects this period almost always occurs during the larval stage (Danks 1987, Nijhout 2003). Indeed, for *B. anynana* it has been shown for a long time that the

environmental induction of the seasonal adult wing patterns occurs mainly during the late larval stage (Kooi & Brakefield 1999).

In the ecdysteroid treated females, the onset of oviposition was accelerated, similar to the naturally induced wet season morph (Brakefield & Zwaan 2011). However, fecundity after peak egg laying was reduced, while the natural wet season morph shows generally higher fecundity throughout adult life. This was contrary to our initial expectation that the ecdysteroid treated females would be more wet season-like in all aspects of adult life history. Previously, it was shown that fecundity after peak egg laying is mainly determined by temperature during oviposition, and to a lesser extent by developmental temperature (e.g. Fischer *et al.* 2003). It thus seems likely that, unlike the onset of oviposition, later-life fecundity differences between the naturally occurring wet and dry season morph are not under control of ecdysteroid-mediated developmental plasticity but instead are determined by adult acclimation (Brakefield *et al.* 2007). The reduction in late-life fecundity observed in our experiments likely reflects a fitness cost of the accelerated early oviposition, which in the natural wet season morph would be masked by adult conditions. It remains to be tested whether other traits that commonly trade off with reproductive investment, such as flight ability (*cf.* Zera 2009), are also integrated into the hormone-mediated adult life history. One indication that this might indeed be the case is the observation that larval food stress-induced allocation to thorax at the expense of abdomen increases flight endurance in adults (Saastamoinen *et al.* 2010), which a modeling approach showed to be a potential adaptive response (Van den Heuvel *et al.* 2013).

In contrast to their effects on abdomen size, development time and adult reproductive strategy, exogenously applied ecdysteroids did not affect adult RMR. Previous studies in *B. anynana* and other insects reported a negative effect of developmental temperature on adult RMR (Berrigan 1997, Pijpe *et al.* 2007, Le Lann *et al.* 2011), and in the opposite direction to the positive effect of adult acclimation temperature (Oostra *et al.* 2011). We confirmed the developmental imprint of temperature on adult RMR, but showed that hormone manipulations did not, at any of the tested time points or rearing temperatures, induce significant changes in RMR (Figure 3.2, Annexes 3.3 and 3.5). This result is unlikely to be due to lack of statistical power, as smaller sample sizes than the ones used in our study have been used previously in this species to statistically detect effects of sex, developmental and adult temperature, genetic background, and age on adult RMR (e.g. Pijpe *et al.* 2007).

Indeed, in the present study the negative effect of developmental temperature on adult RMR was clearly detectable, but we observed no pattern in our data even weakly suggesting that pupal ecdysteroids decrease adult RMR, as one would expect if these hormones would mediate the natural seasonal plasticity of RMR. The most parsimonious explanation for our results is that, despite a correlated response with developmental temperature, RMR and pupal ecdysteroid signaling are not functionally linked. Thus, the developmental signature is independent of pupal ecdysteroid signaling and probably originates during the larval stage (*cf*. Pijpe *et al.* 2007). Clearly the RMR reaction norm deserves follow-up studies to uncover what mechanisms underpin the differences in metabolic rate between the seasonal forms and at which stage during development these differences are set.

Adult RMR and SR show a negative phenotypic correlation in *B. anynana*, responding in opposite directions to developmental temperature (Pijpe *et al.* 2007). Nevertheless, here we uncovered independent variation between RMR and SR; virgin females injected with ecdysteroids live longer under starvation despite having unchanged RMR (Figures 3.3, 3.4E). The proximate cause of the increased SR probably lies in the observed increase in abdominal fat content in response to pupal ecdysteroids injections (Annex 3.1). This strongly suggests that under stressful conditions, females can re-allocate these abdominal resources, and in particular fat (*cf.* Zwaan *et al.* 1991), to survival rather than reproduction.

Our findings reveal that not all traits involved in the alternative adult life histories (and responding to developmental temperature) are regulated by pupal ecdysteroids. This underscores the idea that, even when traits are correlated and co-vary with hormonal patterns, a functional study is needed to ascertain whether a particular hormone is indeed mediating these relationships, including potential trade-offs (see Zera & Harshman 2001).

CONCLUSIONS

In conclusion, our results support a functional role for ecdysteroids during *B. anynana* development in translating information on environmental quality into adaptive alterations in the adult. In particular, we show that these hormones act as a switch between developmental pathways that culminate in alternative adult life histories. Although such developmentally restricted hormonal switches have been found in many insects that display phenotypic plasticity, seasonal or otherwise (Hartfelder &

Emlen 2011, Simpson *et al.* 2011), they likely occur in all animals that display condition-dependent alternative life histories or behaviors. Vertebrates show a wide diversity of reproductive traits that can be coupled to alternative reproductive tactics (Oliveira *et al.* 2008). In birds and lizards, among others, it has been shown that hormones are involved in morphological and neuro-organizational changes during development that underpin these alternative tactics (reviewed in Oliveira *et al.* 2008). A more dramatic example of a condition dependent developmental switch between alternative developmental pathways is environmental sex determination, such as occurs in many reptile species, where the sex of the developing embryo is determined by the temperature at which the egg is incubated (Sarre *et al.* 2004). More generally, hormone-mediated developmental switches allow organisms to mount a systemic, integrated and coordinated response to environmental variation, as systemic hormone titers are centrally regulated from the central nervous system in response to signals sensed from the environment. At the same time, how the tissues and cells that ultimately bring about the phenotypic changes respond to the hormone is a local property of those tissues, which can be regulated via a myriad of mechanisms, including variation in expression, intracellular activity or localization of hormone receptors. Such local hormone sensitivity allows for a cell-, tissue- or trait-dependent differentiated response to the circulating hormone. Our results illustrate how organisms can use systemic hormones and their time- and tissue-specific sensitivity to respond to predictive indicators of environmental quality and to make strategic life history decisions that enable them to cope with fluctuating environments.

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ANNEXES

Increased abdominal fat content and fat-free abdomen mass in response to pupal ecdysteroid treatments (Annexes 3.1 and 3.2, Figures A1, A2) and statistical models of developmental, morphological, physiological, and life-history traits in response to pupal ecdysteroid treatments (Annexes 3.3-3.6, Tables A1-A4). All six annexes can be found at the following website:
http://www.jstor.org/stable/suppl/10.1086/677260/suppl_file/54745apa.pdf.

ANNEX 3.1 - Pupal ecdysteroids induce higher abdominal fat content in adult females.
http://www.jstor.org/action/showPopup?citid=citart1&id=fg5&doi=10.1086%2F677260

ANNEX 3.2 - Pupal ecdysteroids induce increase in fat-free abdomen mass.
http://www.jstor.org/action/showPopup?citid=citart1&id=fg6&doi=10.1086%2F677260

ANNEX 3.3 - Minimum adequate models of developmental, morphological, and physiological traits, related to Figures 3.1-3.3 and Annexes 3.1 and 3.2.
http://www.jstor.org/action/showPopup?citid=citart1&id=tba1&doi=10.1086%2F677260

ANNEX 3.4 - Linear regression models of resting metabolic rate (RMR), abdomen mass, abdomen fat content, and fat-free abdomen mass on pupal mass for cohorts injected at 3%, 16%, 29%, or 34% of pupal development, related to Figures 3.2, 3.3 and Annexes 3.1 and 3.2.
http://www.jstor.org/action/showPopup?citid=citart1&id=tba2&doi=10.1086%2F677260

ANNEX 3.5 - Minimum adequate models of developmental, morphological, and physiological traits at 3%, 16%, 29%, or 34% of pupal development, related to Figures 3.1-3.3 and Annexes 3.1 and 3.2.
http://www.jstor.org/action/showPopup?citid=citart1&id=tba3&doi=10.1086%2F677260

ANNEX 3.6 - Statistical models of life-history traits in response to ecdysteroid treatment, related to Figure 3.4.

http://www.jstor.org/action/showPopup?citid=citart1&id=tba4&doi=10.1086%2F677260

REFERENCES

Arpagaus M, Beydon P, Lafont R (1986) Effects of ecdysone injection on diapause termination in *Pieris brassicae* pupae - evidence for a stimulatory action on endogenous ecdysone production. *Reproduction Nutrition Development* **26**, 1305-1317.

Baayen RH (2011) LanguageR: Data sets and functions with "Analyzing Linguistic Data: A practical introduction to statistics", R package version 1.4. http://CRAN.R-project.org/package=languageR.

Bates DM, Maechler M, Bolker B (2011) Lme4: Linear mixed-effects models using S4 classes, R package version 0.999375-39. http://CRAN.R-project.org/package=lme4.

Beldade P, Mateus ARA, Keller RA (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology* **20**, 1347-1363.

Berrigan D (1997) Acclimation of metabolic rate in response to developmental temperature in *Drosophila melanogaster*. *Journal of Thermal Biology* **22**, 213-218.

Braendle C, Heyland A, Flatt T (2011) Integrating mechanistic and evolutionary analysis of life history variation, Pages 3-10 *in* T. Flatt, and A. Heyland, eds. Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs. Oxford, UK, Oxford University Press.

Brakefield PM, Beldade P, Zwaan BJ (2009) The African butterfly *Bicyclus anynana*: a model for evolutionary genetics and evolutionary developmental biology. Cold Spring Harbor Protocols.

Brakefield PM, Kesbeke F, Koch PB (1998) The regulation of phenotypic plasticity of eyespots in the butterfly *Bicyclus anynana*. *American Naturalist* **152**, 853-860.

Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden P, Monteiro A, French V Carroll S (1996) Development, plasticity and evolution of butterfly eyespot patterns. *Nature* **384**, 236-242.

Brakefield PM, Reitsma N. (1991) Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (*Satyridae*) in Malawi. *Ecological Entomology* **16**, 291-303.

Brakefield PM, Zwaan BJ (2011) Seasonal polyphenisms and environmentally-induced plasticity in the Lepidoptera - the coordinated evolution of many traits on multiple levels, Pages 243-252 *in* T. Flatt, and A. Heyland, eds. Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs. Oxford, UK, Oxford University Press.

Brakefield PM, Frankino WA (2007) Polyphenisms in Lepidoptera: multidisciplinary approaches to studies of evolution. *in* D. W. Whitman, and T. N. Ananthakrishnan, eds. Phenotypic Plasticity in Insects. Mechanisms and Consequences. UK, Science Publishers.

Brakefield PM, Pijpe J, Zwaan BJ (2007) Developmental plasticity and acclimation both contribute to adaptive responses to alternating seasons of plenty and of stress in *Bicyclus* butterflies. *Journal of Biosciences* **32**, 465–475.

Brisson JA (2010) Aphid wing dimorphisms: linking environmental and genetic control of trait variation. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* **365**, 605-616.

Danks HV (1987) *Insect Dormancy: An Ecological Perspective*. Biological Survey of Canada, Ottawa.

Dawson A. (2008) Control of the annual cycle in birds: endocrine constraints and plasticity in response to ecological variability. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* **363**, 1621-1633.

de Jong MA, Kesbeke F, Brakefield PM, Zwaan BJ (2010) Geographic variation in thermal plasticity of life history and wing pattern in *Bicyclus anynana*. *Climate Research* **43**, 91-102.

Denlinger DL (2002) Regulation of diapause. *Annual Review of Entomology* **47**, 93-122.

Emlen DJ, Warren IA, Johns A, Dworkin I, Lavine LC (2012) A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. *Science* **337**, 860-864.

Fischer K, Eenhoorn E, Bot AN, Brakefield PM, Zwaan BJ (2003) Cooler butterflies lay larger eggs: developmental plasticity versus acclimation. *Proceedings of the*

Royal Society of London, Series B: Biological Sciences **270**, 2051-2056.

Geister TL, Lorenz MW, Meyering-Vos M, Hoffmann KH, Fischer K (2008) Effects of temperature on reproductive output, egg provisioning, juvenile hormone and vitellogenin titers in the butterfly *Bicyclus anynana*. *Journal of Insect Physiology* **54**, 1253-1260.

Gilbert SF (2012) Ecological developmental biology: environmental signals for normal animal development. *Evolution and Development* **14**, 20-28.

Hartfelder K, Emlen DJ (2012) Endocrine control of insect polyphenism. Pages 464-522 *in* L. Gilbert, ed. Insect Endocrinology. London, UK, Academic Press.

Hofmann GE, Todgham AE (2010) Living in the now: physiological mechanisms to tolerate a rapidly changing environment. *Annual Review of Physiology* **72**, 127-145.

Klowden MJ (2007) Physiological Systems in Insects. London, UK, Elsevier Science / Academic Press.

Koch PB, Lorenz U, Brakefield PM, ffrench-Constant RH (2000) Butterfly wing pattern mutants: developmental heterochrony and co-ordinately regulated phenotypes. *Development Genes and Evolution* **210**, 536–544.

Koch PB (1996) Preadult changes of ecdysteroid and juvenile hormone titers in relation to diapause and pigmental variations in two lepidopteran species, Cerura vinula and Araschnia levana (Lepidoptera: *Notodontidae Nymphalidae*). *Entomologia Generalis* **20**, 143-155.

Koch PB, Brakefield PM, Kesbeke F (1996) Ecdysteroids control eyespot size and wing color pattern in the polyphenic butterfly Bicyclus anynana (Lepidoptera: *Satyridae*). *Journal of Insect Physiology* **42**, 223-230.

Koch PB, Bückmann D (1987) Hormonal control of seasonal morphs by the timing of ecdysteroid release in *Araschnia levana* L. (Nymphalidae: *Lepidoptera*). *Journal of Insect Physiology* **33**, 823-829.

Kooi RE, Brakefield PM (1999) The critical period for wing pattern induction in the polyphenic tropical butterfly *Bicyclus anynana* (*Satyrinae*). *Journal of Insect Physiology* **45**, 201–212.

Kremen C, Nijhout HF (1989) Juvenile hormone controls the onset of pupal commitment in the imaginal discs and epidermis of Precis caenia (Lepidoptera: Nymphalidae). *Journal of Insect Physiology* **35**, 603–612.

Le Lann C, Wardziak T, van Baaren J, van Alphen JJM (2011) Thermal plasticity of metabolic rates linked to life-history traits and foraging behaviour in a parasitic wasp. *Functional Ecology* **25**, 641-651.

Meylan S, Miles DB, Clobert J (2012) Hormonally mediated maternal effects, individual strategy and global change. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* **367**, 1647-1664.

Munyiri FN, Ishikawa Y (2004) Endocrine changes associated with metamorphosis and diapause induction in the yellow-spotted longicorn beetle, *Psacothea hilaris*. *Journal of Insect Physiology* **50**, 1075-1081.

Nijhout HF (1975) A threshold size for metamorphosis in the tobacco hornworm, Manduca sexta (L.). *The Biological Bulletin* **149**, 214 -225.

Nijhout HF (1991) The development and evolution of butterfly wing patterns. Smithsonian Institution Press.

Nijhout HF (2003) Development and evolution of adaptive polyphenisms. *Evolution and Development* **5**, 9-18.

Nylin S, Gotthard K (1998) Plasticity in life-history traits. *Annual Review of Entomology* **43**, 63-83.

Oberlander H (1985). The imaginal discs *in* G. A. Kerkut, and L. I. Gilbert eds. Comprehensive Ultrastructural Effects of Tebufenozide 133 Insect Physiology Biochemistry and Pharmacology, vol. 2. Oxford, Pergamon Press.

Oliveira RF, Canário AVM, Ros AFH (2008) Hormones and alternative reproductive tactics in vertebrates. Pages 132-173 in R.F. Oliveira, M. Taborsky, and H. J. Brockmann, eds. Alternative Reproductive Tactics. Cambridge, UK, Cambridge University Press.

Oostra V, de Jong MA, Invergo BM, Kesbeke F, Wende F, Brakefield PM, Zwaan BJ (2011) Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **278**, 789-797.

Ohtaki T, Yamanaka F, Sakurai S (1986) Differential timing of pupal commitment in various tissues of the silkworm, Bombyx mori . *Journal of Insect Physiology* **32**, 635-642.

Pijpe J, Brakefield PM, Zwaan BJ (2007) Phenotypic plasticity of starvation resistance in the butterfly *Bicyclus anynana*. *Evolutionary Ecology* **21**, 589-600.

R-Core-Team (2012) R - A Language and Environment for Statistical Computing.

Ramaswamy SB, Shu S, Park YI, Zeng F (1997) Dynamics of juvenile hormone-mediated gonadotropism in the Lepidoptera. *Archives of Insect Biochemistry and Physiology* **35**, 539-558.

Riddiford LM (1985) Hormone action at the cellular level. *in* Comprehensive Insect Physiology, Biochemistry, and Pharmacology, vol. 8. G. A. Kerkut, and L. I. Gilbert eds. Oxford, Pergamon.

Rountree DB, Nijhout HF (1995) Hormonal control of a seasonal polyphenism in *Precis coenia* (Lepidoptera: *Nymphalidae*). *Journal of Insect Physiology* **41**, 987-992

Richard DS, Jones JM, Barbarito MR, Stacy C, Detweiler JP, Fisher SJ, Brannigan DM, Scheswohl DM (2001) Vitellogenesis in diapausing and mutant *Drosophila melanogaster*: further evidence for the relative roles of ecdysteroids and juvenile hormones. *Journal of Insect Physiology* **47**, 905-913.

Saastamoinen M, van der Sterren D, Vastenhout N, Zwaan BJ, Brakefield PM (2010) Predictive adaptive responses: condition dependent impact of adult nutrition and flight in the tropical butterfly *Bicyclus anynana*. *American Naturalist* **176**, 686-698.

Schmidt PS (2011) Evolution and mechanisms of insect reproductive diapause: A plastic and pleiotropic life history syndrome, Pages 230-242 *in* T. Flatt, and A. Heyland, eds. Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs. Oxford, UK, Oxford University Press.

Schwedes CC, Carney GE (2012) Ecdysone signaling in adult *Drosophila melanogaster*. *Journal of Insect Physiology* **58**, 293-302.

Shingleton AW, Frankino WA, Flatt T, Nijhout HF, Emlen DJ (2007) Size and shape: the developmental regulation of static allometry in insects. *Bioessays* **29**, 1-13.

Simpson SJ, Sword GA (2009) Phase polyphenism in locusts: mechanisms, population, consequences, adaptive significance and evolution., Pages 147-189 *in* T. N. Ananthakrishnan, and D. W. Whitman, eds. Phenotypic plasticity in insects. Mechanisms and consequences. Plymouth, UK, Science Publishers, Inc.

Simpson SJ, Sword GA, Lo. N (2011) Polyphenism in Insects. *Current Biology* **21**, R738-R749.

Singtripop T, Wanichacheewa S, Tsuzuki S, Sakurai S (1999) Larval growth and diapause in a tropical moth, *Omphisa fuscidentalis* Hampson. *Zoological Science* **16**, 725-733.

Smith CR, Toth AL, Suarez AV, Robinson GE (2008) Genetic and genomic analyses of the division of labour in insect societies. *Nature Reviews Genetics* **9**, 735-748.

Shirai LT (2014) Morphological diversification through the evolution of developmental hierarchies. Ph.D. Thesis. Lisboa: Portugal.

Sommer RJ, Ogawa A (2011) Hormone signaling and phenotypic plasticity in nematode development and evolution. *Current Biology* **21**, R758-R766.

Steigenga MJ, Fischer K (2007) Within- and between-generation effects of temperature on life-history traits in a butterfly. *Journal of Thermal Biology* **32**, 396-405.

Therneau T (2012) A package for survival analysis in S, R package version 2.36-14. http://CRAN.R-project.org/package=survival.

Tsuchida K, Nagata M, Suzuki A (1987) Hormonal control of ovarian development in the silkworm, *Bombyx mori*. *Archives of Insect Biochemistry and Physiology* **5**, 167-177.

Van den Heuvel J, Saastamoinen M, Brakefield PM, Kirkwood TBL, Zwaan BJ, Shanley DP (2013) The predictive adaptive response: modeling the life-history evolution of the butterfly *Bicyclus anynana* in seasonal environments. *American Naturalist* **181**, E28-E42.

Visser ME, Caro SP, van Oers K, Schaper SV, Helm B (2010) Phenology, seasonal timing and circannual rhythms: towards a unified framework. *Philosophical Transactions of*

the Royal Society of London, Series B: Biological Sciences **365**, 3113-3127.

West-Eberhard MJ (2003) Developmental plasticity and evolution. New York, NY.

Wittkopp PJ, Beldade P (2009) Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. Seminars in Cell and Developmental Biology **20**, 65-71.

Zera AJ (2007) Endocrine analysis in evolutionary-developmental studies of insect polymorphism: hormone manipulation versus direct measurement of hormonal regulators. *Evolution and Development* **9**, 499-513.

Zera A (2009) Wing polymorphism in crickets *in* T. N. Ananthakrishnan, and D. W. Whitman, eds. Phenotypic plasticity in insects. Mechanisms and consequences. Plymouth, UK, Science Publishers, Inc.

Zera AJ, Harshman LG (2001) The physiology of life history trade-offs in animals. *Annual Review of Ecology And Systematics* **32**, 95-126.

Zera AJ, Harshman LG, Williams TD (2007) Evolutionary endocrinology: The developing synthesis between endocrinology and evolutionary genetics. Annual Review of Ecology and Systematics **38**, 793-817.

Zijlstra WG, Steigenga MJ, Koch PB, Zwaan BJ, Brakefield PM (2004) Butterfly selected lines explore the hormonal basis of interactions between life histories and morphology. *American Naturalist* **163**, E76-87.

Zwaan BJ, Bijlsma R, Hoekstra RF (1991) On the developmental theory of ageing. I. Starvation resistance and longevity in *Drosophila melanogaster* in relation to preadult breeding conditions. *Heredity* **68**, 123-130.

CHAPTER 4. THERMAL REACTION NORMS FOR PIGMENTATION MUTANTS: G, T AND GXT EFFECTS

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ABSTRACT

Developmental plasticity refers to the ability for the external environment to modulate development leading to the production of different phenotypes from the same genotype. Genotypes can differ in many properties of reaction norms such as height, slope, or shape. Despite being well-known that there is genetic variation for properties of reaction norms, which is the raw material for the evolution of plasticity, too little is known about the genes that contribute to that. Here, we characterized thermal reaction norms in butterfly wing pattern for different pigmentation variants to test the hypothesis that alleles that affect pigmentation also affect plasticity therein. We characterized thermal reaction norms for the eyespot color rings of four *Bicyclus anynana* genetic stocks corresponding to allelic variants affecting eyespot size and color composition. Our results show variation between genetic stocks in the height, slope and shape of reaction norms providing evidence for significant GxE effects. Genotypes with alleles affecting eyespot size and color were the most sensitive to variation in developmental temperature. However, this was true for only one of the wings suggesting organ-specific allelic effects. This study underscores the complexity of GxE interactions and their importance for the evolution of developmental plasticity.

KEYWORDS

Bicyclus anynana, Butterfly wing patterns, Gene-by-environment interaction, Pigmentation, Plasticity genes, Reaction norm

INTRODUCTION

Developmental plasticity refers to the process whereby a single genotype produces distinct phenotypes depending on external conditions experienced during development. This phenomenon reflects the complexity of the interactions between genetic and environmental factors that modulate organismal development (e.g. West-Eberhard 2003, Beldade *et al.* 2011). In alternative seasonal habitats, developmental plasticity may evolve as a result of predictable seasonal selection pressures and can result in alternative phenotypes each adapted to the conditions in the corresponding season (Brakefield & Zwaan 2011).

An important analytical tool in the study of developmental plasticity is the concept of reaction norm. Reaction norms represent the set of phenotypes expressed by a single genotype across a range of environments (Schlichting & Pigliucci 1998, Cheplick 2003, Beldade *et al.* 2011). For any plastic trait, different genotypes can differ in many properties of these reaction norms (Sultan 1995), such as their height, slope, or shape. These properties can be considered as traits for which there is heritable variation and which can evolve. While there are well-known examples of the evolution of plasticity in natural and artificial populations (e.g. Brakefield *et al.* 1996, Suzuki & Nijhout 2006, Wray 2007, Aubret & Shine 2009, Bento *et al.* 2010), little is known about which genes carry allelic variants that underlie those changes (Gibert *et al.* 2007).

Here, we characterized thermal reaction norms for wing pattern in pigmentation variants to test the hypothesis that alleles that affect pigmentation also affect plasticity therein. Previous studies have addressed this topic by exploring the abdominal pigmentation in *Drosophila melanogaster*, a particularly well described plastic trait that exhibits large phenotypic variability depending on growth temperature. They showed that different abdominal segments with differences in color patterns show different shapes of reaction norms across temperature, which suggests that genes involved in pigmentation are also involved in plasticity (David *et al.* 1990, Gibert *et al.* 2000, 2007). However, little is known about the complexity of the interaction between genes and environment, represented by the reaction norms, and other models that show phenotypic plasticity for pigmentation should be considered.

The wing color patterns of *Bicyclus anynana* butterflies are a prime example where the study of the mechanisms regulating developmental plasticity can be combined with knowledge about the ecological significance of that plasticity (e.g.

Brakefield *et al.* 1996, Beldade & Brakefield 2002, Beldade *et al.* 2011). The temperature experienced during development determines the production of alternative phenotypes resembling the natural wet and dry seasonal forms of this seasonally polyphenic species (Brakefield & Frankino 2007). Larvae developing at high temperatures produce a wet-season-like phenotype with large ventral eyespots, while individuals developing at low temperatures produce a dry-season-like phenotype with reduced eyespots and a more or less overall brown wing. The marginal eyespots on the ventral wing surfaces, which are exposed in the butterfly when at rest, are thought to be under selection by natural predators (Brakefield & Larsen 1984, Oliver *et al.* 2009). While the large eyespots of the wet-season butterflies are thought to attract the predators' attention to the wing margin and away from the vulnerable body, the all-brown dry-season butterflies are cryptic against the background of dry leaves characteristic of that season (Brakefield & Frankino 2007, Olofsson *et al.* 2010). In the lab, butterflies with eyespots of intermediate size develop at intermediate temperatures (e.g. Oostra *et al.* 2011, Mateus *et al.* 2014).

A number of studies of genetic variants for *B. anynana* wing patterns have reveald quantitative variation that enabled gradual response to artificial selection on the height, but not the shape, of thermal reaction norms for this trait (Brakefield *et al.* 1996, Wijngaarden & Brakefield 2001). However, it remains unclear about the genes that contribute to the genetic variation for properties of reaction norms and whether the alleles that affect pigmentation also affect plasticity therein. Here, we test the hypothesis that alleles that contribute to variation in pigmentation also contribute to variation in levels of pigmentation plasticity. We do this by characterizing thermal reaction norms in size of eyespot color rings for *B. anynana* spontaneous mutants with altered eyespot size and/or color composition.

MATERIAL AND METHODS

Butterfly material

We used *B. anynana* captive populations with different pigmentation phenotypes (Figure 4.1): an outbred stock representing the "wildtype" phenotype (WT, Brakefield *et al.* 2009), a larval color mutant with wildtype adult pigmentation called Chocolate (Choc, Saenko *et al.* 2012), and eyespot mutants Bigeye (BE, affecting eyespot size) and Frodo (Fr, affecting eyespot color composition, Saenko *et al.* 2010). While the

Choc stock is pure-breeding for the mutant allele, BE and Fr alleles are recessive embryonic lethal with dominant effect on wing pattern, and the corresponding stocks always segregate for mutant and wildtype-looking individuals. All mutant stocks have been maintained with selection in favor of the mutant phenotype and occasionally outcrossed to the laboratory outbred WT stock to avoid inbreeding depression. In order to simplify, we will use the word "genotype" to refer to each of the four stocks, even thought there is genetic variation within stocks.

About 120 first-instar larvae from each stock were grown in each of three climate-controlled rooms (70% relative humidity, 12:12hr light/dark cycle) differing in ambient temperature (± 0.5°C). We chose temperatures that simulate the conditions of the natural dry (19°C) and wet (27°C) seasons and an intermediate temperature (23°C). Larvae were kept in large cages and fed *ad libitum* with young maize plants sprayed with anti-fungic solution. Adults were frozen 24h after eclosion. Their wings were cut and stored in the freezer until analysis. Due to a fungal infection in all stocks, much fewer than 120 adults per genetic stock per temperature were obtained. Mortality was especially elevanted for the BE stock in the low rearing temperature. Sample sizes are provided in Table 4.1 and statistical analysis in the Annexes section.

Image analysis of target eyespot traits

The ventral surface of undamaged right fore- and hindwing of adult females and males were photographed (Leica DC200 digital camera) under a binocular microscope (Leica MZ12) at 10x magnification. This was done with standard light, and including both a ruler for conversion from pixels to millimeters and a color reference card (QPcard 201) for color calibration and background correction. The resulting images were analysed with a custom image processing system (cf. Mateus *et al.* 2014) using the ImageJ-based open-source Fiji software package (Schindelin *et al.* 2012). With this tool, areas of eyespot color rings were calculated by a threshold method in which the image was first converted to black and white and values of intensity under or above user-established threshold values were selected and corresponding areas were calculated. In total, we measured eight areas characterizing eyespot color rings total wing areas. The eight eyespot traits correspond to the middle black ring and external golden ring of the two eyespots on the ventral surface of the forewing (the Anterior eyespot, eA, and the Posterior eyespot, eP), as well as of two of the seven eyespots that typically decorate the

hindwing (the second, e2, and the fifth, e5, eyespots, corresponding to the equivalent positions, cf. wing venation, of eA and eP) (see Figure 4.1).

Table 4.1 - Sample sizes. Number of females (F) and males (M) measured for each of the target traits from each phenotype (WT, BE, Fr, Choc) at each of the three temperatures (in the order: 19°C-23°C-27°C). For BE and Fr the top row represents the mutant phenotype (heterozygous at BFS locus) and the bottom row the wildtype phenotype (homozygous for wildtype allele) for the traits analized in Figure 4.4.

Stock\Trait	eA	eP	e2	e5	FW	HW
WT-F	30-30-30	30-30-30	27-30-30	27-30-30	32-30-31	29-30-30
WT-M	12-30-31	12-30-31	7-29-27	7-28-27	12-30-31	7-29-27
BE-F	9-7-22	10-7-22 5-5-7	8-6-19	9-6-19 4-5-5	10-7-22 5-5-7	9-6-19 4-5-5
BE-M	6-8-15	6-8-15 5-9-4	5-6-12	5-6-13 5-9-4	6-8-15 5-9-4	5-6-13 5-10-4
Fr-F	20-26-44	20-27-44 22-35-21	15-23-40	15-23-40 19-34-18	21-27-45 23-36-21	15-23-40 20-34-18
Fr-M	18-20-17	18-20-27 15-37-16	17-16-15	17-16-15 15-37-15	18-20-17 15-40-16	17-16-15 15-37-33
Choc-F	19-19-29	19-19-29	16-18-26	16-18-26	21-20-32	16-18-26
Choc-M	17-19-21	17-20-21	15-20-20	16-20-20	18-20-22	16-20-20

Statistical analyses

All data analyses were performed with *R* (R Development Core Team 2012) and done separately for females and males because of sexual dimorphism in wing size and pigmentation. In all statistical models, we use "genotype" to refer to the different genetic backgrounds.

We divided our analysis into three parts explained in detail below. First, we ran a Principal Component Analysis (PCA) for all eight eyespot traits in each of four genetic backgrounds to reduce data complexity and identify which traits contribute to the different principal components (PCs). Second, we compared thermal reaction norms for the PCs as well as for the eigth eyespot traits between genetic backgrounds. Finally, we compared mutant and wildtype-like "siblings" from the BE and Fr stocks to assess the impact of single allelic variants on thermal reaction norm. In each of the cases, we tested the impact of temperature (T), genetic background (G), and their interaction (GxT). Before that, parametric assumptions were considered by checking for normality (Shapiro-Wilk test, alpha=0.05) and homoscedasticity (Fligner-Killeen test, alpha=0.05)

of residuals, and transforming data where appropriate. When a significant difference (alpha=0.05) was found for our models, we performed post-hoc comparisons between factor levels using Tukey´s honest significant differences (HSD) tests (alpha=0.01).

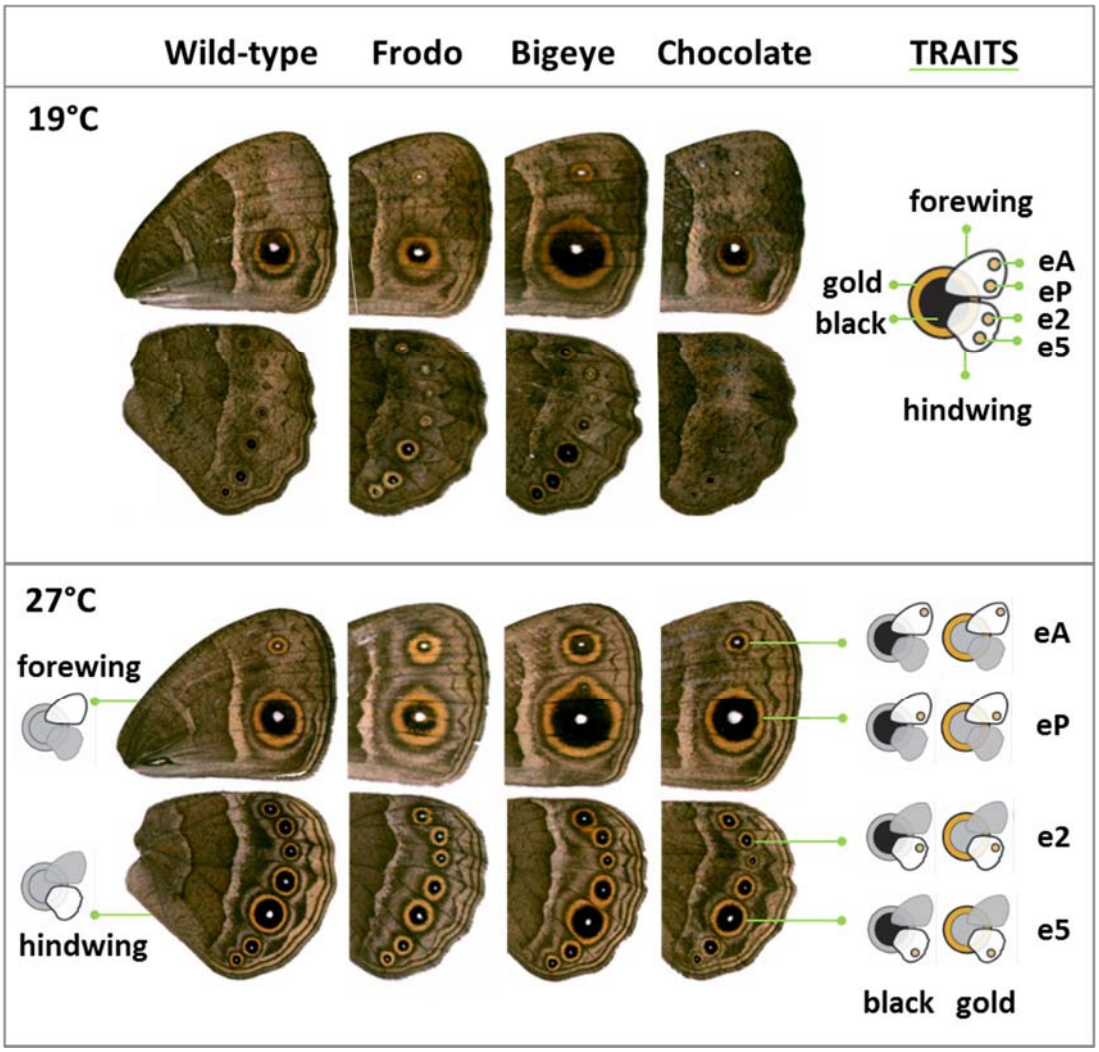

Figure 4.1 - Wing traits measured in adult butterflies from four genotypes. The photos represent the typical phenotype for the four genetic stocks (WT, Fr, BE, and Choc) of female *Bicyclus anynana* reared at 19˚C (top panel) or 27˚C (bottom panel). For each individual, we obtained measurements corresponding to the black and gold areas of two eyespots on the forewing (eA and eP) and two on the hindwng (e2 and e5), as well as forewing (FW) and hindwing (HW) areas. The diagram on the right of the top panel displays the symbols used to refer to each of the traits throughout the chapter. For each of the two eyespots measured on each wing, the more anterior is represented by a circle on the top of the wing, and the more posterior by a circle on the bottom of the wing. The color of the circles at the center of each icone corresponds to either the black or golden rings.

We first used a Principal Component Analysis (PCA) technique (Jolliffe 1986) to reduce and explore the patterns of variation for the eight eyespot rings in same-sex individuals of four genetic stocks. In order to handle missing values, we used the R packages FactoMineR (Lê *et al.* 2008) and missMDA (Husson & Josse 2010). PCA was run using the values of eyespot ring area/wing area. We stored and represented graphically the scores for the first four Principal Components (PCs), hereafter referred to as Dimensions (Dims; terminology in agreement with the package that we used to deal with missing values), for all individuals. We then characterized the reaction norms for each of these Dims and statistically tested the model *Dim~temperature*genotype* (general linear model with Gaussian distribution of the errors) with *temperature* (three levels: 19°C, 23°C, 27°C) and *genotype* (four levels: WT, Fr, BE and Choc) as fixed factors.

Second, for each eyespot trait we tested the model *ring area~wing area+ temperature*genotype*, with *wing area* as covariate. To specifically query eyespot color composition, defined as the proportion of black to gold ring areas, we also tested the model *back/gold ~temperature*genotype*. For both models, we used a general linear model assuming a Gaussian distribution of the error, and with *temperature* (three levels: 19°C, 23°C, 27°C) and *genotype* (four levels: WT, Fr, BE and Choc) as fixed effects.

Thirdly, to avoid confounding effects of variable genetic background within each of the four lab populations differing in pigmentation, we compared wildtype-looking and mutant-looking individuas that segregate within each of the BE and Fr stocks. Note that we did not include the wildtype-looking individuals from the BE and Fr stocks in the previous analyses. We tested the model *ring area~wing area+temperature*phenotype* using a general linear model with a Gaussian distribution of the error.This was done for the BE and Fr stocks separately, with *temperature* (three levels: 19°C, 23°C, 27°C) and *phenotype* (two levels: mutant, wildtype) as fixed factors and using *wing area* as covariate.

RESULTS

In order to explore plasticity in eight wing pigmentation traits in different *B. anynana* pigmentation mutants (Figure 4.1), we collected phenotypic data from individuals of four different genetic stocks reared at three temperatures (Table 4.1). We compared thermal reaction norms between genotypes for PCs that reduce data complexity (Table 4.2, Figure 4.2) and also for the actual eyespot measurements (Figures 4.3 and 4.4). This

analysis allowed us to determine effects of temperature (T), genetic line (G), and their interaction (GxT) on phenotype. Our results show prevalence of temperature effect on phenotype and inter-population variation in the height and, to a lesser extent, the shape of thermal reaction norms.

Principal components contrast different groups of traits

The PCA describing the patterns of variation for the eight eyespot traits in butterflies from four different stocks reared at three temperatures enabled us to reduce the variation to four main Dims together accounting for about 94% of the variation in our data for females and males independently (Table 4.2, Annex 4.1).

The loadings for eyespot traits on Table 4.2 enable us to assess how each of those traits contributes to defining each of the Dims: high absolute values versus values close to zero reflect high versus low contribution, positive versus negative values reflect traits with contrasting contributions. The thermal reaction norms for each of the main Dims (Figure 4.2) allow us to determine how plastic each of them is for different genetic stocks.

For both females and males, all eyespot traits seem to contribute equally to Dim 1, explaining most of the variation in each respective dataset. Dim 1 is significantly affected by developmental temperature (females: F=312.9, df=2, P = $2.2x10^{-6}$; males: F=333.1, df=2, P = $2.2x10^{-6}$), by genotype (females: F=94.9, df=3, P = $2.2x10^{-6}$; males: F=146.6, df=3, P = $2.2x10^{-6}$), and by the interaction betweem these two factors (females: F=19.3, df=6, P = $2.2x10^{-6}$; males: F=6.1, df=6, P = $6.0x10^{-6}$) (see details in Annex 4.2). The genotype that seems less plastic for Dim 1 is WT in females (lower difference between temperature extremes, Figure 4.2A), and the reaction norm that stands out for height is that of BE (eyespot size mutant) for both sexes.

Dim 2 is also similar for the female and male datasets in that it largely contrasts black versus gold eyespot rings (loadings of opposite sign for the two colors) (Table 4. 2). Two traits stand out in both datasets: the black area of eyespot e2 and the gold area of eyespot eP with loadings closer to zero suggestive of little contribution to Dim 2. Dim 2 was significantly affected by genotype (females: F=258.7, df=3, P = $2.2x10^{-6}$; males: F=140.5, df=3, P = $2.2x10^{-6}$) and by genotype x temperature (females: F=4.4, df=6, P = 0.0002; males: F=9.6, df=6, P = $2.6x^{10-9}$) for both sexes, temperature only was significant for females (F=3.1, df=2, P = 0.044) (see detailed results in Annex 4.2). Dim 2 also shows that BE is more responsive across temperature with a steepest reaction

norm (reflected in higher mean differences between temperatures, see Annex 4.2) in relation to Choc and WT, and that Fr has a different reaction norm height from the other genotypes (Figure 4.2).

Table 4.2 - Results of the Principal Component Analysis for females and males. Summary of the loadings for Dims 1- 4 describing 94% of the variation for eight wing traits corrected for wing size: anterior (eA) and posterior (eP) eyespots of the forewing, and second (e2) and fifth (e5) eyespots of the hindwing (with the correspondent trait icon on the left, cf. Figure 4.1). For each Dim, the table displays the Eigenvalues, the proportion of the variation explained, and the contribution of each trait area/wing area.

	FEMALES				MALES			
TRAIT / wing	DIM1	DIM2	DIM3	DIM4	DIM1	DIM2	DIM3	DIM4
eA black	0.839	0.234	-0.367	-0.113	0.903	-0.238	-0.134	0.215
eA gold	0.878	-0.352	-0.127	-0.125	0.836	0.312	0.137	0.414
eP black	0.664	0.672	0.219	0.020	0.858	-0.395	0.191	0.090
eP gold	0.864	-0.059	0.304	-0.367	0.861	0.082	0.424	-0.181
e2 black	0.884	-0.072	-0.324	0.043	0.901	-0.075	-0.337	0.010
e2 gold	0.849	-0.418	0.122	0.183	0.896	0.324	-0.139	-0.112
e5 black	0.846	0.435	-0.004	0.175	0.898	-0.285	-0.027	-0.219
e5 gold	0.884	-0.263	0.223	0.189	0.897	0.285	-0.078	-0.187
Eigenvalue	5.6615	1.0737	0.4611	0.2652	6.2189	0.5894	0.3926	0.3508
% variation	70.8	13.4	5.8	3.3	77.7	7.4	4.9	4.4

Dim 3 is not equivalent between sexes. While in females it contrasts anterior (eA and e2) versus posterior (eP and e5) eyespots, in males it contrasts eyespots on the forewing (eA and eP) versus those on the hindwing (e2 and e5) (Table 4.2). The traits that stand out in their contribution to Dim 3 are: 1) the gold area of eyespot e2 for females, and 2) the black area of eyespot eA in males. For both sexes there is little contribution of the black area of eyespot e5. The analysis of the reaction norms for Dim 3 (Figure 4.2) shows it to be significantly affected by genotype for females ($F=27.6$, $df=3$, $P = 8.199e-16$) and by temperature ($F=3.4$, $df=2$, $P = 0.033$) and genotype for males ($F=3.1$, $df=3$, P

= 0.027) (see details in Annex 4.2). We did not find significant genotype x temperature effects for Dim 3.

Finally, Dim 4 contrasts eyespots on forewing (eA and eP) versus hindwing (e2 and e5) for females, and forewing anterior eyespot (eA) versus all others for males (Table 4.2). The traits that stand out are: 1) the black area of eP and e2, which have very little contribution (loadings close to zero) for Dim 4 of both males and females, and 2) the gold area of eP which has negative loadings like all hingwing eyespot traits and contrary to the other forewing eyespot traits for males. Dim 4 is only significantly affected by temperature (F=5.5, df=2, P = 0.019) and genotype x temperature for females (F=3.2, df=6, P = 0.023), (see detailed results in Annex 4.2).

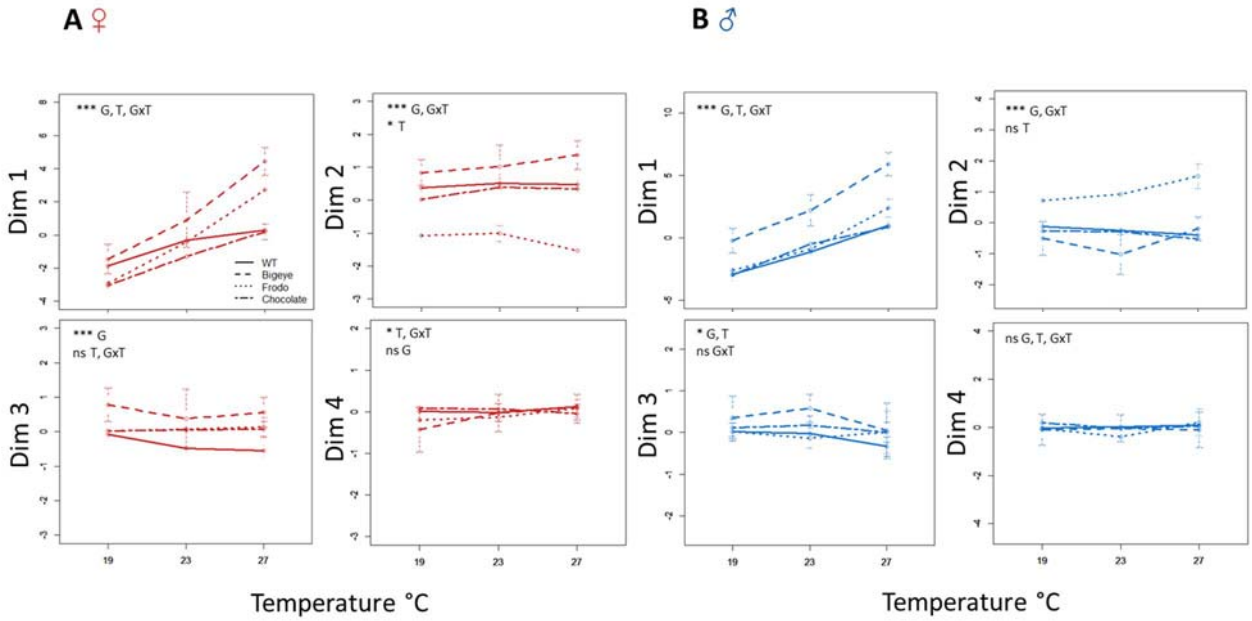

Figure 4.2 - Effects of developmental temperature for the four Dims of wing patterns. For each Dim, we plot the mean value as a function of temperature and use bars to represent the standard deviation (SD) of four genetic stocks (WT, Fr, BE and Choc). Females (left side, red color) and males (right side, blue color) are represented separately. We tested for the effect of temperature and genotype on each Dim using the model *Dim~temperature*genotype* (see Material and Methods and Annex 4.2). Statistical significance for effects of temperature, genotype and GxT on wing traits (see Material and Methods) are indicated on the top left corner of each reaction norm: ns (non-significant) P > 0.05, *P < 0.05, ** P < 0.01, *** P < 0.001. When we found significant effects of temperature or/ and genotype on trait value P < 0.05, we compared across factors (Tukey HDS, P < 0.01), (see Annex 4.2 for details on these analyses).

Eyespot mutants BE and Fr stocks stand out in their response to developmental temperature

We investigated how black and gold eyespot rings changed with temperature for different genetic stocks (Figure 4.3) and tested the effect of genotype (G), temperature (T) and their interaction (GxT). Significant G effect means that genetic backgrounds differ, significant T effect means that traits are thermally plastic, and significant GxT effects reflect differences between genetic stocks in their thermal reaction norms.

Figure 4.3 shows the size of eyespot black and gold areas and color composition across temperature for females and males of WT, Fr, BE and Choc stocks. We quantified these differences for the anterior and posterior eyespot on the forewing (eA and eP) and hindwing (e2 and e5) and found that color composition differed between genotypes across temperatures (GxT) only for the posterior eyespots (except the eP for females), but not for the anterior eyespots (except the eA for females) (see also Annexes 4.3 and 4.5), and that Temperature has a significant effect for all eyespots except for the posterior eyespots (eP and e5) of females (Annexes 4.3 and 4.5).

For all traits except the posterior gold areas for males, there was a significant effect of GxT (Annexes 4.4 and 4.5). BE and Fr genotypes, for both genders, showed the most pronounced differences between temperatures (Annex 4.5). In general, BE showed the highest levels of plasticity for all traits (higher mean differences between temperatures), except for the gold areas for which Fr showed to be more responsive to temperature (Annex 4.5).

These genotypes, as already shown with the PCA (see below), show higher plasticity in comparison with the WT and Choc genotypes (Figure 4.3). The hindwing seems more sensitive to temperature in relation to forewing as judged by their higher F-values for the Temperature effect (Annex 4.5).

All in all, for both genders, there is clear plasticity for both black and gold areas for all the genotypes. There are clear GxE effects and, from all genetic backgrounds, WT seems the least thermally plastic with Fr and BE being the most responsive genotypes.

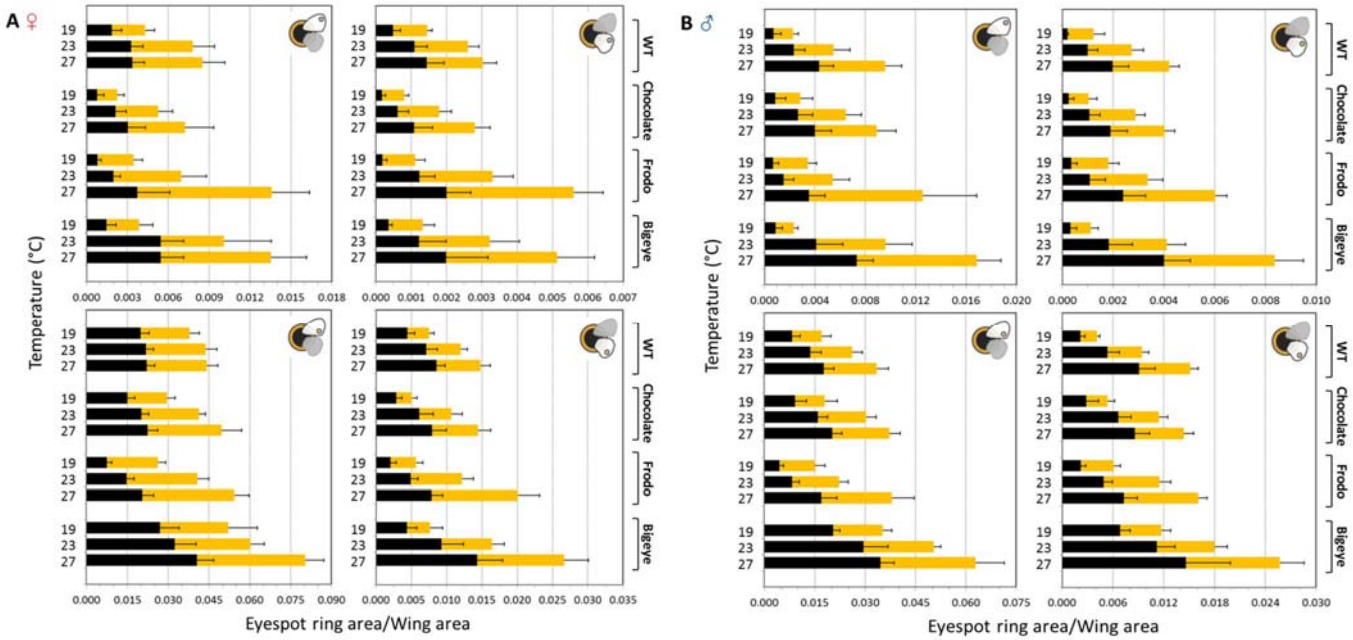

Figure 4.3 - Variation in eyespot ring areas in relation to developmental temperature and genotype. Panel A (females) and panel B (males) show the means of black and gold eyespot areas (relative to corresponding wing area) across temperatures (19, 23, and 27°C). Bars represent standard deviations. Top panels show the results for the anterior eyespots and bottom panels for the posterior eyespots of forewing and hindwing. Genotypes are indicated on the right side of the plots and traits are represented by the respective icons on the top right corner (see Figure 4.1). We tested for the effect of temperature and genotype on eyespot color composition using the model *black/gold~temperature*genotype*, and on ring area using the model *ring area~wing area + temperature*genotype*. When we found significant effects of temperature and/or genotype ($P < 0.05$), we compared across temperatures (Tukey HDS, $P < 0.01$). See Annex 4.5 for details on these statistical analyses, and Annexes 4.3 and 4.4 for the reaction norms of ring and wing size and eyespot color composition.

Alleles affecting pigmentation can affect plasticity therein

We asked if BE and Fr alleles, two alleles at the same locus that affect different aspects of eyespot morphology, have temperature-specific effects resulting in differences in thermal reaction norms for eyespot color rings. For that purpose, we compared siblings within each of those stocks that differ at which allele they have at the BFS locus but not in genetic background (Figure 4.4, Annex 4.5).

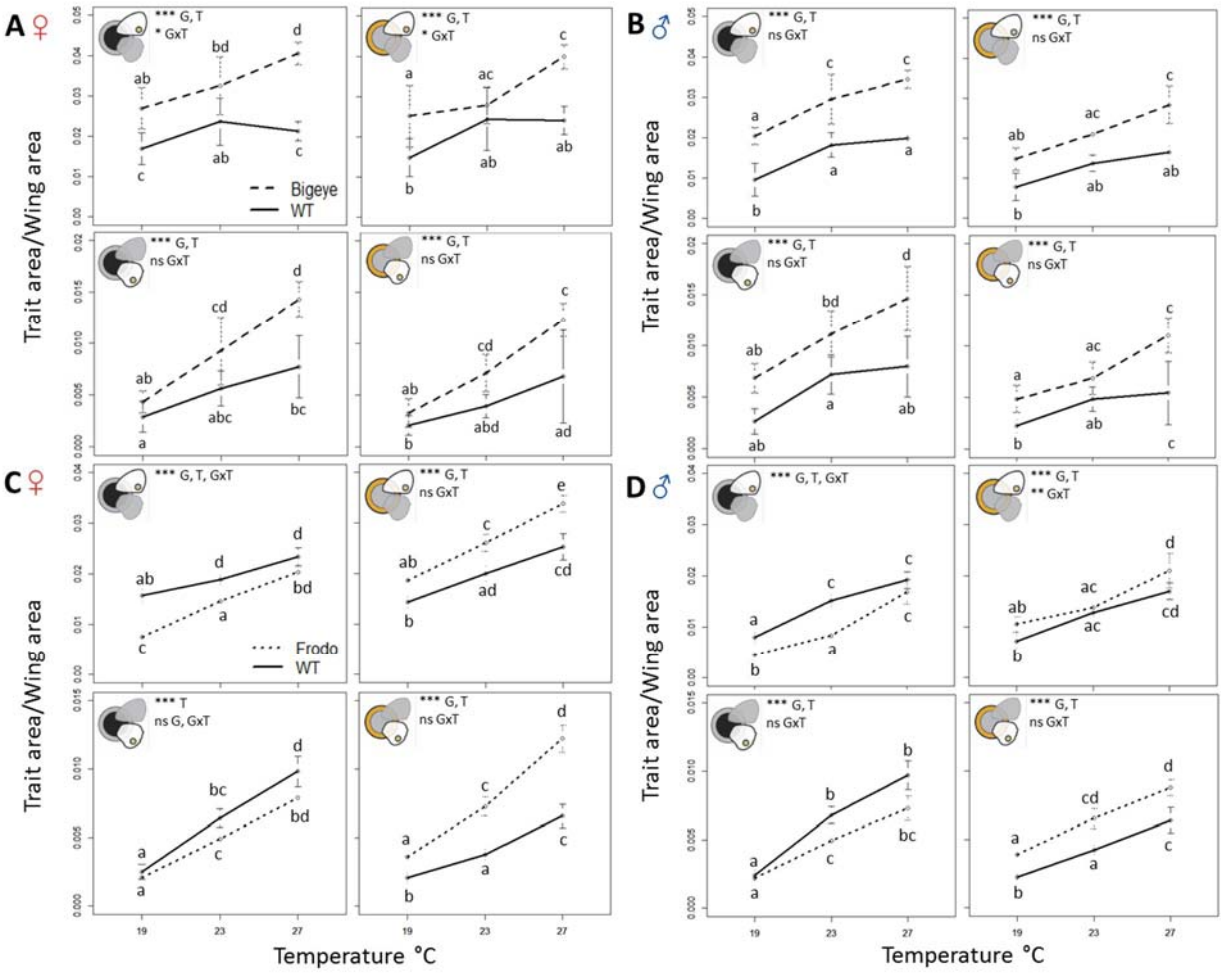

Figure 4.4 - Thermal reaction norms for eyespot traits for mutant (heterozygous at BFS locus) and wildtype (homozygous for wildtype allele) in the BE and Fr genetic stocks. Panels A (females) and B (males) show the means (and standard deviations) for eyespot ring areas (relative to corresponding wing area) across developmental temperatures for BE. Panels C (females) and D (males) show the equivalent plots for Fr. Top panels show the results for eP on the forewing and bottom panels for e5 on the hindwing (icons on the top left corner of each plot cf. Figure 4.1). We tested for the effect of temperature and phenotype on relative eyespot ring area using the model *ring area~wing area+ temperature*phenotype* (see Material and Methods and Annex 4.6). When we found significant effects of temperature or/ and genotype on trait value $P < 0.05$, we compared across temperatures (Tukey HDS, $P < 0.01$). Statistical significance for effects of temperature, genotype and GxT on black and gold areas are indicated on the top left corner of each reaction norm: ^{ns} (non-significant) $P>0.05$, $*P<0.05$, $** P < 0.01$, $*** P < 0.001$ (see Material and Methods and Annex 4.6 for more details on these statistical analyses). For each reaction norm, different letters indicate pairwise comparisons that revealed statistically significant differences.

Figure 4.4 shows that BE reaction norms are always highest and, in most of the cases, steepest, as we can observe by comparing the differences between trait means across temperatures between BE and the sibling wildtype (Tukey HSD tests, Annex 4.6). In BE, temperature and genotype have significant effects for all traits for both sexes (see Figure 4.4A and B and Annex 4.6), and the interaction GxT has significant effect for the forewing traits of females (see Figure 4.4A and Annex 4.6).

For Fr relative to wildtype siblings, the height of the reaction norms is lower for black eyespot rings and higher for gold eyespot rings (Figure 4.4). Temperature and genotype have significant effects for all traits for both sexes (see Figure 4.4A and B and Annex 4.6), except for the black eyespot rings on female hindwings (Figure 4.4C). GxT has significant effect for the black ring of eP in females (Figure 4.4C) and for both rings of the same eyespot in males (Figure 4.4D, details in Annex 4.6) representing significant differences in reaction norm shape.

DISCUSSION

Reaction norms are an important tool in the study of developmental plasticity (Schlichting & Pigliucci 1998). By representing thermal reaction norms of different *B. anynana* genetic stocks we were able to assess the genetic, temperature, and genetic-by-temperature effects on eyespot ring variation. Both black and gold rings, for females and males, from all genotypes show strong thermal plasticity.

For several traits there is evidence for a prevalence of GxE effects, for both genders, seen in principal components (Figure 4.2) and in individual traits (Annexes 4.3 and 4.4). Between siblings differing in a single allele affecting eyespot size (BE) and color composition (Fr) (Figure 4.4) only for eyespots on the forewing did we see GxE effects.

Principal components analysis and trait responses to temperature and genotype

Globally, the differences in the reaction norms slope/shape for each Dim show that BE followed by Fr were more responsive to developmental temperature compared to WT and Choc genotypes (Figure 4.2 and Annex 4.2). Some traits stood out in our analysis. Dim 1 increased with temperature (Figure 4.2) reflecting the increase with temperature of all eyespot ring areas that has been amply described for this species (e.g. Brakefield *et al.* 1996, Oostra *et al.* 2011, Mateus *et al.* 2014, CHAPTER 2). Dim 2 contrasted black versus gold eyespot areas which we had shown to have different patterns of

response to hormone manipulations (Mateus *et al.* 2014, CHAPTER 2). BE and Fr are the genotypes that most contribute to this contrast (see Annex 4.1), probably because BE shows larger black areas and Fr larger gold areas (Figure 4.1) in relation to the other genotypes, especially for higher temperatures. Curiously, the two color rings that contributed little to Dim 2 (black e2 and gold eP) also stood out in their response to our hormone manipulations (Mateus *et al.* 2014, CHAPTER 2). The black area of eyespot e2 showed the highest response within the black areas that were analysed and the gold area of eyespot eP was shown to be the least responsive of all gold rings.

Dim 3 for females contrasts anterior versus posterior eyespots, for which the golden rings also also showed differences in response to hormone manipulations (Mateus *et al.* 2014, CHAPTER 2). The gold area of eyespot e2 for females stands out as it did in our previous study, which also stood out in for being the only exception to the division between forewing versus hindwing in relation to the patterns of response to temperature (Mateus *et al.* 2014, CHAPTER 2). However, while for females just Genotype appears has a significant factor to explain variation in Dim 3, for males Temperature is also contributing significantly. The effect of Temperature on a variable defined by the contrast between male forewing versus hindwing eyespots is consistent with constrasting responses between female forewing versus hinwing eyespots we documented before in relation to developmental temperature (Mateus *et al.* 2014, CHAPTER 2).

Evidence of GxE effects: BE and Fr stand out in their response to temperature

BE, a mutant for eyespot size, showed larger eyespot color rings with increased developmental temperatures. For all genotypes, the hindwing eyespots, e2 and e5, seem more sensitive to temperature, as they show higher differences between means across temperartures, than forewing eyespots, eA and eP (Figure 4.3 and Annexes 4.4 and 4.5). Particularly for eP in females, we had argued before that lower thermal plasticity is probably a reflection of the fact this eyespot is typically hidden by the hindwing and, thus, less exposed to predators in butterflies at rest (Chapter 2, Mateus *et al.* 2014). However, here we only see lower change with temperature for this eyespot in WT females.

For eyespot color composition, measured as the proportion of black to gold areas, Fr, a mutant, characterized by broader eyespot golden rings (Saenko *et al.* 2010), shows a clear distinction from the other phenotypes (see Figure 3 and Annex 3). The proportionally larger golden rings in Fr eyespots are seen across developmental

temperatures but especially for higher temperatures. Curiously, eyespot e2 color composition is similar across genotypes (see Figure 4.3 and Annex 4.3) but the most different across temperatures. Previous work had shown this eyespot to be not only very plastic in relation to temperature but also to hormone manipulations, its gold ring having the largest window of sensitivity to the latter (Chapter 2, Mateus *et al.* 2014).

In general, we find differences in environmental responses between genotypes, however we do not know whether these or other alleles at the same loci contribute to the evolution of plasticity or affect ecdysone dynamics (e.g. regulating hormone titers or the timing of hormone secretion).

Alleles affecting pigmentation can affect plasticity therein

In Annex 4.4 we showed that BE and Fr stand out for thermal plasticity, having different reaction norms for eyespot rings relative to WT and Choc genotypes which have "wild-type" like eyespots. Still, because these stocks differ not only for the allele of strong effect responsible for the pigmentation phenotype but also in genetic background, we proceeded to analyse if the BE and Fr alleles alone resulted in different plasticity. To investigate this, we compared thermal reaction norms between "sibling" mutant and wildtype-looking individuals (wt) segregating in each stock.

For both sexes, our results show differences in the shape and/or height of reaction norms between the mutant and the wildtype individuals. BE reaction norms are always higher in comparison with the sibling wt phenotype, consistent with BE's characteristic effect of enlarging all eyespots. For Fr, the height of the reaction norms for the black areas is lower in comparison with the sibling wt phenotype and is higher for gold areas (Figure 4.4C and D), consistent with Fr's effect of enlarging eyespot golden rings.

GxT effects were not found for all our target traits. In fact, for BE, only eyespots on the forewing of females, and for Fr all eyespots in the forewing except the gold area of females, show significant GxT effects (Figure 4.4 and Annex 4.6). We showed before that there are differences in response to temperature between eyespot rings on different wings (Mateus *et al.* 2014, CHAPTER 2): color elements on the forewing responding to temperature differently from color elements on the hindwing.

Previous work analyzing thermal plasticity for the pigmentation of different abdominal segments of *D. melanogaster* found differences in the shape, height, and slope of reaction norms (Gibert *et al.* 2007). The authors proposed that the spatio-

temporal expression of pigmentation enzymes responsible for melanine production in the abdomen is differentially thermosensitive across body segments (Gibert *et al.* 2007). Our results also show evidence that alleles affecting pigmentation can affect plasticity therein. The fact that we just see this result for the forewing suggests organ-specific effects on temperature sensitivity.

We show that different genotypes have different thermal sensitivities reflected by different reaction norm slopes/shapes. Alleles affecting environmental sensitivity can fuel genetic accommodation and the evolution of plasticity (West-Eberhard 2003, 2005). Increased environmental sensitivity can also enable the revelation of hidden genetic variation and enable further adaptive evolution upon environmental perturbation (Braendle & Flatt 2006, Gibson & Dworkin 2004, Suzuki & Nijhout 2006).

CONCLUSIONS

Developmental plasticity may be described as a phenotypic result of the effects of environmental variation, in interaction with genetic variation, on development, and can play an important role in evolution (West-Eberhard 2003).

Developmental plasticity and different properties of reaction norms are heritable traits that can vary between genotypes and can evolve. Our results (Figure 4.2-4.4, Annexes 4.3-4.4) show evidence for GxE for many *B. anynana* eyespot patterns in the response to developmental temperature. BE and Fr mutants showed to be the most temperature-sensitive genotypes. These or other alleles at this locus might contribute to genetic accommodation and the evolution of plasticity (West-Eberhard 2003, 2005, Gibson & Dworkin 2004), and possibly even mediating the origin of novel adaptive phenotypes (Suzuki & Nijhout 2006).

We show evidence that alleles affecting pigmentation can affect plasticity therein. Genotypes differ in how traits are affected by temperature, including organ-specific effects, however we do not know what the underlying mechanisms (e.g. effects on ecdysone dynamics). The analysis of the interactions between temperature with ecdysone dynamics, developmental genes, and pigmentation genes will help to understand the thermal regulation of pigmentation development. We also do not know to what extent alleles such as these contribute to the evolution of plasticity (de Jong *et al.* 2013).

ACKNOWLEDGEMENTS

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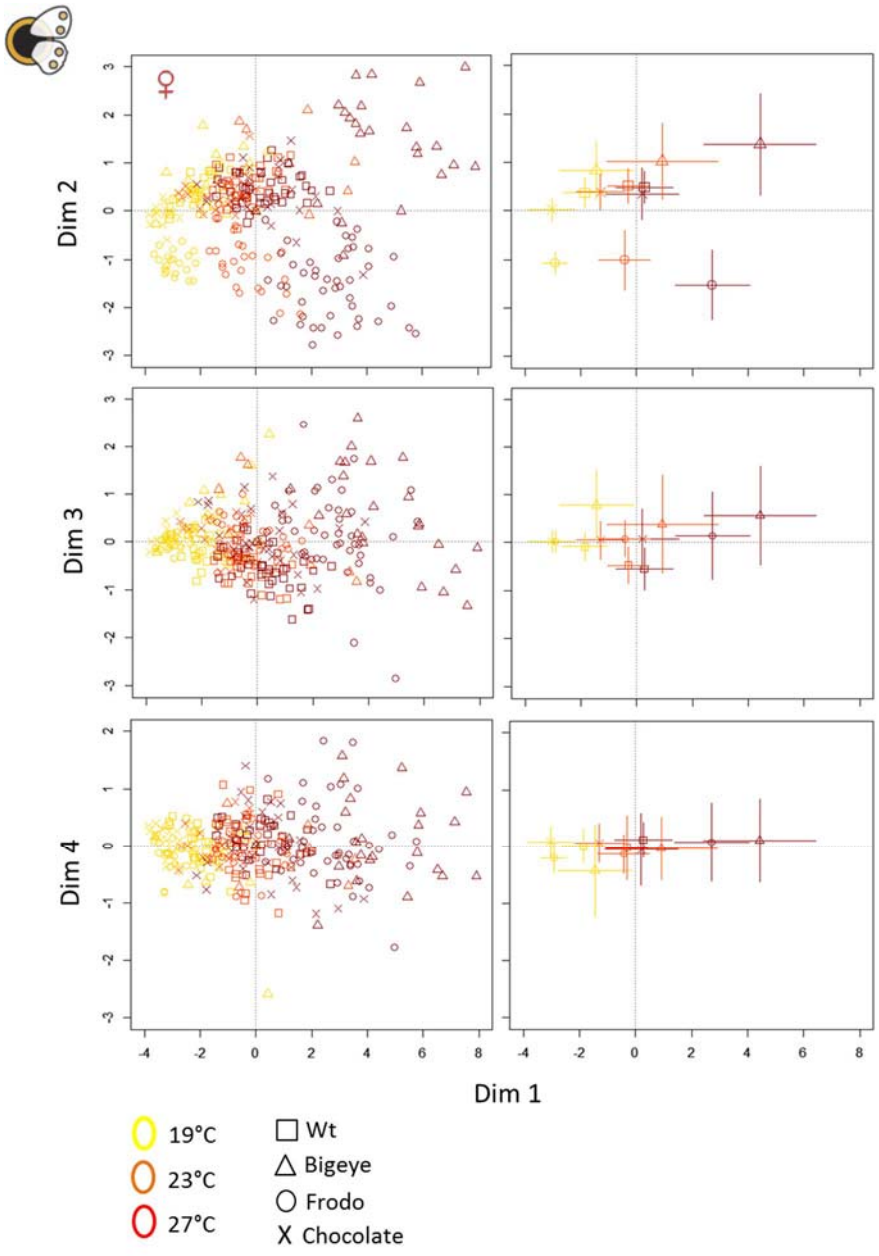
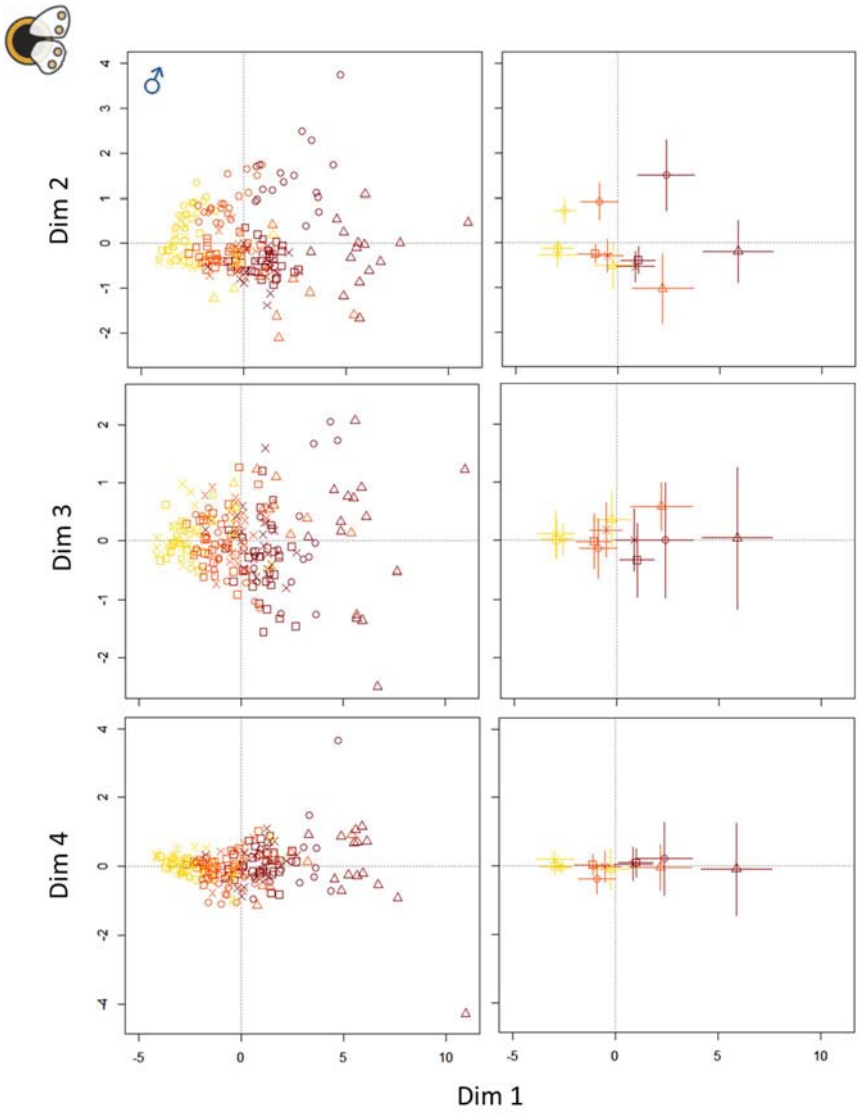
ANNEXES

ANNEX 4.1

PCA for variation in eyespot traits with developmental temperature for different genotypes. The plots represent the scores for all measured individuals along Dims 1-4 separated by developmental temperature (symbol color) and genotype (symbol shape) for females and males. Left panels show all individuals for each group, and right panels show mean group values (± standard error). Similar patterns were found between sexes.




♂
Dim 2
Dim 3
Dim 4
Dim 1
-5
0
5
10

ANNEX 4.2

Summary of the statistical results for the first fourth Dims to test the effect of temperature (T) and genotype (G) for females and males (c.f. Figure 4.2, see sample sizes in Table 4.1). Statistical significance for effects of T, G and G:T is indicated as: *P < 0.05, ** P < 0.01, *** P < 0.001. When we found significant effects of each factor on trait value P < 0.05, we compared across temperatures (Tukey HDS, P < 0.01).

A - FEMALES **Model: Dim~Genotype*Temperature**

		Dim 1			Dim 2			Dim 3			Dim 4		
	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
G	3	377.17	94.91	<2.2e-16 ***	233.26	258.73	<2.2e-16 ***	29.94	27.66	8.19e-16 ***	0.53	0.69	0.55
T	2	829.11	312.96	<2.2e-16 ***	1.88	3.13	0.04 *	1.58	2.19	0.11	1.41	5.51	0.01 *
G:T	6	154.04	19.38	<2.2e-16 ***	8.08	4.48	0.0002 ***	3.78	1.74	0.10	2.48	3.21	0.02 *

HSD	Dim 1		Dim 2		Dim 3		Dim 4	
	Means	Groups	Means	Groups	Means	Groups	Means	Groups
WT_19	-1.852	ef	0.370	bc	-0.082	bc	0.008	a
WT_23	-0.302	cd	0.517	bc	-0.476	bc	-0.013	a
WT_27	0.277	c	0.483	bc	-0.549	c	0.124	a
Choc_19	-3.036	f	0.024	c	0.022	abc	0.088	a
Choc_23	-1.290	de	0.388	bc	0.054	abc	0.069	a
Choc_27	0.204	c	0.350	bc	0.071	ab	-0.049	a
Fr_19	-2.917	f	-1.068	de	0.023	abc	-0.198	a
Fr_23	-0.431	cd	-1.008	d	0.082	ab	-0.129	a
Fr_27	2.722	b	-1.520	e	0.141	ab	0.081	a
BE_19	-1.447	def	0.841	ab	0.779	a	-0.425	a
BE_23	0.914	c	1.025	ab	0.379	ab	-0.029	a
BE_27	4.427	a	1.377	a	0.565	a	0.112	a

B - MALES **Model: Dim~Genotype*Temperature**

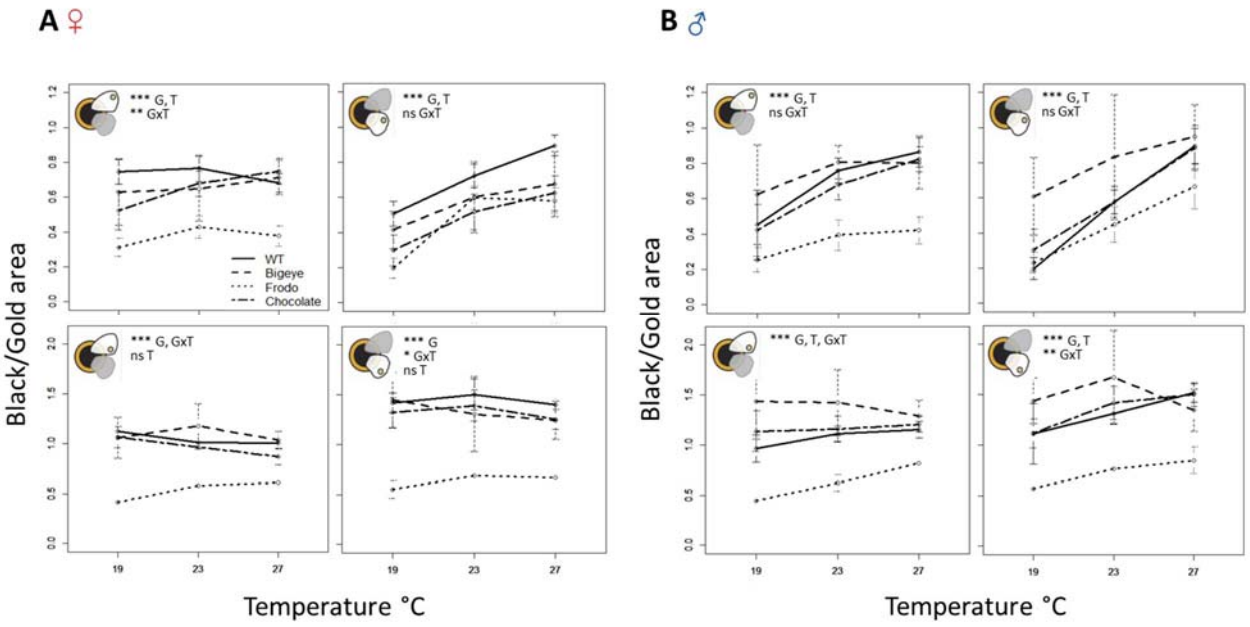
		Dim 1			Dim 2			Dim 3			Dim 4		
	Df	Dev	F	P	Dev[a]	F	P	Dev[a]	F	P	Dev	F	P
G	3	440.34	146.62	<2.2e-16 ***	-	140.50	<2.2e-16 ***	-	3.11	0.02 *	1.10	1.06	0.36
T	2	667.03	333.16	<2.2e-16 ***	-	1.32	0.26	-	3.43	0.03*	1.33	1.93	0.14
G:T	6	36.93	6.14	6.02e-06 ***	-	9.60	2.627e-09 ***	-	0.96	0.45	2.59	1.24	0.28

[a]: For Dims 2 and 3 we had to use **Model: ((Dim+3) ^lambda - 1)/lambda ~Genotype*Temperature**. With this model we have no Deviance.

HSD	Dim 1		Dim 2		Dim 3	
	Means	Groups	Means	Groups	Means	Groups
WT_19	-2.929	f	1.522	c	1.618	a
WT_23	-1.103	e	1.441	c	1.592	a
WT_27	1.015	cd	1.336	c	1.358	a
Choc_19	-2.943	f	1.424	c	1.682	a
Choc_23	-0.501	e	1.407	c	1.725	a
Choc_27	0.867	cd	1.231	cd	1.606	a
Fr_19	-2.596	f	2.082	b	1.619	a
Fr_23	-0.892	e	2.210	ab	1.507	a
Fr_27	2.378	b	2.565	cd	1.582	a
BE_19	-0.230	de	1.241	cd	1.846	a
BE_23	2.210	bc	0.825	d	1.995	a
BE_27	5.903	a	1.456	c	1.572	a

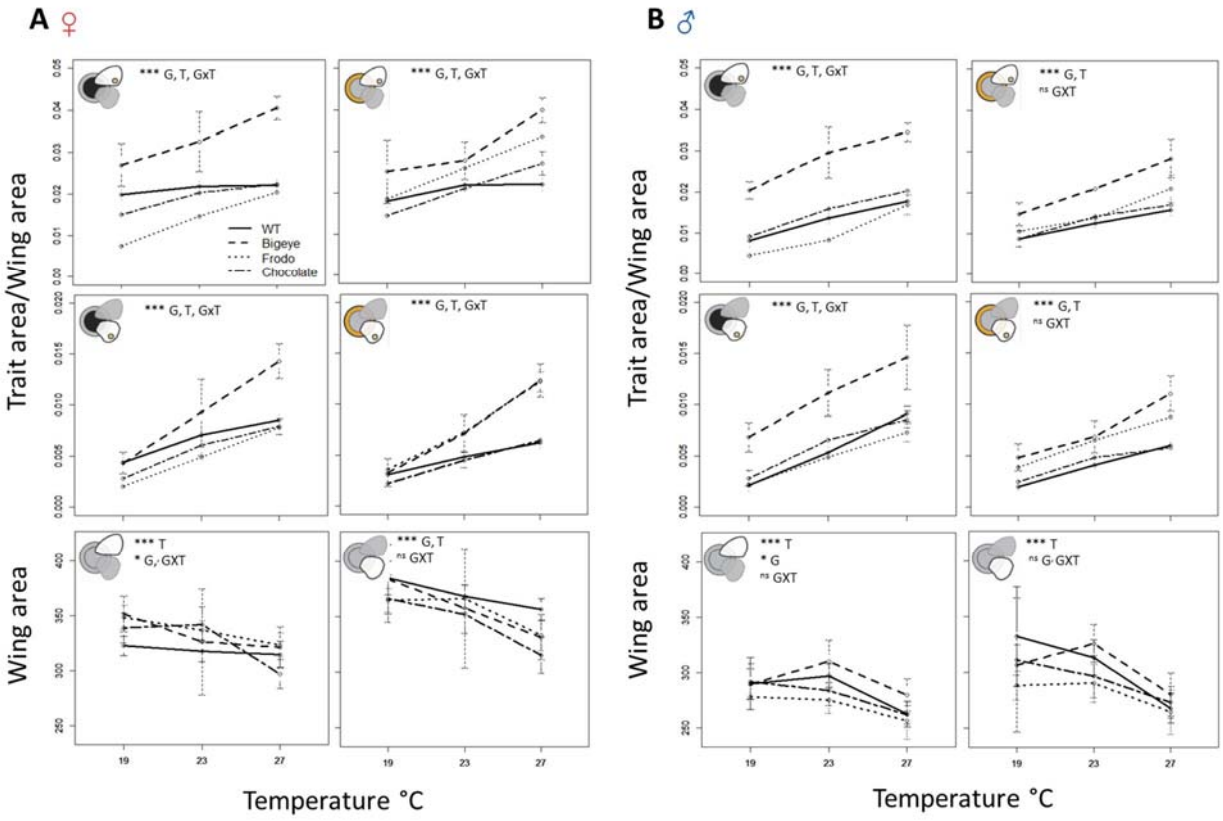
ANNEX 4.3

For each eyespot, we plotted the mean value of the proportion of the black/gold area as a function of temperature and use bars to represent the standard deviation of four genotypes (WT, Fr, BE and Choc). Females (panel A) and males (panel B) are represented separately and the different traits are represented by the respective icon (top left corner). We tested for the effect of temperature and genotype using the model *black/gold ~ temperature*genotype* (see Material and Methods and Annex 4.5). Statistical significance for the effects of temperature, genotype and GxT on wing traits (see Material and Methods) is indicated on the top left corner of each reaction norm [ns] (non-significant) P>0.05, *P<0.05, ** P < 0.01, *** P < 0.001. When we found significant effects of temperature and/ or genotype on trait value, we compared across temperatures (Tukey HDS, P < 0.01), (see Annex 4.5 for more details on these statistical analyses).



ANNEX 4.4

For each eyespot, we plotted the mean value of the black and gold areas as a function of temperature and use bars to represent the standard deviation for four genotypes (WT, Fr, BE and Choc). Females (panel A) and males (panel B) are represented separately and the different traits are represented by the respective icon (top left corner). We tested for the effect of temperature and genotype using the model *ring area ~ wing area+ temperature*genotype* (Annex 4.5, see Material and Methods). Statistical significance for the effects of temperature, genotype, and GxT on wing traits (see Material and Methods) is indicated on the top left corner of each reaction norm: ^{ns} (non-significant) P>0.05, *P<0.05, ** P < 0.01, *** P < 0.001. When we found significant effects of temperature and/ or genotype on trait value, we compared across temperatures (Tukey HDS, P < 0.01), (see Annex 4.5 for more details on these statistical analyses). For both sexes, BE and Fr genotypes show the most pronounced response across temperatures with black and gold areas showing similar levels of plasticity. Because results for trait size were similar between anterior and posterior eyespots (see Annex 4.5) we chose to show the reaction norms for the posterior traits to exemplify.



ANNEX 4.5

Summary of the statistical results for the size and color composition of black and gold areas and size of wing area (WA) to test the effect of temperature (T) and genotype (G) for females and males (c.f. Figure 4.3, Annexes 4.3 and 4.4, see sample sizes in Table 4.1). Statistical significance for effects of T, G and G:T are indicated as: *P < 0.05, ** P < 0.01, *** P < 0.001. When we found significant effects of temperature and/ or genotype on trait value, we compared across temperatures (Tukey HDS, P < 0.01)

TRAIT SIZE

A - FEMALES ♀ **Model: TraitArea~WingArea+Temperature*Genotype**

	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
WA	1	0.31	8.37	0.004 **	0.02	0.95	0.32	0.02	3.69	0.05	0.07	10.99	0.001 **
G	3	2.72	24.33	5.58e-14 ***	5.73	77.37	<2.2e-16 ***	3.73	192.05	<2.2e-16 ***	2.00	98.96	<2.2e-16 ***
T	2	11.46	153.79	<2.2e-16 ***	8.25	167.23	<2.2e-16 ***	1.87	144.10	<2.2e-16 ***	1.43	106.36	<2.2e-16 ***
G:T	6	1.38	6.17	4.34e-06 ***	0.83	5.64	1.53e-05 ***	1.04	26.90	<2.2e-16 ***	0.28	7.10	4.73e-07 ***

HSD	Means	Groups	Means	Groups	Means	Groups	Means	Groups
WT_19	2.517	a	-0.124	d	0.798	d	5.822	ef
WT_23	2.492	a	0.130	bc	0.830	d	6.958	de
WT_27	2.424	b	0.183	b	0.843	cd	6.929	de
Choc_19	2.491	a	-0.323	e	0.701	e	4.937	f
Choc_23	2.468	ab	0.002	cd	0.835	d	7.249	cde
Choc_27	2.434	ab	0.042	bcd	0.811	d	7.903	cd
Fr_19	2.458	ab	-0.047	d	0.410	f	6.481	def
Fr_23	2.460	ab	0.197	b	0.684	e	8.748	cd
Fr_27	2.419	b	0.484	a	0.806	d	10.910	b
BE_19	2.483	ab	-0.124	de	0.954	bc	9.254	bc
BE_23	2.513	a	0.102	bcd	1.008	ab	9.014	bcd
BE_27	2.445	ab	0.386	a	1.105	a	12.720	a

	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
WA	1	0.42	8.36	0.004 **	0.36	29.72	1.21e-07 ***	0.06	4.21	0.04 *	0.62	46.97	5.74e-11 ***
G	3	5.18	33.80	<2.2e-16 ***	4.28	117.81	<2.2e-16 ***	2.13	48.43	<2.2e-16 ***	3.96	99.37	<2.2e-16 ***
T	2	19.22	188.20	<2.2e-16 ***	5.30	218.76	<2.2e-16 ***	6.81	232.25	<2.2e-16 ***	6.13	230.59	<2.2e-16 ***
G:T	6	2.30	7.53	1.99e-07 ***	0.95	13.19	6.14e-13 ***	0.67	7.63	1.56e-07 ***	0.50	6.37	2.95e-06 ***

HSD								
	Means	Groups	Means	Groups	Means	Groups	Means	Groups
WT_19	-0.769	d	-0.445	e	0.214	c	0.068	e
WT_23	-0.420	abc	-0.267	c	0.406	b	0.243	cd
WT_27	-0.317	ab	-0.263	c	0.478	b	0.335	bc
Choc_19	-1.259	e	-0.656	f	0.001	d	-0.110	f
Choc_23	-0.724	cd	-0.402	de	0.308	bc	0.174	de
Choc_27	-0.528	bc	-0.286	cd	0.379	b	0.289	bcd
Fr_19	-1.248	e	-0.485	e	-0.172	e	0.103	e
Fr_23	-0.375	ab	-0.135	bc	0.242	c	0.414	b
Fr_27	-0.218	a	0.063	a	0.407	b	0.594	a
BE_19	-0.881	de	-0.459	e	0.195	c	0.048	ef
BE_23	-0.376	abc	-0.128	bc	0.494	ab	0.392	bc
BE_27	-0.274	ab	-0.008	ab	0.658	a	0.591	a

	Df	Dev	F	P	Dev	F	P
G[a]	3	11695	3.83	0.01 *	34993	8.92	1.23e-05 ***
T	2	33935	16.68	1.40e-07 ***	70406	26.93	2.62e-11 ***
G:T	6	17024	2.79	0.01 *	8162	1.04	0.39

[a]: For wing areas we used **Model: WingArea~Temperature*Genotype**.

HSD				
	Means	Groups	Means	Groups
WT_19	322.5	ab	384.8	a
WT_23	317.6	ab	368.1	a
WT_27	314.7	ab	356.3	abc
Choc_19	338.8	a	365.9	ab
Choc_23	341.6	a	351.8	abc
Choc_27	296.6	b	315.2	c
Fr_19	348.4	a	364.7	ab
Fr_23	336.7	a	336.7	ab
Fr_27	323.3	ab	323.3	bc
BE_19	351.5	a	351.5	a
BE_23	326	ab	326	abc
BE_27	321	ab	321	bc

B - MALES ♂

Model: TraitArea~WingArea+Temperature*Genotype

	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
WA	1	0.01	0.20	0.65	0.01	0.04	0.83	43.37	47.16	7.89e-11 ***	0.10	8.84	0.003 **
G	3	24.54	89.39	<2.2e-16 ***	2.59	38.30	<2e-16 ***	690.30	250.20	<2.2e-16 ***	1.57	42.42	<2.2e-16 ***
T	2	25.79	140.93	<2.2e-16 ***	6.47	143.44	<2e-16 ***	287.62	156.37	<2.2e-16 ***	1.57	42.42	<2.2e-16 ***
G:T	6	1.68	3.07	0.006 **	0.35	2.59	0.01	17.22	3.12	0.006 **	0.08	1.08	0.37

HSD							
	Means	Groups	Means	Groups	Means	Groups	Means	Groups
WT_19	0.207	g	2.566	f	0.355	e	2.566	e
WT_23	0.697	ef	3.720	cd	0.594	d	3.720	de
WT_27	1.133	bc	4.107	b	0.657	cd	4.107	de
Choc_19	0.240	g	2.484	ef	0.389	e	2.484	e
Choc_23	0.758	def	4.093	bcd	0.646	cd	4.093	de
Choc_27	1.054	bcd	4.467	bc	0.718	c	4.467	cd
Fr_19	0.190	g	2.949	de	0.083	f	2.949	e
Fr_23	0.419	fg	3.792	bcd	0.340	e	3.792	de
Fr_27	0.890	cde	5.508	a	0.618	cd	5.508	bc
BE_19	0.665	efg	4.250	bcd	0.770	bc	4.250	cde
BE_23	1.426	b	6.468	ab	0.948	ab	6.468	ab
BE_27	2.052	a	7.911	a	0.979	a	7.911	a

	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
WA	1	0.17	6.38	0.01 *	0.03	2.41	0.12	0.01	0.25	0.61	0.05	6.23	0.01 *
G	3	4.66	57.16	<2e-16 ***	1.83	42.49	<2e-16 ***	3.62	85.83	<2.2e-16 ***	1.78	68.51	<2e-16 ***
T	2	7.01	128.97	<2e-16 ***	3.78	131.92	<2e-16 ***	6.13	218.05	<2.2e-16 ***	3.24	186.45	<2e-16 ***
G:T	6	0.42	2.62	0.01 *	0.24	2.86	0.01 *	0.28	3.32	0.003 **	0.10	1.96	0.07

HSD								
	Means	Groups	Means	Groups	Means	Groups	Means	Groups
WT_19	0.069	e	-0.236	de	0.214	e	-0.194	g
WT_23	0.312	d	-0.284	bc	0.406	d	0.099	ef
WT_27	0.530	b	0.530	d	0.478	bc	0.198	cd
Choc_19	0.075	e	-0.663	e	0.001	e	-0.134	g
Choc_23	0.314	d	-0.280	bc	0.308	cd	0.140	def
Choc_27	0.519	bc	-0.245	b	0.379	bc	0.188	cde
Fr_19	0.101	de	0.101	cd	-0.213	e	0.041	f
Fr_23	0.318	cd	0.318	b	0.140	d	0.265	bc
Fr_27	0.637	b	-0.026	a	0.273	cd	0.359	ab
BE_19	0.300	de	-0.308	bcd	0.315	bcd	0.160	cdef
BE_23	0.668	b	-0.095	ab	0.553	ab	0.341	abc
BE_27	1.112	a	0.075	a	0.590	a	0.476	a

	Df	Dev	 F	P	Dev	 F	P
G[a]	3	8026	3.32	0.02 *	0.01	1.76	0.15
T	2	35218	21.89	2.4e-09 ***	0.13	25.71	1.52e-10 ***
G:T	6	3798	0.78	0.58	0.01	1.18	0.31

[a]: For wing areas we used **Model: WingArea~Temperature*Genotype**.

HSD				
	Means	Groups	Means	Groups
WT_19	290	ab	2.517	a
WT_23	297	a	2.492	a
WT_27	262.4	b	2.424	b
Choc_19	291.9	ab	2.491	a
Choc_23	283.7	ab	2.468	ab
Choc_27	261.8	b	2.434	ab
Fr_19	256.2	ab	2.458	ab
Fr_23	275.3	ab	2.460	ab
Fr_27	256.2	b	2.419	b
BE_19	289.5	ab	2.483	ab
BE_23	309.9	a	2.513	a
BE_27	279.8	ab	2.445	ab

COLOR COMPOSITION

A - FEMALES ♀ **Model: EyespotColor~Temperature*Genotype**

	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
G	3	4.91	67.72	<2.2e-16 ***	13.32	140.78	<2.2e-16 ***	2.26	17.37	2.92e-10 ***	5.87	177.16	<2e-16 ***
T	2	0.35	7.41	0.0007 ***	0.05	0.89	0.41	5.02	57.74	<2.2e-16 ***	0.04	2.02	0.13
G:T	6	0.43	3.02	0.007 **	1.30	6.89	8.14e-07 ***	0.38	1.47	0.18	0.15	2.26	0.03 *

HSD								
	Means	Groups	Means	Groups	Means	Groups	Means	Groups
WT_19	-0.148	a	0.145	a	0.506	cd	0.145	a
WT_23	-0.130	a	0.162	ab	0.722	ab	0.162	a
WT_27	-0.174	ab	0.143	ab	0.896	a	0.143	a
Choc_19	-0.348	bcd	0.112	ab	0.298	de	0.112	a
Choc_23	-0.179	ab	0.134	ab	0.518	bcd	0.134	a
Choc_27	-0.139	a	0.090	b	0.624	bc	0.090	a
Fr_19	-0.530	d	-0.285	d	0.196	e	-0.285	b
Fr_23	-0.396	cd	-0.172	cd	0.597	bcd	-0.172	b
Fr_27	-0.460	d	-0.187	c	0.582	bcd	-0.187	b
BE_19	-0.231	abc	0.146	ab	0.414	cde	0.146	a
BE_23	-0.215	abc	0.102	a	0.600	bcd	0.102	a
BE_27	-0.164	ab	0.066	ab	0.675	abc	0.066	a

A - MALES ♂ **Model: EyespotColor~Temperature*Genotype**

	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
G	3	4.30	34.61	<2.2e-16 ***	3.58	98.15	<2.2e-16 ***	3.05	21.13	1.04e-11 ***	2.97	109.25	<2.2e-16 ***
T	2	3.48	41.99	5.62e-16 ***	0.35	14.48	1.32e-06 ***	6.83	70.99	< 2.2e-16 ***	0.44	24.58	3.73e-10 ***
G:T	6	0.34	1.38	0.22	0.40	5.51	2.56e-05 ***	0.36	1.27	0.27	0.16	3.09	0.006 **

HSD								
	Means	Groups	Means	Groups	Means	Groups	Means	Groups
WT_19	0.452	bcd	-0.027	ab	0.198	c	0.030	bcd
WT_23	0.760	ab	0.038	a	0.577	bc	0.108	ab
WT_27	0.863	a	0.053	a	0.893	a	0.176	a
Choc_19	0.421	cd	0.027	ab	0.303	c	0.033	bc
Choc_23	0.677	abc	0.051	a	0.577	bc	0.138	ab
Choc_27	0.824	a	0.077	a	0.885	a	0.169	a
Fr_19	0.255	d	-0.367	c	0.231	c	-0.255	e
Fr_23	0.394	d	-0.231	c	0.449	bc	-0.124	d
Fr_27	0.421	cd	-0.092	b	0.666	ab	-0.085	cd
BE_19	0.623	abcd	0.145	a	0.609	abc	0.154	ab
BE_23	0.807	ab	0.138	a	0.832	ab	0.212	a
BE_27	0.805	ab	0.097	a	0.949	a	0.113	ab

ANNEX 4.6

Summary of the statistical results for the size of black and gold areas and size of wing area (WA) to test the effect of temperature (T) and phenotype (P) for females and males of BE and Fr mutant and wt phenotypes respectively (c.f. Figure 4.4). Statistical significance for effects of T, P and P:T are indicated as: *P < 0.05, ** P < 0.01, *** P < 0.001. When we found significant effects of temperature and/ or genotype on trait value, we compared across temperatures (Tukey HDS, P < 0.01)

A - BE FEMALE ♀ **Model: TraitArea~WingArea+Temperature*Phenotype**

FW- BE: 19C N=10, 23C N=7, 27C N=22; **wt**: 19C N=5, 23C N=5, 27C N=7
HW- BE: 19C N=9, 23C N=6, 27C N=19; **wt**: 19C N=4, 23C N=5, 27C N=5

	Df	 Dev	F	P	 Dev	F	P	 Dev	F	P	 Dev	F	P
WA	1	63.16	15.70	0.0002 ***	0.03	3.48	0.06	0.41	0.41	0.52	0.11	4.48	0.04 *
P	1	279.12	69.37	6.02e-11 ***	0.53	53.13	2.36e-09 ***	36.30	36.79	3.49e-07 ***	1.29	50.56	1.15e-08 ***
T	2	127.81	15.88	4.81e-06 ***	0.34	17.29	2.07e-06 ***	68.17	34.54	1.60e-09 ***	1.81	35.44	1.15e-09 ***
P:T	2	26.67	3.31	0.04	0.06	3.17	0.05 *	6.21	3.15	0.05	0.02	0.49	0.61

HSD	 Means	Groups	 Means	Groups	 Means	Groups	 Means	Groups
wt_19	5.456	c	0.671	b	-0.018	a	-0.151	b
wt_23	7.383	ab	0.874	ab	0.249	abc	0.094	cd
wt_27	6.694	c	0.871	ab	0.366	bc	0.277	c
BE_19	9.372	ab	0.944	ab	0.195	ab	0.048	ab
BE_23	10.69	bd	0.945	a	0.494	cd	0.392	cd
BE_27	13	d	1.097	c	0.658	d	0.591	c

	Df	 Dev	F	P	 Dev	F	P
P[a]	1	1431.8	1.02	0.31	5798.0	3.69	0.06
T	2	6088.8	2.17	0.12	18592.5	5.93	0.005 **
P:T	2	813.5	0.29	0.74	848.9	0.27	0.76

[a]: For wing areas we used **Model: WingArea~Temperature*Phenotype**.

HSD				
	Means	Groups	Means	Groups
wt_19	-	-	343.7	a
wt_23	-	-	324.2	a
wt_27	-	-	313	a
BE_19	-	-	384.1	a
BE_23	-	-	357.5	a
BE_27	-	-	331.4	a

B - BE MALE ♂

Model: TraitArea~WingArea+Temperature*Phenotype

FW- BE: 19C N=6, 23C N=8, 27C N=15; **wt**: 19C N=5, 23C N=9, 27C N=4
HW- BE: 19C N=5, 23C N=6, 27C N=13; **wt**: 19C N=5, 23C N=9, 27C N=4

	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
WA	1	11.21	6.87	0.01 *	0.01	1.46	0.23	0.06	0.03	0.84	0.01	0.88	0.35
P	1	194.53	19.23	1.43e-13 ***	0.93	106.03	8.24e-13 ***	1.23	68.15	7.98e-10 ***	1.25	82.43	7.67e-11 ***
T	2	79.98	24.51	1.12e-07 ***	0.49	27.92	2.57e-08 ***	0.81	22.44	4.70e-07 ***	0.54	17.87	4.05e-06 ***
P:T	2	1.71	0.52	0.59	0.05	0.32	0.72	0.06	1.72	0.19	0.03	1.09	0.34

HSD								
	Means	Groups	Means	Groups	Means	Groups	Means	Groups
wt_19	0.428	b	2.304	b	-0.100	c	-0.159	b
wt_23	0.689	a	3.762	ab	0.280	a	0.111	ab
wt_27	0.708	a	4.231	ab	0.301	ab	0.120	ab
BE_19	0.770	a	4.250	ab	0.315	ab	0.160	a
BE_23	0.948	c	6.468	ac	0.553	bd	0.341	ac
BE_27	0.979	c	7.911	c	0.590	d	0.476	c

	Df	Dev	F	P	Dev	F	P
P[a]	1	1901.1	3.16	0.08	1128.7	1.11	0.29
T	2	5159.1	4.29	0.02 *	12864.9	6.37	0.004 **
P:T	2	2622.5	2.18	0.12	3997.5	1.98	0.15

[a]: For wing areas we used **Model: WingArea~Temperature*Phenotype**.

HSD				
	Means	Groups	Means	Groups
wt_19	294.8	a	316	a
wt_23	275.6	a	283.7	a
wt_27	258	a	257.9	a
BE_19	289.5	a	306.6	a
BE_23	309.9	a	325.9	a
BE_27	279.8	a	280.2	a

C - Fr FEMALE ♀

Model: TraitArea~WingArea+Temperature*Phenotype

FW- Fr: 19C N=20, 23C N=27, 27C N=44; **wt**: 19C N=22, 23C N=35, 27C N=21
HW- Fr: 19C N=14, 23C N=23, 27C N=40; **wt**: 19C N=19, 23C N=34, 27C N=18

	Df	 Dev	F	P	 Dev	F	P	 Dev	F	P	 Dev	F	P
WA	1	0.05	7.50	0.006 **	2.78	1.25	0.26	0.10	4.53	0.03 *	0.20	15.36	0.0001 ***
P	1	0.63	92.74	<2.2e-16 ***	251.07	112.81	<2e-16 ***	0.03	1.72	0.19	4.20	308.14	<2.2e-16 ***
T	2	2.18	159.04	<2.2e-16 ***	448.43	100.74	<2e-16 ***	7.05	158.11	<2e-16 ***	5.03	184.60	<2.2e-16 ***
P:T	2	0.43	31.58	2.6e-12 ***	9.18	2.06	0.13	0.05	0.01	0.98	0.01	0.58	0.55

HSD								
	Means	Groups	Means	Groups	Means	Groups	Means	Groups
wt_19	0.410	ab	5.044	b	-0.068	a	-0.124	b
wt_23	0.817	d	7.074	ad	0.334	bc	0.414	a
wt_27	0.880	d	8.376	cd	0.526	d	0.349	c
BE_19	0.410	c	6.480	ab	-0.172	a	0.103	a
BE_23	0.684	a	8.748	c	0.242	c	0.414	c
BE_27	0.806	bd	10.910	e	0.407	bd	0.594	d

	Df	 Dev	F	P	 Dev	F	P
P[a]	1	6948.2	7.62	0.006 **	0.009	4.63	0.03 *
T	2	14495.9	7.95	0.0005 ***	0.03	8.51	0.0003 ***
P:T	2	1048.0	0.57	0.56	0.009	2.19	0.11

[a]: For wing areas we used **Model: WingArea~Temperature*Phenotype**.

HSD				
	Means	Groups	Means	Groups
wt_19	350.3	a	376.5	a
wt_23	336.7	a	357.7	ab
wt_27	323.3	ab	353.7	ab
BE_19	348.4	ab	364.7	ab
BE_23	336.7	ab	365.9	ab
BE_27	323.3	b	333.1	b

D - Fr MALE ♂

Model: TraitArea~WingArea+Temperature*Phenotype

FW- Fr: 19C N=18, 23C N=20, 27C N=17; **wt**: 19C N=15, 23C N=37, 27C N=16
HW- Fr: 19C N=17, 23C N=16, 27C N=15; **wt**: 19C N=15, 23C N=37, 27C N=15

	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
WA	1	0.01	1.13	0.28	0.02	2.24	0.13	0.001	0.12	0.72	0.004	0.004	0.94
P	1	1.43	128.75	<2.2e-16 ***	0.17	18.40	3.72e-05 ***	0.58	44.53	1.11e-09 ***	0.79	96.37	<2e-16 ***
T	2	3.53	158.25	<2.2e-16 ***	1.74	90.79	<2.2e-16 ***	5.08	194.08	<2.2e-16 ***	2.20	133.17	<2e-16 ***
P:T	2	0.23	10.34	7.35e-05 ***	0.10	5.30	0.006 **	0.05	1.96	0.14	0.02	1.55	0.21

HSD								
	Means	Groups	Means	Groups	Means	Groups	Means	Groups
wt_19	0.338	a	1.998	b	-0.167	a	-0.180	b
wt_23	0.601	c	3.475	ac	0.140	b	0.077	a
wt_27	0.673	c	4.267	cd	0.383	b	0.198	c
BE_19	0.083	b	2.949	ab	-0.213	a	0.041	a
BE_23	0.340	a	3.792	ac	0.140	c	0.265	cd
BE_27	0.618	c	5.508	d	0.273	bc	0.358	d

	Df	Dev	F	P	Dev	F	P
P [a]	1	242.2	0.29	0.58	250.4	0.23	0.62
T	2	12418.1	7.66	0.0007 ***	20491.5	9.69	0.0001 ***
P:T	2	361.1	0.22	0.80	838.2	0.39	0.67

[a]: For wing areas we used **Model: WingArea~Temperature*Phenotype**.

HSD				
	Means	Groups	Means	Groups
wt_19	280.3	a	280.3	a
wt_23	270.1	a	270.1	a
wt_27	249.7	a	249.7	ab
BE_19	278.3	a	288	ab
BE_23	275.3	a	290	ab
BE_27	256.2	a	264.2	ab

REFERENCES

Aubret F, Shine R (2009) Genetic assimilation and the post-colonization erosion of phenotypic plasticity in island tiger snakes. *Current Biology* **19**, 1932.

Beldade P, Brakefield PM (2002) The genetics and evo–devo of butterfly wing patterns. *Nature Review Genetics* **3**, 442-452.

Beldade P, Mateus ARA, Keller RA (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology* **20**, 1347-1363.

Bento G, Ogawa A, Sommer RJ (2010) Co-option of the hormone-signaling module dafachronic acid-DAF-12 in nematode evolution. *Nature* **466**, 494-497.

Brakefield PM, Frankino WA (2007) Polyphenisms in Lepidoptera: Multidisciplinary approaches to studies of evolution. In Phenotypic Plasticity in Insects. Edited by Ananthakrishnan TN, Whitman DW, pp 121-151. Plymouth: Science Publishers.

Brakefield PM, Larsen TB (1984) The evolutionary significance of dry and wet season forms in some tropical butterflies. *Biological Journal of the Linnean Society* **22**, 1-12.

Brakefield PM, Kesbeke F, Koch PB (1998) The regulation of phenotypic plasticity of eyespots in the butterfly *Bicyclus anynana*. *American Nat*uralist **152**, 853-860.

Brakefield PM, Zwaan BJ (2011) Seasonal polyphenisms and environmentally-induced plasticity in the Lepidoptera - the coordinated evolution of many traits on multiple levels. pp. 243–252 in T. Flatt and A. Heyland, eds. Mechanisms of life history evolution: the genetics andphysiology of life history traits and trade-offs. Oxford Univ.Press, Oxford, UK.

Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden P, Monteiro A, French V, Carroll S (1996) Development, plasticity and evolution of butterfly eyespot patterns. *Nature* **384**, 236-242.

Brakefield PM, Beldade P, Zwaan BJ (2009) The African butterfly *Bicyclus anynana*: a model for evolutionary genetics and evolutionary developmental biology. In Emerging Model Organisms: A Laboratory Manual. Edited by Behringer RR, Johnson AD, Krumlauf RE. New York: Cold Spring Harbor Laboratory Press: 291-329.

Braendle C, Flatt T (2006) A role for genetic accommodation in evolution? *Bioessays* **28**, 868-873.

Cheplick GP (2003) Evolutionary significance of genotypic variation in developmental reaction norms for a perennial grass in competition. *Evolutionary Ecology* **17**, 175-196.

David JR, Capy P, Gauthier JP (1990) Abdomen pigmentation and growth temperature in *Drosophila melanogaster*. Similarities and differences in the norms of reaction of successive segments. *Journal of Evolutionary Biology* **3**, 429-445.

de Jong M, Collins S, Beldade P, Brakefield PM, Zwaan BJ (2013) Footprints of selection in wild populations of *Bicyclus anynana* along a latitudinal cline. *Molecular Ecology* **22**, 341-353.

Gibert P, Moreteau B, David JR (2000) Developmental constraints on an adaptive plasticity: Reaction norms of pigmentation in adult segments of *Drosophila melanogaster*. *Evolution & Development* **2**, 249-260.

Gibert JM, Peronnet F, Schlötterer C (2007) Phenotypic Plasticity in *Drosophila* Pigmentation Caused by Temperature Sensitivity of a Chromatin Regulator Network. *PLoS Genetics* **3**, e30.

Gibson G, Dworkin I (2004) Uncovering cryptic genetic variation. *Nature Review Genetics* **5**, 681-690.

Jolliffe IT (1986) Principal Component Analysis. Springer, New York: Springer.

Husson F, Josse J (2010) missMDA - Handling missing values with/in multivariate data analysis (principal component methods). R package version 1.2.

Lê S, Josse J, Husson F (2008) FactorMineR: An R package for multivariate analysis. *Journal of Statistical Software* **25**, 1-18.

Mateus ARA, Marques-Pita M, Oostra V, Lafuente E, Brakefield PM, Zwaan BJ, Beldade P (2014) Adaptive developmental plasticity: Compartmentalized responses to environmental cues and to corresponding internal signals provide phenotypic flexibility. *BMC Biology* **12**, 97.

Oliver JM, Robertson KA, Monteiro A (2009) Accommodating natural and sexual selection in butterfly wing pattern evolution.

Proceedings of the Royal Society of London, Series B: Biological Sciences **276**, 2369-2375.

Olofsson M, Vallin A, Jakobsson S, Wiklund C (2010) Marginal Eyespots on Butterfly Wings Deflect Bird Attacks Under Low Light Intensities with UV Wavelengths. *PLoS One* **5**, e10798.

Oostra V, de Jong MA, Invergo B, Kesbeke F, Wende F, Brakefield PM, Zwaan BJ (2011) Translating environmental gradients into discontinuous reaction norms via hormone signaling in a polyphenic butterfly. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **278**, 789-797.

Oostra V, Mateus ARA, van der Burg KR, Piessens T, van Eijk M, Brakefield PM, Beldade P, Zwaan BJ (2014) Ecdysteroid hormones link the juvenile environment to alternative adult life histories in a seasonal insect. *American Naturalist* **184**, E79-E92.

R-Core-Team (2012) R - A Language and Environment for Statistical Computing.

Saenko SV, Brakefield PM, Beldade P (2010) Single locus affects embryonic segment polarity and multiple aspects of an adult evolutionary novelty. *BMC Biology* **8**, 111.

Saenko SV, Jerónimo MA, Beldade P (2012) Genetic basis of stage-specific melanism: a putative role for a cysteine sulfinic acid decarboxylase in insect pigmentation. *Heredity* **108**, 594-601.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji - an open source platform for biological image analysis. *Nature Methods* **9**, 676-682.

Schlichting CD, Pigliucci M (1998) Phenotypic Evolution: A Reaction Norm Perspective. Massachusetts: Sinauer Associates, Inc.

Sultan SE (1995) Phenotypic plasticity and plant adaptation. *Acta Botanica Neerlandica* **44**, 363-383.

Suzuki Y, Nijhout HF (2006) Evolution of a polyphenism by genetic accommodation. *Science* **311**, 650-652.

West-Eberhard MJ (2003) Developmental plasticity and evolution. Oxford, UK: Oxford University Press.

West-Eberhard MJ (2005) Phenotypic accommodation: adaptive innovation due to developmental plasticity. *Journal of Experimental Zoology* **304**, 610-618.

Wijngaarden PJ, Brakefield PM (2001) Lack of response to artificial selection on the slope of reaction norms for seasonal polyphenism in the butterfly *Bicyclus anynana*. *Heredity* **87**, 410-420.

Wray GA (2007) The evolutionary significance of cis-regulatory mutations. *Nature Review Genetics* **8**, 206.

Zijlstra WG, Steigenga MJ, Koch PB, Zwaan BJ, Brakefield PM (2004) Butterfly selected lines explore the hormonal basis of interactions between life histories and morphology. *American Naturalist* **163**, E76-87.

CHAPTER 5. THERMAL PIGMENTATION PLASTICITY: PRELIMINARY RESULTS AND FUTURE DIRECTIONS ON THE SHAPE OF REACTION NORMS AND COLOR ANALYSIS

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ABSTRACT

Developmental plasticity, the ability of a single genotype to express different phenotypes in different environments, may evolve as an adaptive response to seasonality and is typically characterized by reaction norms. Temperature, one of the most important and common environmental factors regulating development is of extreme importance in regulating seasonal plasticity of insect´s pigmentation patterns, namely in butterflies. Here, we would like to explore the genotype (G), temperature (T), and GxT effects on *Bicyclus anynana* pigmentation patterns. *B. anynana* butterflies exhibit developmental plasticity for pigmentation patterns as an adaptive response to the alternating wet and dry seasons in their natural environment. In addition, this system also shows developmental plasticity for life-history traits. In order to explore GxT effects on *B. anynana* pigmentation patterns we derived artificial selected lines expressing extreme wet season-like or dry-season-like phenotypes at intermediate temperatures and characterized thermal reaction norms for several traits for a wide range of temperatures. Finally, for the first time in this species, we performed qualitative analysis of color and color patterns across temperature. Our preliminary results show that, for both sexes, there is a significant GxT interaction which confirms mean differences between the unselected stock and artificial selected lines responses in shape and height of reaction norms across temperature. Future directions include developing a detailed formal mathematical treatment of the influence of external

environment on development to characterize shape of thermal reaction norms. Curiously by selecting on extreme pigmentation patterns we were able to change other traits such as survivorship and pupal development time. These correlated responses to selection likely reflect genetic pleiotropy. However, we should be cautious about interpreting correlated responses between wing pattern (target of selection) and life-history traits, as we have no replication of the selection lines (see Material and Methods). We also show, for wing background color, that for low temperatures there are three groups of pigments and for high temperatures four well distinct groups. Our preliminary results also revealed a possible new color appearing at the most extreme low temperatures. We do not know what causes these differences, but we suggested that the orange color might correspond to a pigment from a different type or to a modification of a product of the melanin biosynthesis pathway. Our future work includes developing a general method to quantify color patterns possible to apply to most of the organisms.

INTRODUCTION

Coping with fluctuating external conditions is an important challenge for many organisms, such as those living in seasonal environments. Developmental plasticity, the ability of a single genotype to produce distinct phenotypes depending on the conditions experienced during development, can be a solution to cope with environmental fluctuations. The alternative phenotypes resulting from developmental plasticity include changes in behaviour, physiology, morphology, growth, and life-history traits which can result in a better match between the adult form and the conditions the organism will live in (e.g. Schlichting & Pigliucci 1998, Pigliucci 2005, West-Eberhard 2003, Beldade *et al.* 2011). Plasticity can be represented graphically by reaction norms describing phenotypic variation as a function of the environment. These provide an important tool for studying developmental sensitivity to the environment (e.g. Debat & David 2001, Lewontin 2006, Sultan 2007). The shape and height of reaction norms differ between traits and genotypes, and heritable variation for these properties of reaction norms provide the raw material for natural selection to shape the evolution of plasticity.

Plastic traits do not need to vary continuously along a gradient of the environmental cue responsible for the plasticity. In fact, reaction norms can be nonlinear, as in the case of threshold polyphenisms (e.g. Nijhout 2003, Beldade *et al.* 2011), or can have complex shapes, as in the case of pigmentation variation in adult mesothorax and abdomen segments of *Drosophila melanogaster* in response to

temperature (e. g. Gibert *et al.* 2000). Such shapes have been observed especially for environmental values outside the range organisms have adapted to (Neyfakh & Hartl 1993, reviewed in Pigliucci 2001).

One of the environmental cues most often associated to developmental plasticity is temperature, a key environmental factor in eco-evo-devo studies. In this context, thermal sensitivity of ectotherm performance has been extensively studied (e.g. Van der Have & de Jong 1996, Sinclair *et al.* 2003, Hoffmann *et al.* 2003, Fischer *et al.* 2010) being well known that temperature has a large impact on insects, from direct effects on enzymatic reactions to physiological effects that affect development (Lee Jr. 1991). In many cases, the temperature experienced during development is predictive of the environment where the adult forms will live, and of a number of important ecological parameters that can impact fitness.

Thermal plasticity in insect pigmentation is common in nature (e.g. Beldade *et al.* 2011, Gibert *et al.* 2007) and of extreme importance in visual communication (e.g. mate choice, camouflage), thermoregulation or photo-protection (reviewed in True 2003, Wittkopp & Beldade 2009). Additionally, insect pigmentation has been the target of many evo-devo studies that have attempted to characterize the regulatory genes and enzymes responsible for pigmentation development and its evolution (e.g. Jeong *et al.* 2006, Gibert *et al.* 2004, 2007, Wittkopp & Beldade 2009). Still, the sophistication and extent of the genetic analysis has not been matched by detail in quantitative methods for characterizing pigmentation phenotypes, in term of colors and color patterns. In regarding to that, here we are putting a large effort into the analysis of wing color and color pattern beyond measuring eyespots or band widths.

The tropical Nymphalid *B. anynana* has been established as a laboratory model for research on developmental plasticity (e.g. Beldade & Brakefield 2002, Brakefield *et al.* 2009, Beldade *et al.* 2011). This African butterfly exhibits phenotypic plasticity in pigmentation in response to natural wet–dry seasonality which is externally cued principally by temperature in the final larval and early pupal stages (Brakefield & Reitsma 1991, Brakefield *et al.* 1996, Kooi & Brakefield 1999). Resulting changes in adult wing patterns are associated to seasonal changes in the resting background color and different strategies to minimize predation. The wet season form has conspicuous wing patterns with large eyespots and lighter wing background color, whereas the dry season form has very reduced eyespots and a more cryptic appearance with darker wing

color resembling the brown background of dry leafs (Brakefield 1997, Beldade *et al.* 2011, Mateus *et al.* 2014). In addition to wing pattern, *B. anynana* adults of the wet and dry seasons also differ in life-history strategies (e.g. Brakefield & Reitsma 1991, Brakefield & Frankino 2009, Oostra *et al.* 2011, 2014). In the field, adults spend the harsh dry season being relatively inactive and delay reproduction until the beginning of the wet season. Conversely, the relatively short lived adults of the wet season form are more active and reproduce rapidly.

In the laboratory, individuals that develop at warmer temperatures show wet season-like phenotype with eyespots of large size, while individuals that develop at cooler temperatures show a dry season-like form with eyespots of reduced size (Brakefield *et al.* 1996). At intermediate temperatures, lab reared butterflies show intermediate phenotypes (e.g. Brakefield *et al.* 1998). Standing genetic variation for *B. anynana* wing patterns enabled researcher to derive artificial selected lines that, at intermediate temperature, are similar to one or the other of the natural dry and wet season forms (Brakefield *et al.* 1996). This strategy achieved gradual response to artificial selection on the height, but not the shape, of thermal reaction norms for *B. anynana* wing patterns (Brakefield *et al.* 1996, Wijngaarden & Brakefield 2001) and, unfortunately, lines were lost before the full characterization of the basis of phenotypic differences. Here, in order to better explore genotype (G), environmental (E), and GxE effects on *B. anynana* pigmentation development, we have invested in 1) re-deriving lost artificial selected lines expression extreme wet season-like or dry-season-like phenotypes at intermediary temperatures, and 2) characterizing thermal reaction norms for a wider range of temperatures that is usually explored in this species (including more intermediate temperatures to better assess reaction norms shape, and also more extreme temperatures). As only the outer ends of the reaction norms are thought to be exposed to selection in the field, by using intermediate and extreme values we expect to be able to explore how "hidden" parts of the response curves are affected by this thermal gradient.

The results presented here are still preliminary. We hope can fuel future work on: 1) changes in wing color beyond eyespot or band size, 2) shape of reaction norms of artificially selected lines, 3) characterization of correlated response to selection on wing pattern for other seasonally-varying traits.

MATERIAL AND METHODS

Artificial selection lines for wing pattern wet and dry-season phenotypes

We used a large outbred laboratory stock (WT) of *B. anynana* butterflies established from about 80 gravid females captured in Malawi (Brakefield *et al.* 2009). The lab population has been maintained at an adult population size of about 500 individuals/generation under controlled conditions. Larvae were reared on young maize plants sprayed with anti-fungic solution and adults fed on mashed banana.

We re-derived artificial DRY and WET selection lines by selecting individuals that were most similar to either the dry-season form or the wet-season form, respectively. These artificial lines were selected from a single large population of about 2000 individuals (G0), reared from the stock at 23°C (cf. Wijngaarden & Brakefield 2001). Initially, we were using two replicate selection lines in each direction. However, a microsporidia infection in our laboratory populations resulted in the loss of one line per direction resulting in no replication for the artificial selection.

For the first three generations, butterflies from both sexes were selected on the basis of the total diameter of the large fifth eyespot on the ventral hindwing relative to the distance between the second and fifth eyespot white centers (measurement highly correlated with overall wing size, e.g. Zijlstra *et al.* 2003), (Figure 5.1A) and on color patterns characteristic of the natural dry- and wet-season forms, respectively. Smallest eyespot butterflies were used as DRY parents, and largest eyespot butterflies were used as WET parents respectively. Measurements were made in Image-J software (Abramoff *et al.* 2004) using a digitizing tablet and a micrometer eyepiece in a binocular microscope at 10x magnification. To determine measurement error, repeatability was calculated by measuring 50 individuals (25 females and 25 males) three times randomly. Repeatability was calculated from Analysis of Variance (ANOVA) with the formula $r = S^2A/(S^2 + S^2A)$, where S^2A is the between group variance and S^2 is the within group variance (c.f. Falconer & Mackay 1996). Repeatability which ranges from 0 to 1 was of 0.96 *i.e.*, the measurement error is negligible. From a total of 600 G0 females, that survived from the initial population of 2000 individuals, the 222 that showed the wet-like most (large ventral eyespots, lighter background) and those with dry-like most (smaller eyespots and darker background) were measured, and from a total of 985 G0 males 424 were measured (each individual three times, final

measurement corresponds to the average). The 40 most extreme individuals of each sex and phenotype were selected to produce the next generation.

After G0, smaller populations of about 500 larvae per generation were reared at 23°C. From the resulting G1 individuals, 171 extreme females and 229 males were measured, and in G2 207 females and 224 males were measured. For each line, the 40 most extreme females and 40 most extreme males were allowed to mate to produce the next generation. After generation G3, upon obvious reduction of phenotypic variation within line, we started selecting by eye, targeting eyespot size and also background color, the 40 most extreme individuals of each sex.

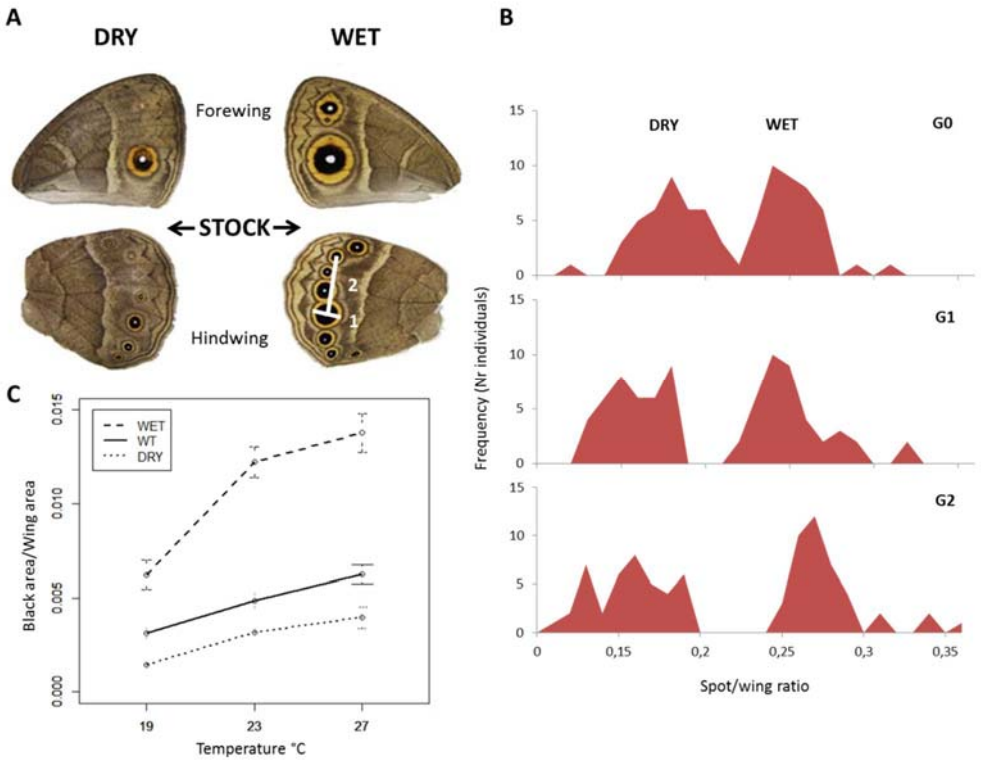


Figure 5.1 - Selection of DRY and WET lines. A) The photos correspond to representative female wings after 10 generations of selection (reared at 23˚C). **B)** Frequency distributions of the size of the fifth eyespot on the hindwing relative to the wing size for 40 selected female butterflies of the DRY and WET lines for the three first generations of artificial selection. After that we applied selection by eye targeting eyespot size and also background color. **C)** Reaction norms for the black area (corrected for total wing area) across developmental temperatures after 10 generations of artificial selection for eyespot size of female *B. anynana* (individuals reared at the same conditions as individuals of CHAPTER 4, however we did not measure the white center size not being possible to represent total eyespot size). This shows that after 10 generations (individuals reared at the same time as those from different pigmentation lines analyzed in CHAPTER 4) it was already possible to distinguish completely different phenotypes

across temperature. Error bars represent 95% of Confidence Interval (CI) for the mean and the sample sizes are 29-30 for WET line, 30-38 individuals for WT (unselected controls), and 29-30 for DRY line.

Thermal reaction norms

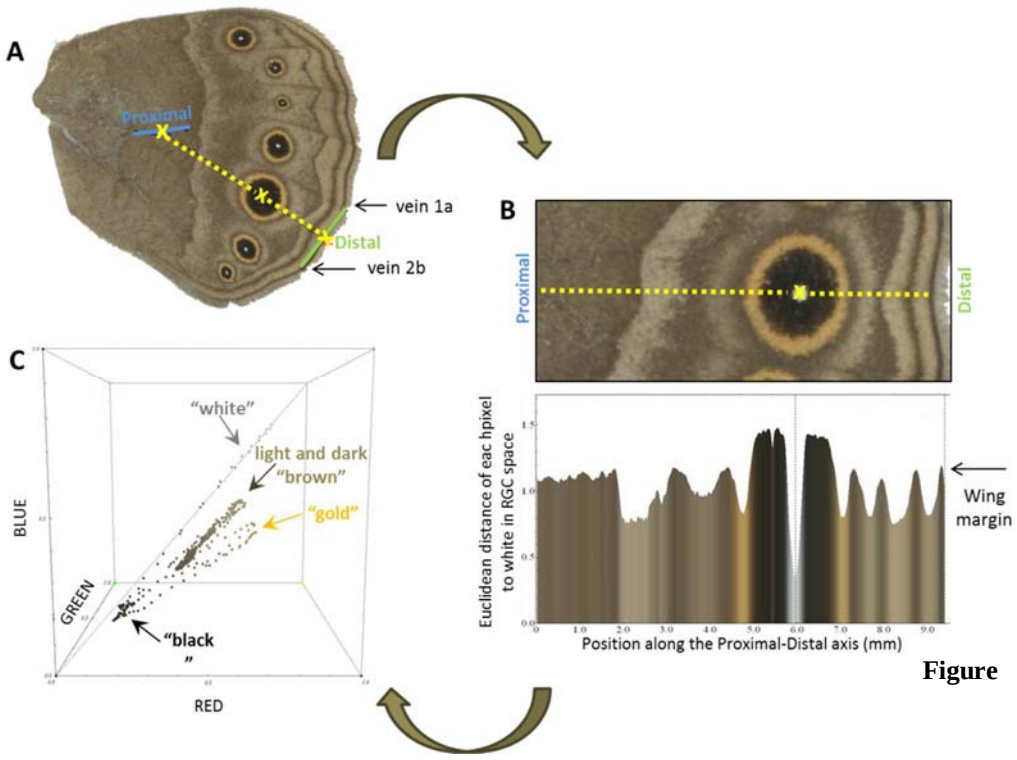
To characterize thermal reaction norms, we reared 120 first-instar larvae from the WT, DRY and WET lines at nine different temperatures and measured wing pattern in the resulting adults. For the artificially selected lines, we collected eggs after 19 generations of selection intensity at 23°C. First-instar larvae from each stock were transferred in batches of 40 individuals onto separate net sleeves, with two maize plants each. Sleeves with larvae were placed in climate-controlled chambers set at 15°C, 17°C, 19°C, 21°C, 23°C, 25°C, 27°C, 29°C or 31°C ± 0.5°C, with 65-70% relative humidity and 12:12hr light/dark cycle. The lowest and the highest temperatures are below and above, respectively, the temperatures typically used in the lab to induce the formation of dry-season (19°C) and wet-season (27°C) phenotypes and which are believed to natural conditions (Brakefield & Reitsma 1991). Sleeves were monitored each two days, plants were watered or replaced when necessary, and pre-pupae were collected and transferred to individual pots until adult eclosion. Pupation and eclosion days were recorded each two days. Adults were frozen 24h after eclosion and their wings were cut and stored in the freezer until analysis.

Phenotypic measurements

We developed a new method to obtain and process high quality images of wing pattern in a standard and semi-automated manner (F Alves and P Beldade, *manuscript in preparation*). With this method color- and light-calibrated image acquisition of flat adult wings are taken by using a high-resolution photographic scanner (Epson Perfection v600 Photo scanner), (pictures available in http://wingpatterns.igc.gulbenkian.pt). VueScan 9x32 9.3.18 software (Steinhoff 2011) was used for setting color-calibration (white point for Red=0.5, Blue=0.5, and Green=0.52; black point for Red, Blue, and Green=0; curve low=0.25, and high=0.75; brightness of 1; and TIF in 24RGB). Images are then processed and analyzed using custom-code in *Mathematica* software (Wolfram 1996).

The total size (diameter) of the fifth eyespot on the ventral surface of the right hindwing as well as the background color of each individual image are "sampled" by

drawing a transect through three visible landmarks: the eyespot center, intersections of veins 1a and 1b with the margin (Distal), and the cross-vein between 1a and 1b (Proximal), (Figure 5.2). We then extract images up to 11 pixels-high centered on the transect and obtained average color values (RGB scale) for each pixel along the transect. These color values are plotted on three-dimensional RGB color space to visualize different color "qualities" and to quantify pixels corresponding to different colors (density of points within defined RGB limits). To assess color pattern, we plotted the Euclidean distances between each of the transect´s pixels RGB to the white reference (1, 1, 1) in the RGB space (hereafter "distance to white"). Total transect size was used as a measurement to correct for overall wing size.



Figure

5.2 - Wing color analysis. A) Transect (yellow line), drawn through the fifth eyespot of a scanned image of a hindwing from a female reared at 27°C, defined by three wing landmarks (marked x), with the Proximal wing cell reference represented in blue and the Distal in green, respectively. **B)** Detail of the wing region including the transect defined in panel A (on the top) with the respective plot of the Euclidean distance between the color value of each of the transect's pixels to RGB-scale white (on the bottom). This type of graphical representation can be used to quantify different aspects of the wing pattern phenotypes. **C)** RGB values (average of 11 pixels from the transect's middle line) were visualized in the 3D RGB space allowing

distinguishing between color "groups": "white", "black", "gold" and "brown". Pixels plotted in respective color.

Statistical analyses

All data analyses were performed with R (R Development Core Team 2012). Eyespot size and pupal development time analyses were done separately for females and males because of sexual dimorphism in *B. anynana* wing size and life history traits (e.g. Zwaan *et al.* 2008, de Jong *et al.* 2010, Oostra *et al.* 2011). In all statistical models, we use genotype to refer to the different genetic backgrounds (DRY, WET and WT).

We analyzed eyespot total size, pupal development time and survival changes with temperature for each of the three genotypes. Before that, for eyespot size and pupal development time, parametric assumptions were considered by checking normality (Shapiro-Wilk test, alpha=0.05) and homoscedasticity (Fligner-Killeen test, alpha=0.05) of residuals, and transforming data when appropriate. When significant differences were found for the different factors in the overall models (ANOVA, alpha=0.05), we performed post-hoc comparisons between factor levels using Tukey´s honest significant differences (HSD) tests (alpha=0.01).

For eyespot size, we tested the model *eyespot size ~ transect size + genotype * temperature*, with transect size as covariate. Pupal development time was log transformed and we tested the general linear model *development time ~ genotype * temperature*. For both cases we used general linear models assuming a Gaussian distribution of the error, and with temperature (nine levels: 15°C, 17°C, 19°C, 21°C, 23°C, 25°C, 27°C, 29°C and 31°C) and genotype (three levels: DRY, WET and WT) as fixed effects. Samples sizes are not equal for both traits in general samples sizes are higher for wing pattern analysis because even when we missed pupation and/or eclosion days the individuals were still used for eyespot size measurement (see detailed sample sizes in Annex 5.1).

Survival differences were compared using the model *survival proportion ~ genotype * temperature * sex*. We used a general linear model assuming a weighted (total N) Binomial distribution of the error, and with temperature (nine levels), genotype (three levels), and sex (two levels) as fixed effects. Here, we used sex as fixed effect because we did not know from previous works (as in the case of eyespot size and development time) if there is sexual dimorphism for this life history trait.

PRELIMINARY RESULTS AND DISCUSSION

We collected phenotypic data from females and males of three different genetic lines (Figure 5.1) reared at nine temperatures. In our preliminary analyses, we show the thermal reaction norms for the total size of the fifth eyespot on the hindwing (Figure 5.3). We also show reaction norms for pupal development time (Figure 5.4) and survival rate (Figure 5.5). This analysis allowed us to determine effects of temperature (T), genetic background (G), and sex (for the survival analysis) and the interaction between them. Finally, we also illustrate differences in wing background color along the gradient of temperatures (Figure 5.6 and 5.7). We found significant effects of T, G and G x T interaction on eyespot width and pupal development time for both sexes. For survival rate, genotype appears as the main factor explaining the high rates of mortality, especially for extreme temperatures. Finally, we show wing background color changes across temperature and for extreme low temperature a different pigment color appears.

Artificial selection lines differ in height and shape (GxT) of thermal reaction norms for eyespot size

Our artificial selection at intermediate temperature produced differences in wing pattern across all temperatures leading, with the lines having well separated reaction norms (at G10 Figure 5.1C and G19 Figure 5.3). The WET line shows the highest and DRY line the lowest phenotype for all temperatures, respectively (reflected in reaction norms of different height) in agreement with what was expected after the artificial selection procedure on both phenotypic directions (Brakefield *et al.* 1996).

Reaction norms are an important tool to quantify the degree of phenotypic variance and magnitude of plasticity of morphometric and life-history traits (DeWitt *et al.* 1998, Karan *et al.* 1998, Pertoldi *et al.* 2014). By measuring thermal reaction norms of *B. anynana* unselected and selected DRY and WET lines across a range of temperatures we were able to assess the G, T and GxT effects on eyespot size variation. We could also assess possible correlated responses, to the artificial selection on wing pattern; notably, for pupal development time and survivorship. However, as we did not have replicate lines for each selection direction, the interpretation of these correlated responses should be taken as indicative rather than definitive. Significant G effect means that genetic backgrounds differ, significant T effect means that trait responses are thermally plastic, and significant GxT effects reflects differences between genetic stocks in thermal reaction norms.

Figure 5.3 and 5.4 underlies seasonal polyphenism in WT and selected lines showing the total size of the fifth eyespot on the hindwing across nine different temperatures, including intermediate and extreme values. We quantified these differences and we found that total eyespot size was significantly affected by temperature (females: F=238.49, df=8, P < 0.001; males: F=225.0730, df=8, P < 0.001), by genotype (females: F=898.43, df=2, P < 0.001; males: F=743.1025, df=2, P < 0.001), and by genotype x temperature (females: F=8.49, df=16, P < 0.001; males: F=8.2658, df=16, P < 0.001) for both sexes (see Annex 5.1).

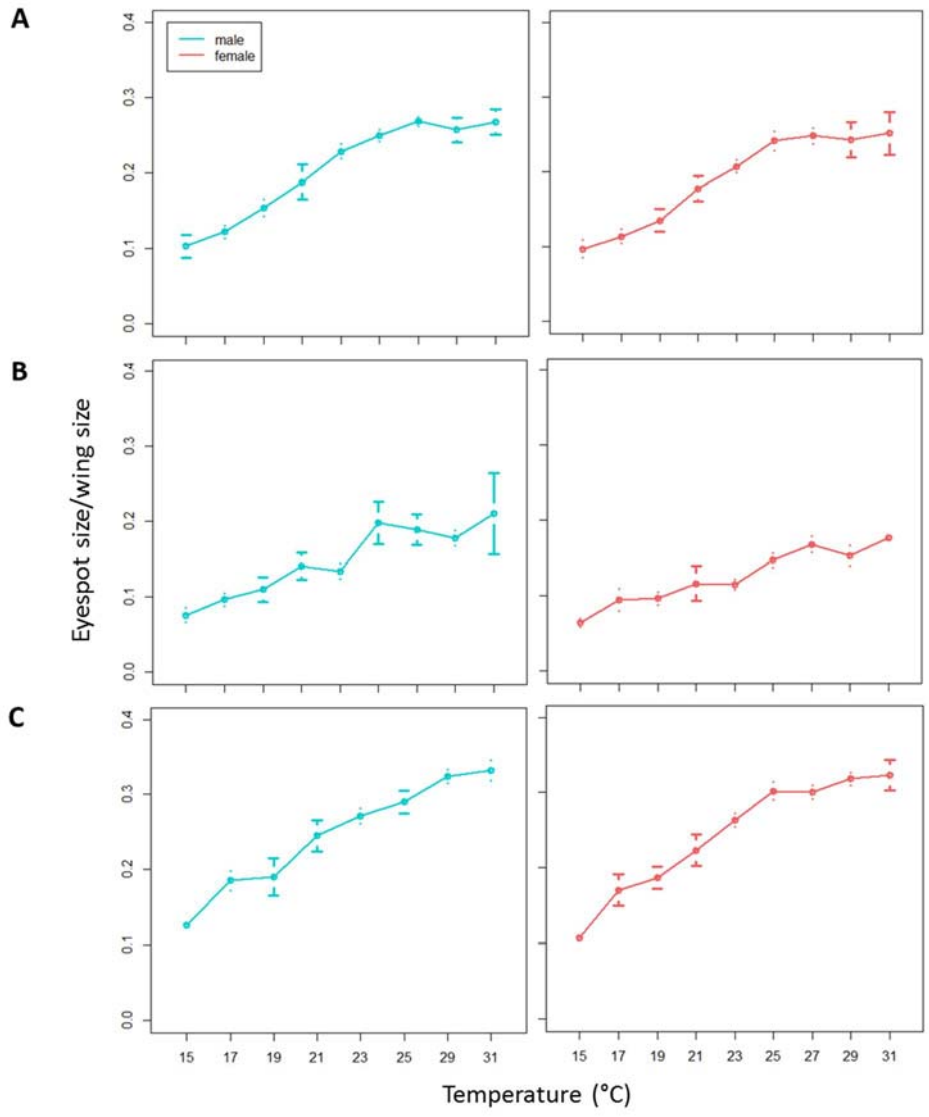


Figure 5.3 - Thermal reaction norms for eyespot size for unselected (WT-A) and artificial selected lines (DRY-B and WET-C). For the total width of the fifth eyespot on the hindwing (relative to corresponding wing size) of three genotypes (A ,B,C), we plotted the mean value as a function of temperature and use bars to represent the standard deviation. Females (right, pink)

and males (left, blue) are represented separately because of the already known sexual dimorphism in *B. anynana* wing size and patterns. We tested for the effect of temperature and genotype using the model *eyespot size ~ transect size + genotype * temperature* (see Material and Methods and Annex 5.1). When we found significant effects of temperature or/and genotype on trait value $P < 0.05$, we compared across temperatures (Tukey HSD, $P < 0.01$, see Annex 5.1 for sample sizes and statistical details). There is a significantly GxT interaction which confirms differences between lines in thermal reaction norms.

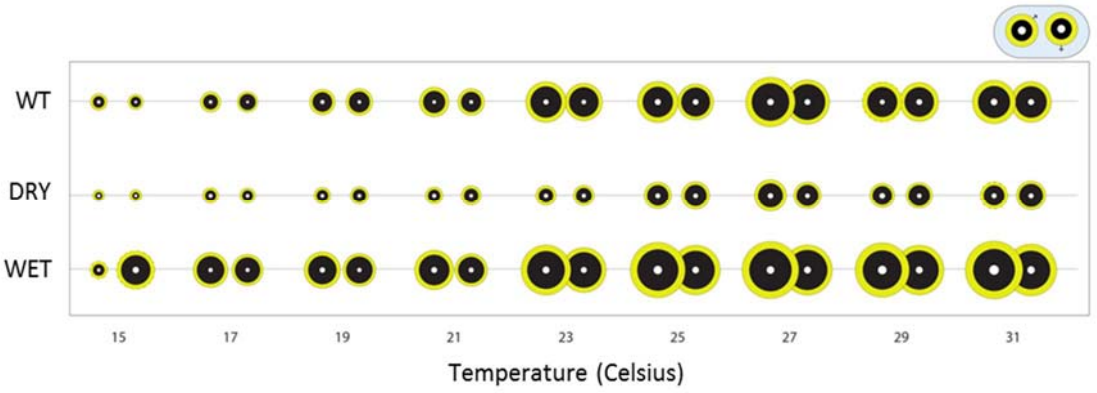


Figure 5.4 - Developmental plasticity for eyespot different ring sizes across temperature, for females and males, of the WT, DRY and WET genotypes. The figure depicts the central tendencies computed as the median of the (unimodal) distributions for the size of the different rings of the fifth eyespot on the hindwing: white, black and gold. For each temperature (15°C to 31°C), males are represented always by the left eyespot and females by the right eyespot (see legend on the right top corner of the figure). Eyespots represented by dashed lines correspond to small sample sizes (N<5 individuals). In general this quantitative approach shows that eyespot size increases with increasing developmental temperature, while DRY and WET lines have smaller and larger eyespots across temperature.

For both sexes, there was a significant interaction GxT which suggests variation for phenotypic plasticity between lines (Figure 5.3 and 5.4). In general, WET and WT lines seem to show higher levels of plasticity with most pronounced eyespot size differences between low and high temperatures relative to the DRY line (see difference between means in Tukey HSD results in Annex 5.1). These results are in agreement with previous works where lines selected for wet-like phenotype at intermediate temperature showed higher sensitivity to temperature in comparison with the line selected for dry-like phenotype (Brakefield *et al.* 1996). A previous study had described an artificial selected line of *B. anynana* that only produced wet season-like large eyespots across all temperatures (from 17°C to 27°C) but still did have larger eyespots at higher

temperatures, which means that phenotypic plasticity is still retained (Brakefield *et al.* 1996).

The existence of phenotypic plasticity demonstrates that the eyespot developmental pathway is under environmental control. The DRY and WET artificial selection lines show that plasticity can be changed trough selection (see also Brakefield *et al.* 1996). Wijngaarden & Brakefield 2000 demonstrated, through different combinations of crosses between genetically different selection lines, that those lines differed in 5 to 10 polymorphic genes that would have contributed to the evolution of these divergent phenotypes. The separable genetic and environmental effects on eyespot size development show that the unselected stock contains allelic variation for influencing eyespot size, and that selection could change those allele frequencies to produce genetically divergent lines. It is unclear how that allelic variation impacts hormone dynamics.

Development time showed correlated response to artificial selection on wing pattern

Because we know that pupal development time and wing pattern show strong genetic and phenotypic correlations, due to shared hormonal effects (Zijlstra *et al.* 2004, Oostra *et al.* 2011) we also explored thermal plasticity in pupal development time for our lines.

Figure 5.5 illustrates thermal plasticity in WT and selected lines for pupal development time. We found that pupal development time was significantly affected by temperature (females: $F=1282.2820$, $df=8$, $P < 2.2e-16$; males: $F=1193.2487$, $df=8$, $P < 2.2e-16$), by genotype (females: $F=227.1087$, $df=2$, $P < 2.2e-16$; males: $F=257.0595$, $df=2$, $P < 2.2e-16$), and by genotype x temperature (females: $F=2.6512$, $df=16$, $P < 0.000504$; males: $F=3.0861$, $df=16$, $P < 5.138e-05$) for both sexes (see Annex 5.1). Figure 5.5 shows that for all genotypes pupal development time decreases with increasing temperature, similarly for both sexes (as in Oostra *et al.* 2011). For total pupal development time, the DRY line shows the highest reaction norms (i.e. longer pupal development across temperature) in comparison with the WET line and the unselected stock that are similar (Figure 5.5). This means that artificial selection on ventral eyespot size at intermediate temperature lead to correlated responses in pupal development time.

Previous works demonstrated that pupal development time and wing pattern show strong genetic correlations due to shared hormonal underpinnings (Zijlstra *et al.* 2004, Oostra *et al.* 2011). However, it was also shown that there was substantial genetic

variation allowing antagonistic selection to uncouple the two traits (Zijlstra *et al.* 2003, 2004). It was suggested that response to selection on development time resulted from shifts in hormone dynamics, while response to selection on eyespot size resulted from later changes in developmental mechanisms of pattern determination (Zijlstra *et al.* 2004). It is unclear what the mechanisms are for our response to selection on eyespot size and correlated changes in development time.

Survivorship differs between lines at extreme temperatures

In Figure 5.6 we show survival rate for different genotypes at different temperatures. We found that survivorship was not significantly affected by temperature and sex (see results in Annex 5.1).

Figure 5.6 shows that at cooler temperatures (<19°C) the DRY line shows higher survival rate in comparison with the WET line, while at warmer temperatures (>27°C) the WET line shows higher survivorship in comparison to the DRY line (Figure 5.6, Annex 5.1). This is especially visible for extreme low and extreme warm temperatures. At 15°C, the DRY shows a noticeably higher proportion of survival in comparison with the WET line, and at 29°C the opposite can be seen. At 31°C mortality is very high for all genotypes (Figure 5.6). For the unselected stock mortality is lower in comparison with the artificial selected lines. However, above 27°C there is an accentuated mortality. *B. anynana* occurs across sub-Saharan Africa where different populations live in very different environments (Roskam & Brakefield 1999). The lab stock, derived from a population in Malawi and adapted to the lab for many generations, represents only some of the species ability to survive an extended temperature range. Also by obtaining different results between DRY and WET lines for survivorship for different temperatures means that we were successfully once more in getting indirect correlated responses for different traits to our artificial selection on wing pattern. The fact that DRY line shows lower mortality at lower temperatures means that by artificial selection on wing pattern we probably also affected genes related with development, specifically in this case with the sensitivity to temperature.

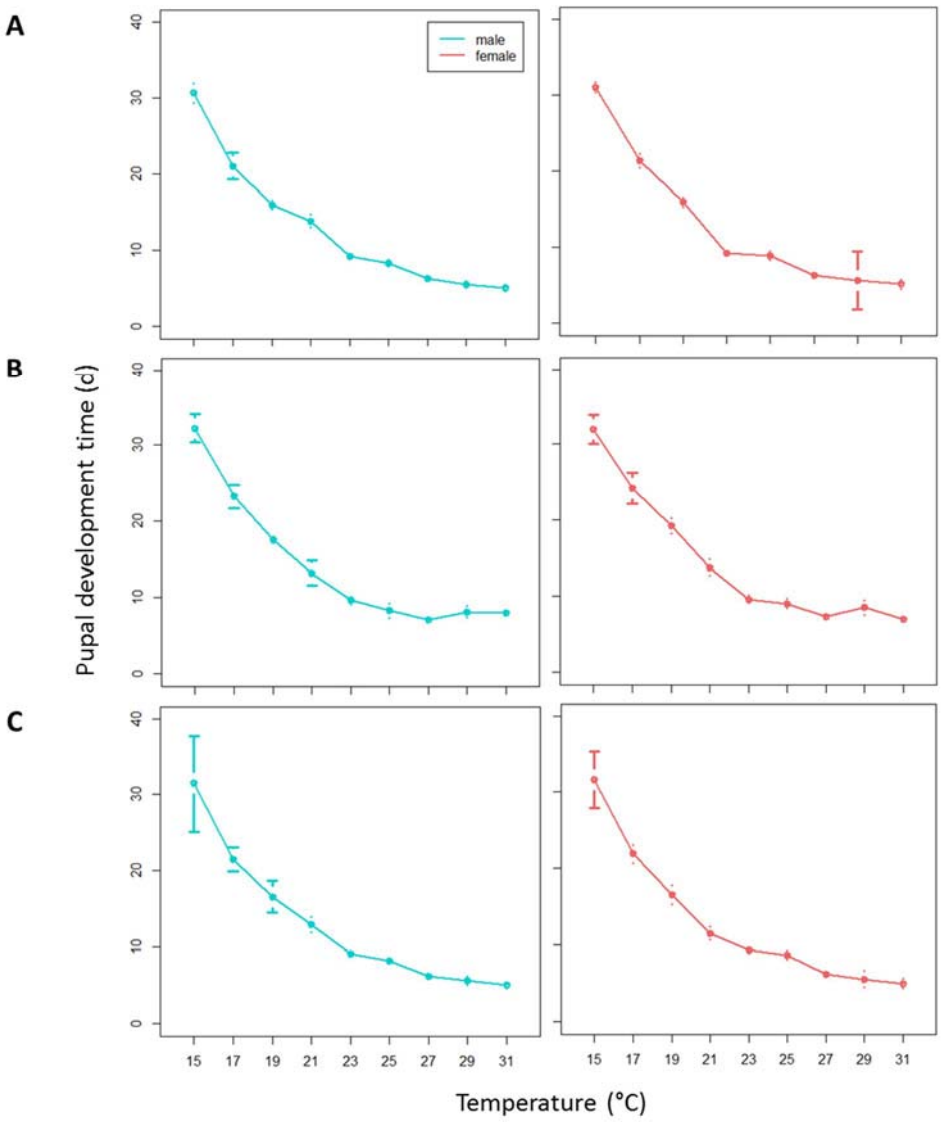


Figure 5.5 - Thermal reaction norms for pupal development time for unselected stock (WT-A) and artificial selected lines (DRY-B and WET-C). For pupal development time in days (d) we plotted the mean value as a function of temperature and used bars to represent standard deviation (SD) of WT (A), DRY (B) and WET (C) genotypes. Females (right, pink) and males (left, blue) are represented separately because of the already known sexual dimorphism in *B. anynana* development time. For each sex, we tested for the effect of temperature and genotype using the model *Days ~ temperature * genotype* (see Material and Methods and Annex 5.1). When we found significant effects of temperature or/and genotype on trait value $P < 0.05$, we compared across temperatures (Tukey HSD, $P < 0.01$, see Annex 5.1 for sample sizes and statistical details). Differences between genotypes are mainly due to DRY (B) line with longer pupal development relative to WET (C) and WT (A).

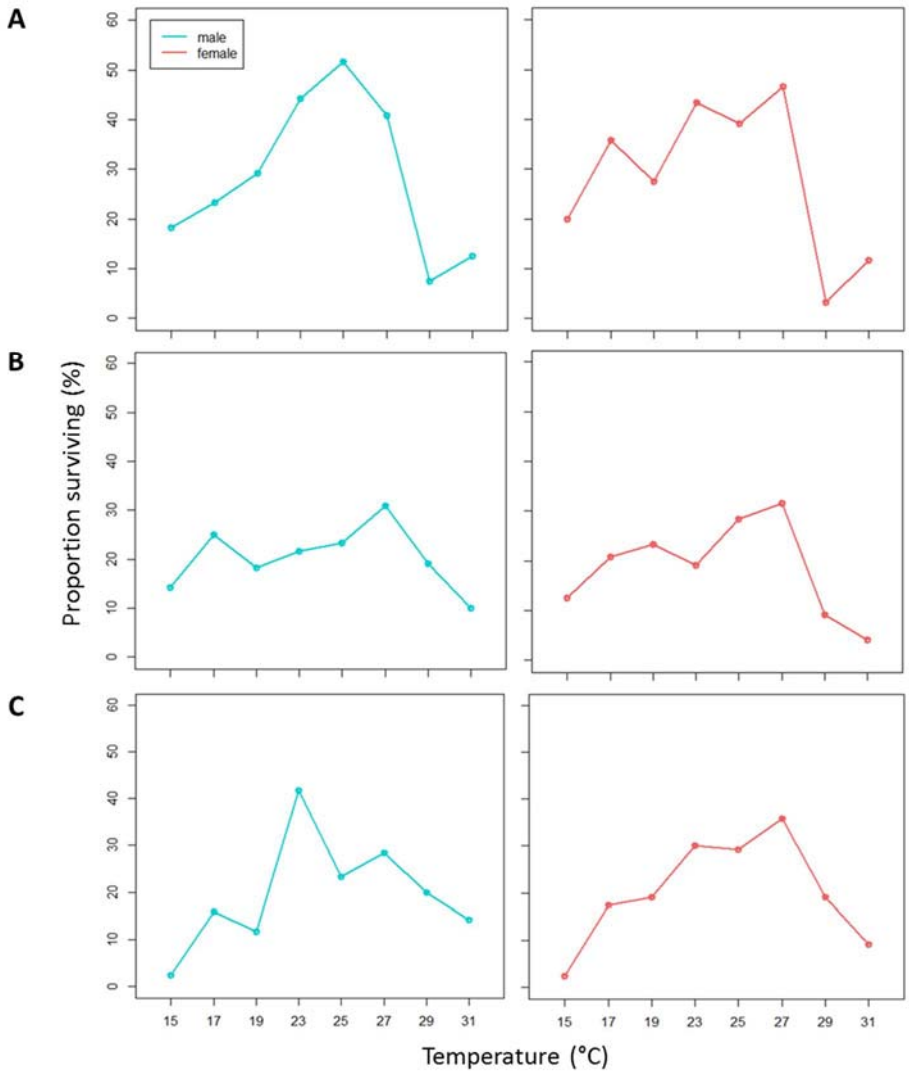


Figure 5.6 - Survival rates for unselected stock (A) and selected DRY (B) and WET (C) artificial selected lines across temperatures. We plotted proportion of survival (%) of 120 individuals (60 per sex) from each genotype, for each temperature (see detailed sample sizes of survival in Annex 5.1). Females (right, pink) and males (left, blue) are represented separately, however because we do not know if sex is a factor that influences survival we included it in the final model. We tested for the effect of temperature, genotype and sex on survival using an ANOVA with a Chi-square test and the model *SurvivalProportion ~ Genotype * Temperature * Sex* assuming a weighted Binomial distribution of the error (see Material and Methods and Annex 5.1). Statistical significance of each factor is represented on the left top corner of the plot with: ^{ns} (non-significant) P>0.05, *P<0.05, ** P < 0.01, *** P < 0.001 (see Annex 5.1 for more details on sample sizes and statistical analysis). We have higher levels of mortality at extreme temperatures especially for the artificially selected lines. We did not plot the survivorship for 21°C because all our lab stocks got a severe fungic infection with notable effects at this temperature, and we needed to use part of the individuals from the experiment to rescue the

stocks. Therefore, because the sample size at 21°C was largely reduced but not due to natural mortality, we decided to not present the results for this temperature.

B. anynana reactions norms show different shapes across an extended temperature range

We tested different genotypes at a large range of temperatures, including intermediate and extreme values. With our results we would like to see how intermediate and extreme points of the reaction norms respond in relation to the already "well known" points that are common in representations of reaction norms for this species (e.g. Brakefield *et al.* 1996, Wijngaarden *et al.* 2002, Zijlstra *et al.* 2004, de Jong *et al.* 2010, Oostra *et al.* 2011, Mateus *et al.* 2014).

In general, intermediate temperatures show less difference between genotypes and between the effects of environments than more extreme temperatures. These intermediate temperatures are considered as a zone of canalization with the range of environments that have been historically most common in the species (Lewontin 2006), but in new environments much greater variance between genotypes appears.

Eyespot size increases with temperature and it seems that it reaches a plateau at 27°C (Figure 5.3 and 5.4). We did not identify a lower limit plateau for lower temperatures (Figure 5.3 and 5.4). For pupal development time there are also fluctuations across temperatures, again with less pronounced response above 27°C in comparison with cooler temperatures (Figure 5.5). Finally for survivorship (Figure 5.6), despite the higher mortality at extreme low and high temperatures, all genotypes seem to show less resistance to warmer temperatures. Our results suggest *B. anynana* might not be well equiped to respond to higher temperatures, even thought much higher then our highest test temperatures being possible in natural populations (e.g. cf. Fischer *et al.* 2010 during solar radiation temperatures of 45°C are possible). While adults of this species might be able to cope with such higher temperatures, pre-adult stages might not.

Wing background color changes with temperature

Pigmentation is involved in intra- and interspecific communication (e.g. camouflage, mate recognition), structural protection (e.g. temperature and light), and chemical defense (Needham 1974). One of the best examples is butterfly wing patterns (Needham 1974, Nijhout 1991). In butterflies, wing scales show only a single pigment. These monochromatic cells are juxtaposed in parallel rows in a two-dimensional layer of the wing tissue and wing patterns are formed by colored scales arranged to produce pattern elements such as bands or concentric rings (Nijhout 1991, 2010, Koch & Kaufmann 1995). In order to explore differences in plasticity of wing color patterns we plotted the RGB values of *B. anynana* wings for the nine temperatures for both sexes and the three genotypes.

In Figure 5.7, it seems that not only eyespot size changes across temperature increasing with warmer temperatures, but also wing background color changes. In general, while for cool temperatures (15°C-21°C) we see mostly three groups of pigments: "white", "black" and "brown", for warm temperatures (23°C-31°C) we see the four pigment groups (Figure 5.2): "white", "black", "brown", and also the "gold" that was almost not present at lower temperatures. This "gold" pigment corresponds mainly to the large "gold" eyespot ring which almost does not exist at lower temperatures. It also seems there are differences for the group of the "brown" pigment. The "cloud" of "brown" is larger, darker, and with different intensities for low temperatures (15°C-21°C). Finally, it also seems there is heterogeneity between Proximal and Distal sides not only in terms of wing color background, that seems to show different color intensities, but also at the level of the eyespot color rings size. These results are similar for both sexes and for the three genotypes (Annex 5.2).

At lower temperatures, by eye, wings seem more heterogeneous in color, seen by the different intensities of "brown" pigments that cover most of the wing background, in comparison with what seems to be lighter and almost uniform color for the warm temperatures (Figure 5.7). These possible differences in color are probably related to the adaptive strategy of *B. anynana*. The adaptive benefit of the cryptic form in the dry season as response to the lower temperatures has been previously demonstrated (Lyytinen *et al.* 2004, Brakefield & Frankino 2007). In the dry season habitats, adult *B. anynana* butterflies typically express a cryptic wing pattern allowing them to rest undetected among the dried vegetation. In the wet season, vegetation is green and

abundant and the individuals instead express prominent concentric eyespots along the distal margin of their wings to protect the fragile body against the attacks of the predators (e.g. Brakefield & Frankino 2007, Oliver *et al.* 2009, Beldade *et al.* 2011). Adittionaly to the mechanism of defense, it was already shown for other species that according to the thermal budget hypothesis, darker phenotypes are observed in cooler environments to favor the absorption of the light radiations to increase the internal temperature, and light body color prevents overheating in warm environments (David *et al.* 1990, Capy *et al.* 1988, Goulson 1994, Gibert *et al.* 1996, 2000). Previous studies, based on the RGB analysis, also confirm that Dry season adults, both males and females, are generally darker than the wet season form (de Jong *et al.* 2010).

Our imaging of wing background color suggests that the artificial selection fot wet- and dry-season like phenotypes altered that phenotype too. As we can see in Annex 5.2 the DRY line seems to show, at low and high temperatures, a more heterogeneous wing color background in comparison with the WET line and the unselected WT stock. WET line for both temperatures seems to show lighter and more uniform wing color.

We also observed that the "white" eyespot centers are sometimes not really "white", appearing almost "yellow" (e.g. WT 15°C in Figure 5.7), or almost "brown" (e.g. DRY 17°C in Annex 5.2). We do not know the mechanism underlying this, but hypothesize that this different color at the eyespot centers might result from some scales of different color being mixed with the colorless scales. For example, "gold" scales under the "white" scales can make the eyespot center look almost "yellow" rather than "white". The density of cells in the eyespot center might also be different in animals developed at different temperatures being low at cooler temperatures. That being the case could mean that the wing background (non-"white" scales) is more visible and affects "white". In order to confirm either of our hypotheses we should analyse eyespot centers under very high magnification to analize individual scale color and density.

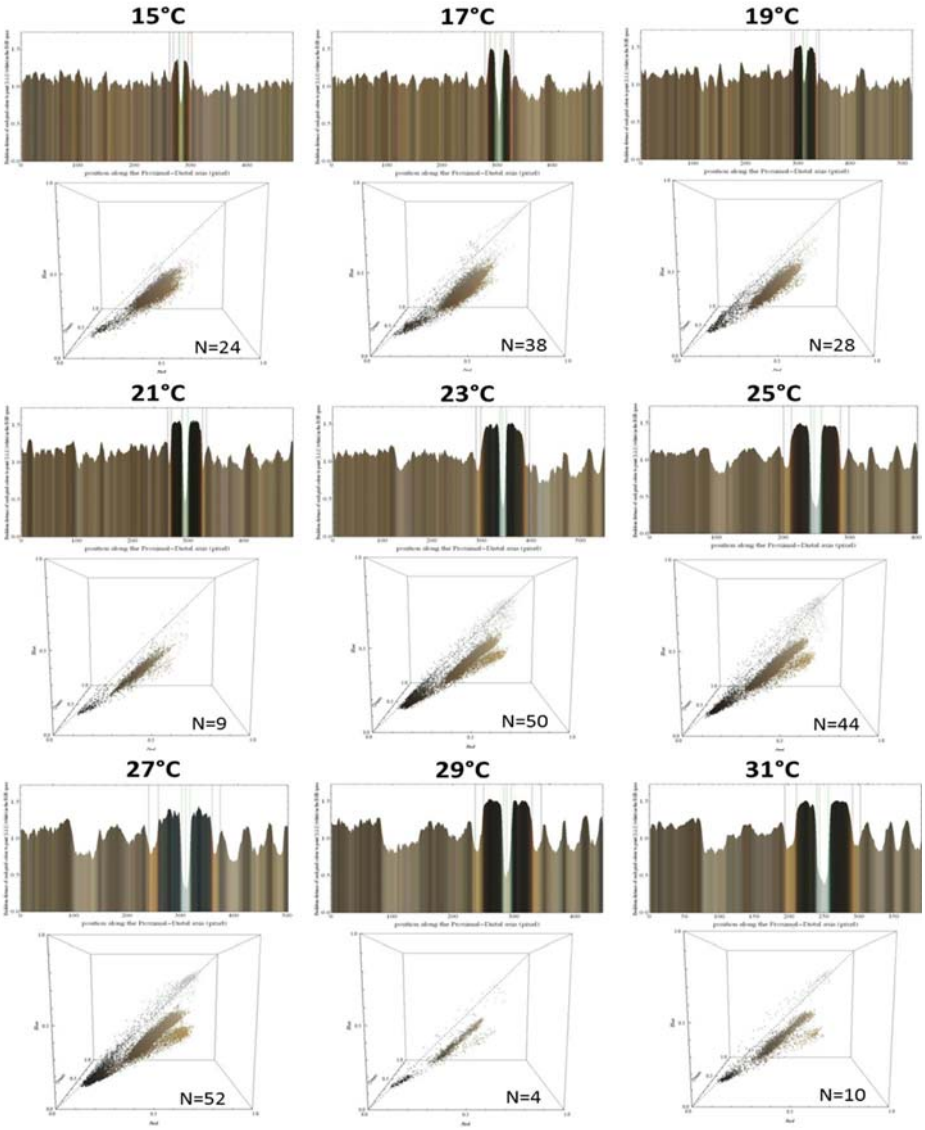


Figure 5.7 - Wing background color changes across temperature. For females of each temperature, we show two different plots representing the pixels along a particular transect of the fifth wing cell of the hindwing. The top plots show distances to white with each pixel along the wing transect. In the xx axis we have "Position along the Proximal-Distal axis (mm)", and in the yy axis we have "Euclidean distance of each pixel to white in RGB space" (axis cf. Figure 5.2C). Each plot represents the typical transect for that temperature. The bottom plots represent the 3D RGB space visualization of the averaged RGB values that allow distinguishing between different wing background pigment groups. Each plot represents the RGB values (see Figure 5.2 to detail on name of axis) of all individuals for that temperature (total N inside each 3D cube). We chose to represent females because wing background is lighter allowing a better visualization of the pigments involved. For higher temperatures samples sizes are very small, in particular at 29°C, due to the low survival observed for these temperatures (see Figure 5.6).

Distal and Proximal wing sides show asymmetry and a novel color seems to appear

Our color analysis also shows clear differences between Distal and Proximal sides of the wing. This goes beyond the light band found only proximally and on the marginal chevrons found distally (for more detail see Figure 5.2). Proximal-distal color asymmetry is more visible for higher than lower temperatures (Figure 5.7). We can clearly see also asymmetry in eyespot ring width between proximal and distal half of the eyespot sides. In fact the rings on the Proximal side seems to be thinner that the ones from the Distal side. This is more obvious for the "gold" ring, but it can also happen for the "black" ring, and it is especially visible at lower temperatures when sometimes the "gold" ring for the Proximal side is almost inexistent (e.g. 19°C in Figure 5.7). We do not know the reason for this asymmetry, but suggest it might be due to the wing developing tissue process. During wing development the distal wing side expands more in surface in comparison with the Proximal side (Nijhout 1991). This could originate that the size of the eyespot rings also follows this process and enlarge also asymmetrically.

Finally, in Figure 5.8, we see the appearance of color pixels of a possibly new color, somewhat more distant from the "brown" pigments group, at the extreme low temperatures mainly for DRY individuals from the line.

For very low developmental temperatures, adult wings seem to display what is possibly a new "orange" color, between the "brown" and "gold" pigment groups, (Figure 5.8). This happens mostly for DRY line (Figure 5.8). During selection for dry-season appearance at intermediate temperature (23°C), we seem to have favored alleles that can now produce a different pigment when at lower temperature. In the forewing this color appears mainly in the Distal part of the wing, next to the margin, and in the hindwing it appears mainly next to the almost inexistent white band (Figure 5.8). We do not know if the orange color corresponds to a pigment from a different type (e.g. ommochromes can be yellow, orange, red), or to a modification of a product of the melanin biosynthesis pathway. We also do not know if there could be any adaptive value for the appearance of this extra color at low temperatures.

In order to explore if this orange color appears in related species that live in the same seasonal environments, we compared this color with that found in other *Bicyclus* and *Heteropsis* species (Annex 5.3). *B. campina*, *B. condamini,* and *Heteropsis perspicua* captured in the wild at their natural temperature, seem to, indeed, display a

similar color (see orange arrows in Figure 5.8 and Annex 5.3). Unfortunately we just had access to one individual from each species and with almost no information about the temperature that they grew in the field and their seasonal form. It would be interesting to analyze color in more individuals of more species, including both seasonal forms.

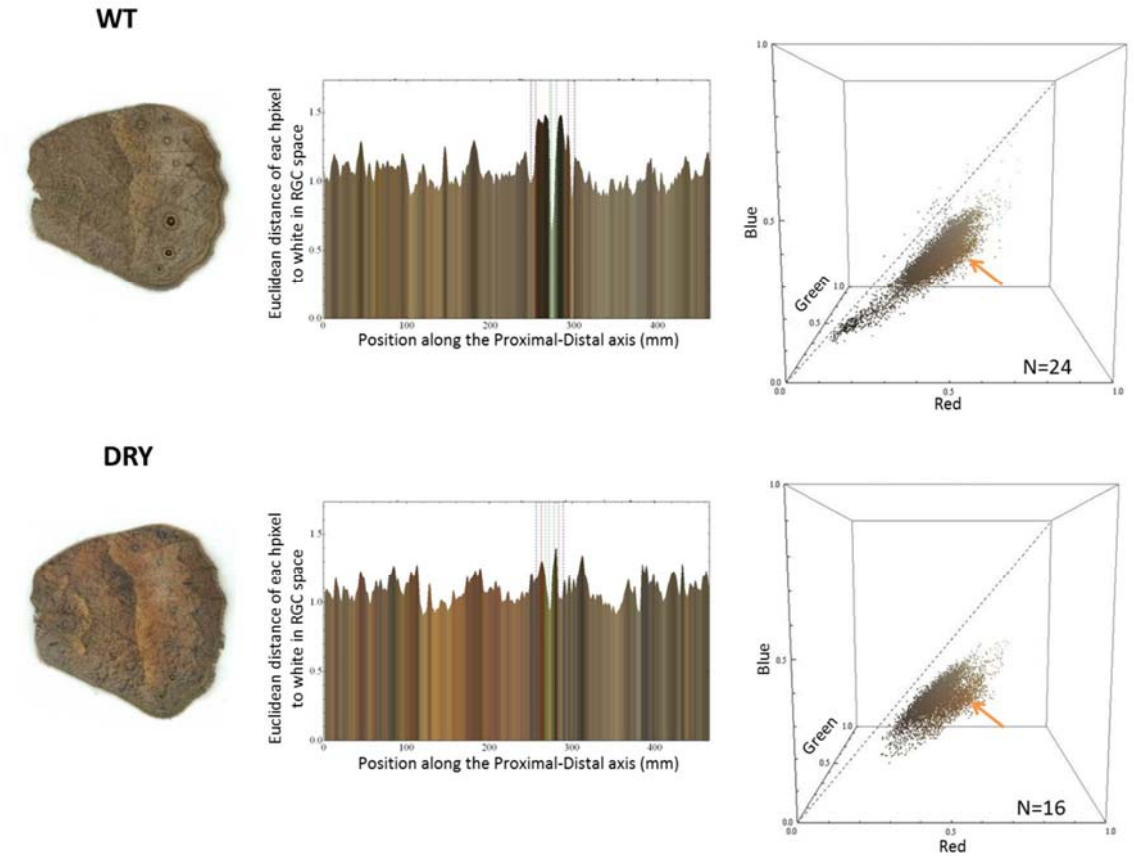


Figure 5.8 - Effect of extreme low temperature in wing background color of different genotypes. In this figure we show differences between genotypes in background color at 15°C. On the top we show the results for the unselected WT stock and on the bottom for the DRY artificial line. From the left to right in the figure we have: the adult hindwing of one individual female that represents the corresponding typical phenotype at 15°C, the plot for that individual showing the distance to white for each pixel along the wing transect (cf. Figure 5.2), and the 3D RGB space visualization of the pixels on the transect for various females together (total N inside each 3D cube). Orange arrows point to orange pixels which we suggest might correspond to different color pigment. Results are similar for both sexes, however we chose to represent females because wing background is lighter allowing a better visualization of the pigments involved (e.g. de Jong *et al.* 2010).

CONCLUSIONS

Even though linear reaction norms are the simplest way to represent graphically phenotypic plasticity, more complex shapes could arise and are also expected to evolve under specific environmental conditions (Gavrilets & Scheiner 1993a, de Jong 1999).

Temperature is of special importance during development of ectotherms because it can pose substantial challenges for survival and development. Thermal plasticity can offer quick and effective ways to cope with environmental fluctuations and even perturbations such as climate change (Chevin *et al.* 2013). Here, we characterized thermal reaction norms in a *B. anynana* wildtype genotype as well as two genotypes artificially selecteded for expression of DRY- or WET-season like wing patterns at intermediate temperature. We used a range of temperature including intermediary values between those typically used to study plasticity in this species (to better assess reaction norm shape), as well as beyond those (to explore extremes). We followed thermal plasticity for an indicative eyespot, pupal development time, and survivorship as well as for wing background color. This could inform about the nature of GxT effects (comparing reaction norms shapes) and allow us to investigate possible novel/extreme phenotypes and increased range of phenotypic variation that might result from exposure of cryptic genetic variation.

Our preliminary analysis show that artificial selection lines for wing patterns at intermediate temperatures resulted in genotypes with different reaction norms, height and possibly also shape. We see evidence of significant GxT effects (Figure 5.3). For both sexes, response to selection seems to have been most extreme for the WET direction. We see that WET reaction norm is heighest and DRY reaction norm is lowest and flattest in comparison with the WT (Figure 5.3). Previous studies targeting *B. anynana* eyespot plasticity were able to change reaction norms height but not shape (Brakefield *et al.* 1996, Wijngaarden & Brakefield 2001).

We show that our artificial selection on wing pattern also could be indicative of differencesin other traits such as pupal development time and survivorship. These correlated responses to selection could possibly reflect genetic pleiotropy. For all genotypes pupal development time decreases with increasing developmental temperature similarly for both sexes (Figure 5.5); in agreement with previous work (e.g. Zijlstra *et al.* 2004, Oostra *et al.* 2011). For both sexes, both temperature and genotype factors had significantly affected development time. The significantly GxT interaction

effect on development time indicates differences between the unselected stock and artificial selected lines in how they respond to temperature. We see that the DRY line shows the highest reaction norms (i.e. longer pupal development across temperature) in comparison with the WET line and the unselected stock that are similar (Figure 5.5). For survivorship DRY, WET and WT genotypes show differences in survival that depend on temperature, with higher levels of mortality at extreme temperatures especially for the artificial selected lines at warmer temperatures (Figure 5.6). Because we had a microspordian infection in our laboratory populations, we only had one replicate line of each selection direction. Therefore, all the correlated responses between the wing pattern (target of our selection) and life-history traits should be interpreted very carefully, and seen as possibilities to explore rather than definitive.

For wing background pigmentation, our results show for low temperatures three groups of pigments and for high temperatures four well distinct groups, with "gold" pigment detected pnly for the latter. There also seems to be a difference in the group of "brown" pigment that is darker and with different tones for lower temperatures. Finally, we found differences between Proximal and Distal sides not only in terms of wing color background but also at the width of eyespot color rings (Figure 5.7). Our analysis also revealed what is possibly a new color appearing at the most extreme low temperatures and mainly for DRY artificial line (Figure 5.8). We do not know what causes these differences, but suggested that the orange color corresponds to a pigment from a different type (e.g. ommochromes can be yellow, orange, red), or to a modification of a product of the melanin biosynthesis pathway.

The results present here are still from preliminary analyses and future work needs to be done in order to explore the data in more detail. This will include: 1) quantitative analysis of plasticity in background color, 2) formal mathematical treatment of the influence of external environment on development to characterize shape of thermal reaction norms, 3) mechanisms underlying the plasticity we document, and 4) extend analysis to other species.

ACKNOWLEDGEMENTS

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ANNEX 5.1

Summary of the statistical results for survival rate to test the effect of temperature, genotype and sex (S), for the total size of the fifth eyespot on the hindwing, corrected for wing size (WA), and pupal development time to test the effect of temperature (T) and genotype (G) for females and males (c.f. Figures 5.3, 5.4, 5.5 and 5.6). Statistical significance for the effects of T, G, S and their interactions is indicated as: *P < 0.05, ** P < 0.01, *** P < 0.001. When we found significant effects of each factor on trait value P < 0.05, we compared across temperatures (Tukey HDS, P < 0.01).

SURVIVAL ANALYSIS

Model: SurvivalProportion~Genotype*Temperature*Sex, weight=totalN

SAMPLE SIZE[a]										
	Females			**Males**				Df	Dev	Pr(>Chi)
	DRY	WET	WT	DRY	WET	WT				
15°C	15	3	24	17	3	22	**G**	1	54.1	1.78e-12 ***
17°C	25	21	43	30	19	28	**T**	2	0.04	0.82
19°C	28	23	33	22	14	35	**S**	1	0.1	0.75
23°C	23	36	52	26	50	53	**G:T**	2	25.7	2.62e-06 ***
25°C	34	35	47	28	28	62	**S:T**	1	2.62	0.1
27°C	38	43	56	37	34	49	**G:S**	2	1.07	0.58
29°C	11	23	4	23	24	9	**G:S:T**	2	0.13	0.93
31°C	5	11	14	12	17	15				

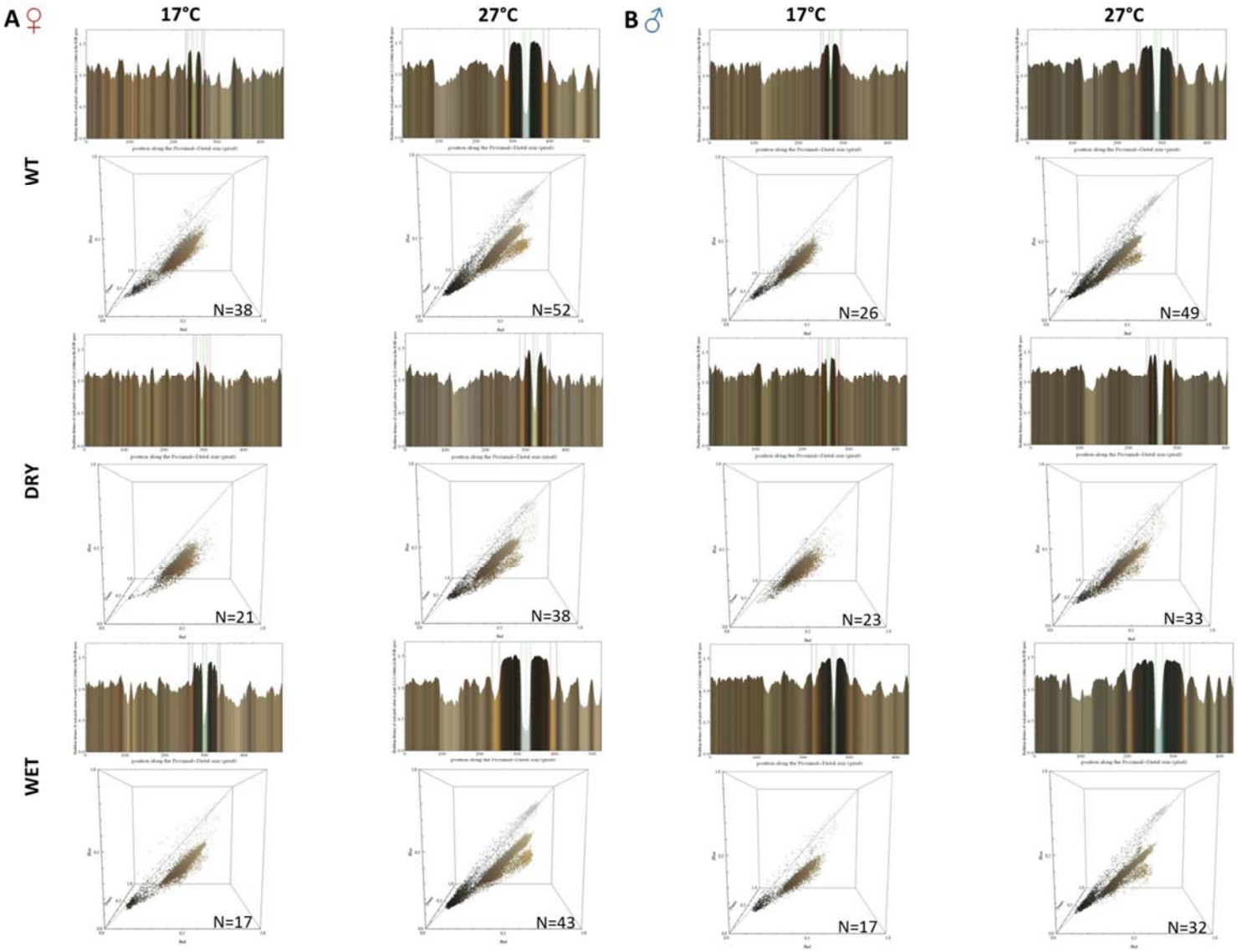
[a] Sample size at 21°C was largely reduced not due to natural mortality, but because we used the individuals to rescue the lab stock. Therefore, we decided to not present the results for survivorship at this temperature.

		EYESPOT SIZE Model: EyespotSize~TransectSize+Genotype*Temperature						DEVELOPMENT TIME Model: log(Days)~Genotype*Temperature					
		A-FEMALES			B-MALES			A-FEMALES			B-MALES		
	Df	Dev	F	P	Dev	F	P	Dev	F	P	Dev	F	P
WA	1	9.79	135.01	<2.2e-16 ***	1.6	31.11	<3.7e-08 ***	-	-	-	-	-	-
G	2	195.54	898.43	<2.2e-16 ***	114.6	743.1	<2.2e-16 ***	3.73	192.5	<2.2e-16 ***	2.00	98.96	<2.2e-16 ***
T	8	138.42	238.49	<2.2e-16 ***	92.6	225.07	<2.2e-16 ***	1.87	144.1	<2.2e-16 ***	1.43	106.3	<2.2e-16 ***
G:T	16	9.24	8.49	<2.2e-16 ***	6.37	8.26	<2.2e-16 ***	1.04	26.9	<2.2e-16 ***	0.28	7.10	4.7e-07 ***

HSD	EYESPOT SIZE						DEVELOPMENT TIME					
	A-FEMALES			B-MALES			A-FEMALES			B-MALES		
	Sample size (N)	Means	Groups	Sample size (N)	Means	Groups	Sample size (N)	Means	Groups	Sample size (N)	Means	Groups
DRY_15	16	0.635	h	13	0.664	h	15	3.458	a	17	3.468	a
DRY_17	21	0.934	h	23	0.918	gh	14	3.178	bc	12	3.142	bc
DRY_19	24	0.959	h	19	0.979	gh	19	2.953	de	15	2.863	de
DRY_21	18	0.919	h	6	1.076	gh	10	2.620	fg	5	2.576	fg
DRY_23	22	1.101	h	20	1.118	fg	22	2.260	hi	15	2.264	h
DRY_25	28	1.414	gh	22	1.473	ef	20	2.192	ij	26	2.096	ij
DRY_27	38	1.718	fg	33	1.474	ef	38	1.991	k	36	1.962	jk
DRY_29	10	1.417	gh	22	1.395	ef	11	1.793	l	15	1.822	kl
DRY_31	1	1.485	fgh	6	1.522	ef	2	1.946	kl	3	2.079	ijk
WET_15	1	0.948	h	2	2.055	bcde	3	3.454	ab	2	3.450	ab
WET_17	17	1.758	efg	17	1.668	e	11	3.084	cd	8	3.065	bcd
WET_19	22	1.909	def	13	1.734	de	11	2.800	ef	10	2.795	ef
WET_21	17	2.113	cde	20	1.815	de	9	2.442	gh	12	2.558	g
WET_23	34	2.684	ab	43	2.385	b	30	2.228	ij	45	2.202	hi
WET_25	29	2.951	a	24	2.652	a	32	2.111	j	27	2.100	i
WET_27	43	3.054	a	32	2.622	a	43	1.826	l	33	1.831	kl
WET_29	21	2.945	a	19	2.651	a	5	1.713	l	17	1.714	lm
WET_31	6	3.067	a	12	2.709	a	8	1.599	l	17	1.611	m
WT_15	24	0.929	h	22	0.887	gh	24	3.434	ab	22	3.419	ab
WT_17	38	1.147	h	26	1.084	g	17	3.061	cd	9	3.045	cd
WT_19	28	1.423	gh	31	1.402	ef	30	2.765	f	29	2.766	ef
WT_21	9	1.583	fg	12	1.448	ef	7	2.633	fg	19	2.624	fg
WT_23	50	2.161	cd	52	2.037	cde	51	2.223	ij	53	2.217	h
WT_25	44	2.225	c	58	1.944	cde	27	2.132	ij	33	2.113	hi
WT_27	52	2.673	ab	49	2.342	b	56	1.841	l	48	1.837	kl
WT_29	4	2.094	cdef	9	2.067	bcd	3	1.708	l	9	1.711	lm
WT_31	10	2.395	bc	11	2.169	bc	13	1.622	l	14	1.611	m

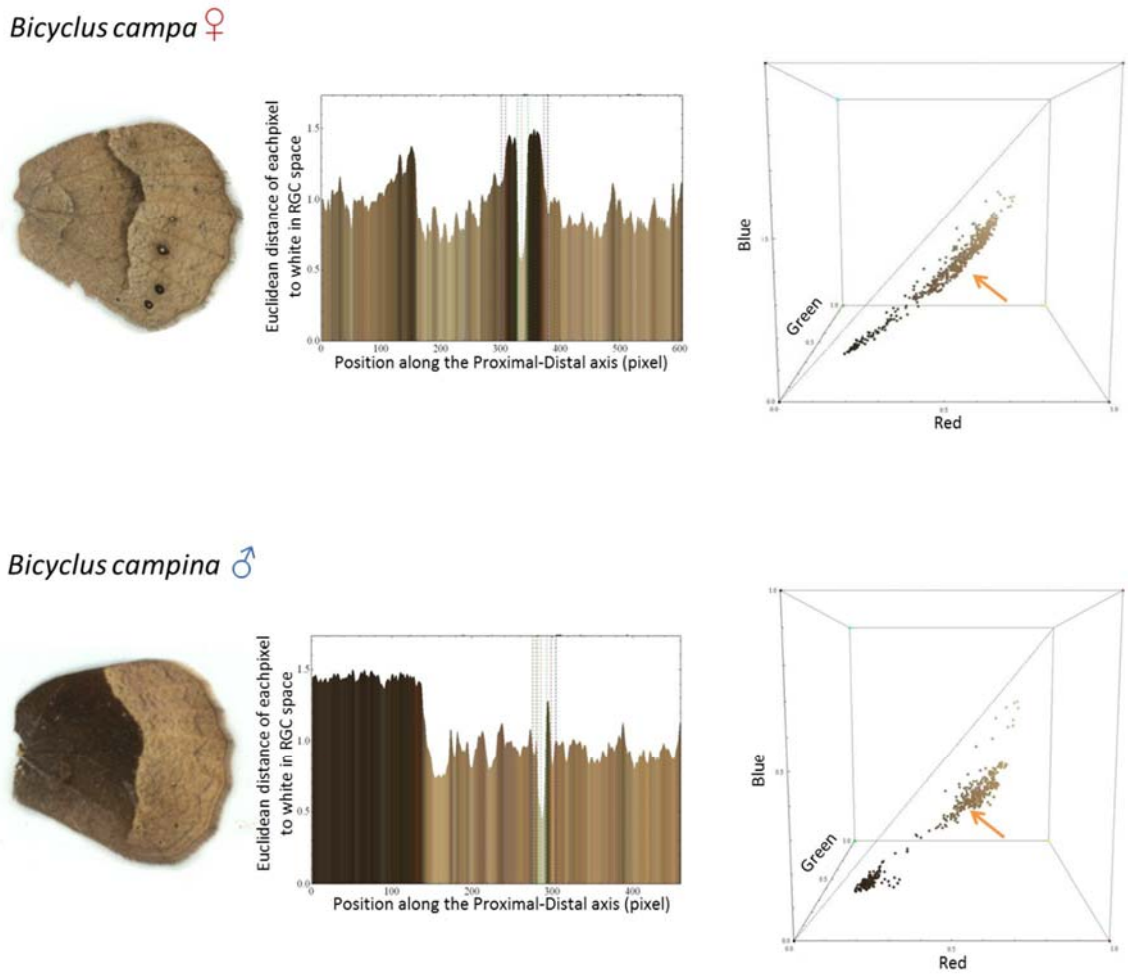
ANNEX 5.2

Wing background color at 17°C and 27°C for the DRY, WET and WT genotypes for females (A) and males (B). We chose these two temperatures of 17°C to represent low and 27°C to represent high, because these were temperatures with large sample sizes. When sample sizes are small some of the pigment groups (see Figure 5.2C) are difficult to distinguish. For each temperature, we show two different plots to characterize the transect through a hindwing (cf. Figure 5.2B): 1) distances to white of each pixel is color along the transect of one typical individual for that temperature, 2) 3D RGB space visualization of the RGB values of all pixels on transect of various individuals (sample size in each 3D) that allow distinguishing between different pigment groups.

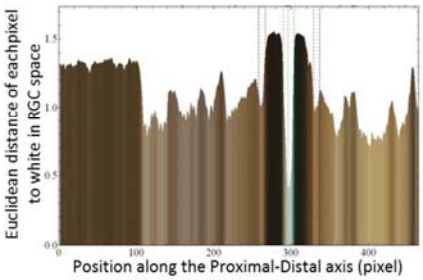


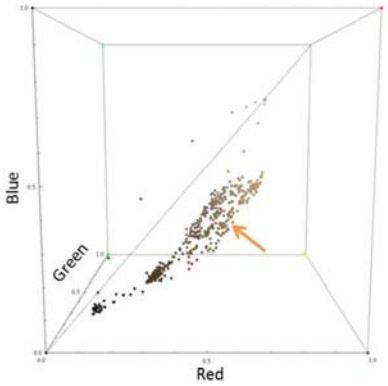
ANNEX 5.3

Wing background color for four different species of *Bicyclus* and *Heteropsis perspicua*. From the left to right in the figure we have: the adult hindwing of one individual that represents the typical phenotype, the plot for that individual that shows distances to white with each pixel along the wing transect illustrated by the average RGB value of that pixel, and the 3D RGB space visualization of the averaged RGB values that allow distinguishing between different wing background pigment groups for all individuals. Orange arrows point to the correspondent position where we find the orange pigment for *B. anynana* found at this temperature (Figure 5.8). For each species we just measured one individual that represents each characteristic phenotype, as it is very difficult to capture these species in the field. We used females and males because for some species females were not available.

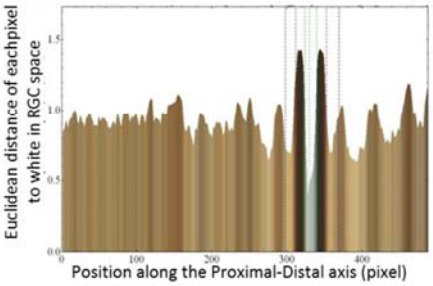


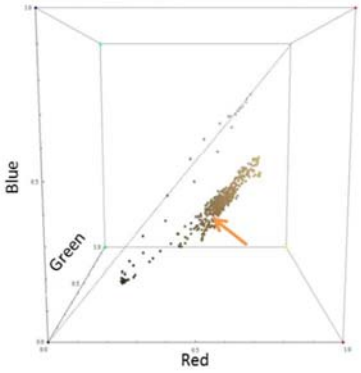
Bicyclus condamini ♂



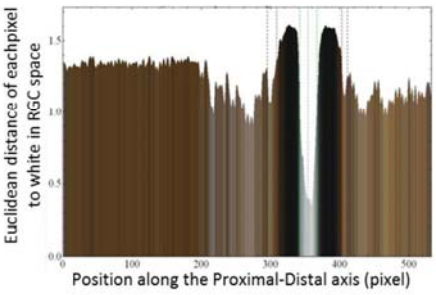


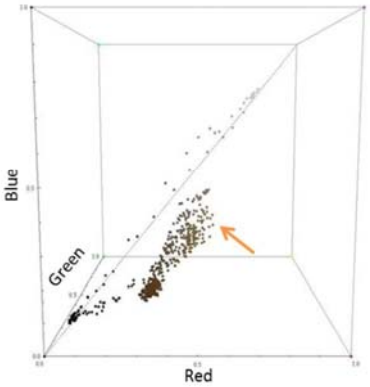
Heteropsis perspicua ♂





Bicyclus smithi ♀





REFERENCES

Abramoff M, Magalhaes P, Ram S (2004) Image processing with ImageJ. *Biophotonics International* **11**, 36-42.

Beldade P, Brakefield PM (2002) The genetics and evo-devo of butterfly wing patterns. *Nature Review Genetics* **3**, 442-452.

Beldade P, Mateus ARA, Keller RA (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology* **20**, 1347-1363.

Brakefield PM, Reitsma N (1991) Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* (Satyridae) in Malawi. *Ecological Entomology* **16**, 291-303.

Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden P, Monteiro A, French V, Carroll S (1996) Development, plasticity and evolution of butterfly eyespot patterns. *Nature* **384**, 236-242.

Brakefield PM (1997) Phenotypic plasticity and fluctuating asymmetry as responses to environmental stress in the butterfly Bicyclus anynana. In: Environmental stress, adaptation and evolution (ed. Bijlsma R, Loeschke V), pp. 65-78, Basel: Birkhäuser.

Brakefield PM, Kesbeke F, Koch PB (1998) The regulation of phenotypic plasticity of eyespots in the butterfly Bicyclus anynana. *American Naturalist* **152**, 853-860.

Brakefield PM, Beldade P, Zwaan BJ (2009) The African butterfly Bicyclus anynana: a model for evolutionary genetics and evolutionary developmental biology. In: Emerging Model Organisms: A Laboratory Manual (ed. Behringer RR, Johnson AD, Krumlauf RE), pp. 291-329, New York: Cold Spring Harbor Laboratory Press.

Brakefield PM, Frankino WA (2009) Polyphenisms in Lepidoptera: multidisciplinary approaches to studies of evolution. In: Phenotypic Plasticity in Insects. Mechanisms and Consequences (ed. Whitman DW, Ananthakrishnan TN), pp. 281-312, UK: Science Publishers.

Capy P, David JR, Robertson A (1988) Thoracic trident pigmentation in natural populations of Drosophila simulans: a comparison with D. melanogaster. *Heredity* **61**, 263-268.

Chevin LM, Collins S, Lef'evre F (2013) Phenotypic plasticity and evolutionary demographic responses to climate change: taking theory out to the field. *Functional Ecology* **27**, 967-979.

David JR, Capy P, Gauthier JP (1990) Abdomen pigmentation and growth temperature in *Drosophila melanogaster*. Similarities and differences in the norms of reaction of successive segments. *Journal of Evolutionary Biology* **3**, 429-445.

Debat V, David P (2001) Mapping phenotypes: canalization, plasticity and developmental stability. *Trends in Ecology and Evolution* **16**, 555-561.

de Jong G (1999) Unpredictable selection in a structured population leads to local genetic differentiation in evolved reaction norms. *Journal of Evolutionary Biology* **12**, 839-851.

de Jong MA, Kesbeke FMNH, Brakefield PM, Zwaan BJ (2010) Geographic variation in thermal plasticity of life history and wing pattern in *Bicyclus anynana*. *Climate Research* **43**, 91-102.

DeWitt TJ, Sih A, Wilson DS (1998) Costs and limits of phenotypic plasticity. *Trends in Ecology and Evolution* **13**, 77-81.

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics. 4th edition, Chicago: The University of Chicago Press.

Fischer K, Dierks A, Franke K, Geister TL, Liszka M, Winter S, Pflicke C (2010) Environmental effects on temperature stress resistance in the tropical butterfly *Bicyclus anynana*. *PLoS ONE* **5**, e15284.

Gavrilets S, Scheiner SM (1993*a*) The genetics of phenotypic plasticity. V. Evolution of reaction norm shape. *Journal of Evolutionary Biology* **6**, 31-48.

Gibert P, Moreteau B, Moreteau JC, David JR (1996) Growth temperature and adult

pigmentation in two Drosophila sibling species: a probably adaptive convergence of reaction norms in sympathetic populations. *Evolution* **50**, 2346-2353.

Gibert P, Moreteau B, David JR (2000) Developmental constraints on an adaptive plasticity: reaction norms of pigmentation in adult segments of Drosophila melanogaster. *Evolution and Development* **2**, 249-260.

Gibert P, Capy P, Imasheva P, Moreteau B, Morin JP, Pétavy G, David JR. (2004) Comparative analysis of morphological traits among Drosophila melanogaster and D. simulans, Genetic variability, clines and phenotypic plasticity. *Genetica* **120**, 165-179.

Gibert J-M, Peronnet F, Schlötterer C (2007) Phenotypic plasticity in *Drosophila* pigmentation caused by temperature sensitivity of a chromatin regulator network. *PLoS Genetics* **3**, e30.

Goulson D (1994) Determination of larval melanization in the moth, Mamestra brassicae, and the role of melanin in thermo-regulation. *Heredity* **73**, 471-479.

Hoffmann AA, Sørensen JG, Loeschcke V (2003) Adaptation of Drosophila to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology* **28**, 175–216.

Jeong S, Rokas A, Carroll SB (2006) Regulation of body pigmentation by the Abdominal-B Hox protein and its gain and loss in Drosophila evolution . *Cell* **125**, 1387-1399.

Karan D, Dahiya N, Munjal AK, Gibert P, Moreteau B, Parkash R, David JR (1998). Desiccation and starvation tolerance of adult *Drosophila* - opposite latitudinal clines in natural populations of three different species. *Evolution* **52**, 825-831.

Koch PB, Kaufmann N (1995) Pattern specific melanin synthesis and DOPA decarboxylase activity in a butterfly wing of *Precis coenia* Hübner. *Insect Biochemistry and Molecular Biology* **25**, 73-82.

Kooi RE, Brakefield PM (1999) The critical period for wing pattern induction in the polyphenic tropical butterfly *Bicyclus anynana* (*Satyrinae*). *Journal of Insect Physiology* **45**, 201-212.

Lee Jr RE (1991) Principles of insect low temperature tolerance. In: Insects at Low Temperature (ed. Lee Jr RE, Denlinger DL), pp. 17-46, New York: Chapman & Hall.

Lewontin RC (2006) The analysis of variance and the analysis of causes. *International Journal of Epidemioly* **35**, 520-525.

Lyytinen A, Brakefield PM, Lindstrom L, Mappes J (2004) Does predation maintain eyespot plasticity in *Bicyclus anynana*? *Proceedings of Biological Sciences* **271**, 279-283.

Mateus ARA, Marques-Pita M, Oostra V, Lafuente E, Brakefield PM, Zwaan, BJ, Beldade P (2014) Adaptive developmental plasticity: Compartmentalized responses to environmental cues and to corresponding internal signals provide phenotypic flexibility. *BMC Biology* **12**, 97.

Needham AE (1974) The Significance of Zoochromes. Berlin: Springer-Verlag.

Neyfakh AA, Hartl DL (1993) Genetic control of the rate of embryonic development: selection for faster development at elevated temperatures. *Evolution* **47**, 1625-1631.

Nijhout HF (1991) The development and evolution of butterfly wing patterns. Smithsonian Institution Press.

Nijhout HF (2003) The control of growth. *Development* **130**, 5863-5867.

Nijhout HF (2010) Molecular and physiological basis of colour pattern formation. *Advances in Insect Physiology* **38**, 219-265.

Oostra V, de Jong MA, Invergo B, Kesbeke F, Wende F, Brakefield PM, Zwaan BJ (2011) Translating environmental gradients into discontinuous reaction norms via hormone signaling in a polyphenic butterfly. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **278**, 789-797.

Oostra V, Mateus ARA, van der Burg KR, Piessens T, van Eijk M, Brakefield PM, Beldade P, Zwaan BJ (2014) Ecdysteroid hormones link the juvenile environment to alternative adult life histories in a seasonal insect. *American Naturalist* **184**, E79-E92.

Oliver JM, Robertson KA, Monteiro A (2009) Accommodating natural and sexual selection in butterfly wing pattern evolution. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **276**, 2369-2375.

Pertoldi C, Bundgaard J, Loeschcke V, Barker JSF (2014) The phenotypic variance gradient – a novel concept. *Ecology and Evolution* **4**, 4230-4236.

Pigliucci M (2001) Phenotypic plasticity: beyond nature and nurture. Baltimore: The John Hopkins University Press.

Pigliucci M (2005) Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology and Evolution* **20**, 481-486.

R-Core-Team (2012) R - A Language and Environment for Statistical Computing.

Roskam JC Brakefield PM (1999) Seasonal polyphenism in Bicyclus (Lepidoptera: Satyridae) Butterflies: Different climates need different cues. *Biological Journal of the Linnean Society* **66**, 345-356.

Schlichting CD, Pigliucci M (1998) Phenotypic Evolution: A Reaction Norm Perspective. Massachusetts: Sinauer Associates, Inc.

Sinclair BJ, Vernon P, Klok CJ, Chown SL (2003) Insects at low temperatures: an ecological perspective. *Trends in Ecology and Evolution* **18**, 257–262.

Steinhoff S (2011) The VueScan Bible: Everything You Need to Know for Perfect Scanning. Santa Barbara: Rocky Nook, Inc.

Sultan SE (2007) Development in context: the timely emergence of eco-devo. *Trends in Ecology and Evolution* **22**, 575-582.

True JR (2003) Insect melanism: the molecules matter. *Trends in Ecology and Evolution* **18**, 640-647.

Van der Have TM, de Jong G. (1996). Adult size in ectotherms: temperature effects on growth and differentiation. *Journal of Theoretical Biology* **183**, 329–340.

West-Eberhard MJ (2003) Developmental plasticity and evolution. New York: Oxford University Press.

Wittkopp PJ, Beldade P. (2009) Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. *Seminars in Cell and Developmental Biology* **20**, 65-71.

Wijngaarden PJ, Brakefield PM (2000) The genetic basis of eyespot size in the butterfly *Bicyclus anynana*: an analysis of line crosses. *Heredity* **85**, 171-179.

Wijngaarden PJ, Brakefield PM (2001) Lack of response to artificial selection on the slope of reaction norms for seasonal polyphenism in the butterfly *Bicyclus anynana*. *Heredity* **87**, 410-420.

Wijngaarden PJ, Koch PB, Brakefield PM (2002) Artificial selection on the shape of reaction norms for eyespot size in the butterfly *Bicyclus anynana*: direct and correlated responses. *Journal of Evoutionary Biology* **15**, 290-300.

Wolfram S (1996) Mathematica 3.0 User's Guide. New York: Cambridge University Press.

Zijlstra WG, Steigenga MJ, Brakefield PM, Zwaan BJ (2003) Simultaneous selection on two fitness related traits in the butterfly Bicyclus anynana. *Evolution* **57**, 1852-1862.

Zijlstra WG, Steigenga MJ, Koch PB, Zwaan BJ, Brakefield PM (2004) Butterfly selected lines explore the hormonal basis of interactions between life histories and morphology. *American Naturalist* **163**, 76-87.

Zwaan BJ, Zijlstra WG, Keller M, Pijpe J, Brakefield PM (2008) Potential constraints on evolution: sexual dimorphism and the problem of protandry in the butterfly *Bicyclus anynana*. *Journal of Genetics* **87**, 395-405.

CHAPTER 6. OVERVIEW: THIS THESIS AND FUTURE RESEARCH DIRECTIONS

CONCLUDING REMARKS

"Intelligence is the ability to adapt to change."

- *Stephen Hawking*

The study of developmental plasticity, the ability of a genotype to produce distinct phenotypes when exposed to different environments during development, has advanced significantly over the past decades. However, despite many advances, there are still many gaps in our understanding of the mechanisms involved. In order to try to contribute with one more piece to this "puzzle", our study intended to explore the genetic and physiological mechanisms underlying adaptive developmental plasticity in *Bicyclus anynana* wing pattern and life-history traits. Our effort involved integrating a broad range of approaches and collaborating with researchers from different areas. We combined information from genes, to development, to physiology, to different phenotypes, and tried to relate our findings with the ecology and evolution in natural populations with particular emphasis in relation to adaptation to changing environments. The experiments described here led to several interesting and new observations. Here I briefly discuss some of the issues which I judge to be especially important to understand the mechanisms involved in adaptive developmental plasticity.

With this thesis we had the opportunity to write and publish a review of the extensive literature on adaptive developmental plasticity contributing with a useful bibliographic tool for future reference (CHAPTER 1).

In general, hormone-mediated developmental switches allow organisms to mount a systemic, integrated and coordinated response to environmental variation, as systemic hormone levels are regulated from the central nervous system in response to signals sensed from the environment. We found that not all organs and groups of cells within organs have equal sensitivities to the external temperature and internal signals that convey information about temperature to developing tissues (ecdysone). In CHAPTER 2 we found unexpected differences between sensitivity to temperature and to hormone levels between traits of the same organ. We also showed that the spatial

compartmentalization of hormone effects is not due to the spatial compartmentalization of the levels of hormone receptor protein as had been suggested before (Brakefield *et al.* 1998). We argued that differences in the way that different groups of cells respond to hormone manipulations must be determined either upstream of the binding of the hormone to its receptor in the cell nucleus or downstream of that. In CHAPTER 3, in a similar way as we had done for wing pattern traits (CHAPTER 2), we found that the response to hormone manipulation is a local property of those tissues. We showed that ecdysteroids have a functional role acting as a switch between developmental pathways by translating information from the external environment into adaptive alterations. This culminates in alternative adult life histories in *Bicyclus anynana*. We concluded that manipulating pupal ecdysteroid levels is sufficient to mimic in direction and magnitude the shifts in adult reproductive resource allocation normally induced by seasonal temperature. Such local hormone sensitivity allows for a cell-, tissue- or trait-dependent differentiated response to the circulating hormone. In general, we argued that the compartmentalization of these effects reflects what has been called phenotypic integration to imply tight connections between traits, or phenotypic independence to refer to connections that are readily uncoupled (Hau 2007). The integration between traits can be a factor constraining future responses to selection if integrated traits are selected to change in opposite ways. On the other hand, having traits responding independently to systemic hormone or external input can allow more rapid evolution of new arrangements of traits. This possible "reorganization" of traits produced by exposure to novel environmental conditions can lead to the production of new phenotypic variants and even differences between species, illustrating a process that has been called developmental recombination (West-Eberhard 2005). Together CHAPTERS 2 and 3 illustrate how organisms can use systemic hormones and their time- and tissue-specific sensitivity to respond to predictive indicators of environmental quality to make strategic life history decisions that enable them to cope with fluctuating environments.

Developmental plasticity may be described as a phenotypic result of the effects of environmental variation, in interaction with genetic variation, on development. It is generally represented by reaction norms. We revealed variation in reaction norms properties, such as height and shape, between different genetic stocks representing spontaneous pigmentation mutants of *B. anynana* (CHAPTER 4). We showed evidence for GxE effects on wing pattern with alleles affecting eyespot color and size displaying

larger sensitivity to temperature. Alleles such as these might contribute to genetic accommodation and the evolution of plasticity (Suzuki & Nijhout 2006).

Finally, we show the preliminary results for data that hopefully will bring new exciting conclusions (CHAPTER 5). During years in which I developed my thesis, we re-derived artificial selected lines expressing extreme wet season-like or dry-season-like phenotypes at intermediary temperatures. Using these lines and the unselected stock of *B.* anynana, we characterized thermal reaction norms for a wide range of temperatures and for several traits including eyespot size, pupal development time, survivorship and, for the first time, of wing background color. Our artificial selection lines differ in eyespot size and wing color across temperatures. We show evidence for GxE effects on eyespot size, suggesting differences in reaction norms between lines. Further analysis can show the extent to which we changed reaction norms shape. For wing background color we conclude that for lower temperatures we have more differences in color intensities and very few yellow scales. We also documented asymmetry between Proximal and Distal half of eyespots, not only in terms of wing color background, but also at the width of the eyespot color rings. Our preliminary analysis also showed a possibly new orange color appearing at extreme low temperatures, mainly for the DRY artificial line. We introduced what we hope will become a method for quantitative analysis of color and color patterns. In the future we hope to expand our dataset to explore a detailed formal mathematical treatment of thermal reaction norms. Our artificial selection procedure targeting wing pattern, also seemed to be indicative of effects in other traits such as pupal development time and survival rate, however we have the limition of not having individual replicate lines.

We hope that the conclusions of this thesis could be in the future a beginning for many other research works and the inspiration for many scientists interested in adaptive developmental plasticity. Some ideas and even data collected during this work, and not analyzed yet, will be refered into the next section. Recently, there has been growing interest in understanding various aspects of developmental plasticity and its importance in evolutionary adaptation by trying to understand how populations cope with changing environmental conditions (e.g. Forsman 2014, Murren *et al.* 2015). Still, there are few examples where the relative contributions of plasticity and evolutionary adaptation have been explored, especially in a climate change context (e.g. Gienapp *et al.* 2008, Merilä & Hendry 2014). In an environment rapidly changing, narrowly adapted populations without the necessary genetic variation in selectively important characters to cope with

environmental perturbations, might be at a higher risk of extinction (Willi *et al.* 2006, Mäkinen *et al.* 2015). In this context, we expect that our results help to increase the current knowledge about the role of developmental plasticity in how organisms can cope with environmental changes and in predicting future evolutionary scenarios.

FUTURE RESEARCH DIRECTIONS

The present thesis took an integrated approach in order to explore the mechanisms underlying adaptive developmental plasticity and combined studies at the genetic, physiological, phenotypic, ecological, and evolutionary level. 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. The results presented in this thesis contribute to our general understanding of the mechanisms of adaptation to environmental variation.

There are many other issues that we would like to explore and we did not have to opportunity such as the role of epigenetics in developmental plasticity. A full understanding of gene-environment interactions requires that epigenetic as well as classical genetic mechanisms should be taken into account. Unlike the genome that is mainly identical in all cells and stable throughout the life-time of an individual, the epigenome differs from cell to cell and is plastic by changing with time and with exposure to the environment (Jirtle & Skinner 2007). The epigenome is particularly vulnerable to environmental influences during certain stages of development and that could influence the phenotype of the adult. Therefore research into the epigenetic regulation of gene expression in the context of developmental plasticity should be of high priority and *B. anynana* has a large potential to be used as biological model.

Developmental plasticity frequently also involves parental effects, which might enable adaptive and context-dependent transgenerational transmission of phenotypic strategies. Recent studies of plants and animals show how studies of parental effects in an ecological context provide important insights into the origin and evolution of adaptation under variable environmental conditions (Uller 2008). We started to explore parental effects in order to check for the effects of parental rearing temperature on progeny thermal plasticity. For that purpose we run a pilot experiment of two generations of *B. anynana* individuals. In the first parental generation we reared larvae from three different genetic stocks (unselected WT stock, DRY and WET artificial lines) at three different temperatures and chose randomly pairs of adults from each stock

to mate and lay eggs for next generation. The progeny from each genetic stock, at each temperature, was then split by the three different temperatures. The adult wings from both generations were frozen and kept in envelops for further analysis. Depending on the pilot results we would like to explore deeply the mechanisms behind parental effects into the context of adaptation to fluctuating environments.

Finally, the formation of species has long represented one of the most central, but also one of the most elusive subjects in evolutionary biology (e.g. Palumbi 1994). Speciation involves the evolution of reproductive barriers between populations, and those barriers ultimately must be maintained if species are to remain distinct entities (e.g. Mayr 1942, Coyne & Orr 2004). A reproductive barrier may be considered important if, by acting alone, it is a strong impediment to gene flow. After so many generations of artificial selection, we would like also to explore the possibility of "reproductive isolation" between different genetic stocks: unselected stock (WT) and the selected artificial DRY and WET lines in the context of development plasticity. This would allow us to start to explore the possibility of a species that show different seasonal forms, depending on different environmental conditions, become in the future different species. So far we performed a small preliminary experiment where we isolated few couples of each of these different genetic stocks in all possible combinations. After, we checked for the total number of larva (or absence of that) for each of the couples, in order to have an idea of the total progeny. A bigger and improved experiment, if the observations from the pilot experiment give exciting results, would be worth to do it because if "reproductive isolation" could be confirmed we could use it to explore the mechanisms behind speciation, which is one of the most important and also one of the most elusive subjects in evolutionary biology.

We did a large effort to explore as much as possible the mechanisms that underlying developmental plasticity and, so far, this thesis is not a conclusion of our work as there are still many questions that need to be answered. For that reason we collected so many extra data and we have in mind to continuous our research on the subject. What are the mechanism(s) that species use to sense the external environmental cues? How is that environmental information translated into internal signals? Which are the genes involved in developmental plasticity? What is the role of developmental plasticity in evolutionary innovation? It is clear that developmental plasticity will continue to be an active area of research and will greatly profit from the availability of sophisticated molecular, genetic or even computational methods.

REFERENCES

Brakefield PM, Kesbeke F, Koch PB (1998) The regulation of phenotypic plasticity of eyespots in the butterfly Bicyclus anynana. *American Naturalist* **152**, 853-860.

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, MA.

Falconer DS (1952) The problem of environment and selection. *American Naturalist* **86**, 293-298.

Forsman A (2014) Rethinking phenotypic plasticity and its consequences for individuals, populations and species. *Heredity*. Advance Access published October 8, doi: 10.1038/hdy.2014.92.

Gienapp P, Teplitsky C, Alho JS, Mills JA, Merillä J (2008) Climate change and evolution: disentangling environmental and genetic responses. *Molecular Ecology* **17**, 167-178.

Gilbert SF, Epel D (2009) Ecological Developmental Biology: Integrating Epigenetics, Medicine, and Evolution. Massachusetts: Sinauer Associates.

Hau M (2007) Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. *Bioessays* **29**, 133-144.

Jirtle RL, Skinner MK (2007) Environmental epigenomics and disease susceptibility. *Nature Review Genetics* **8**, 253-262.

Mäkinen H, Papakostas S, Vøllestad LA, Leder EH, Primmer CR (2015) Plastic and Evolutionary Gene Expression Responses Are Correlated in European Grayling (*Thymallus thymallus*) Subpopulations Adapted to Different Thermal Environments. *Journal of Heredity*, esv069.

Mayr E (1942) Systematics and the origin of species. Columbia Univ. Press, New York.

Merillä J, Hendry AP (2014) Climate change, adaptation, and phenotypic plasticity: the problem and the evidence. *Evolutionary Applications* **7**, 1-14.

Murren CJ, Auld JR, Callahan H, Ghalambor CK, Handelsman CA, Heskel MA, Kingsolver JG, Maclean HJ, Masel J, Maughan H, Pfennig DW, Relyea RA, Seiter S, Snell-Rood E, Steiner UK, Schlichting CD (2015) Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. *Heredity*. Advance Access published February 18, doi: 10.1038/hdy.2015.8.

Palumbi SR (1994) Genetic divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology and Systematics* **25**, 547-572.

Suzuki Y, Nijhout HF (2006) Evolution of a polyphenism by genetic accommodation. *Science* **311**, 650-652.

Uller T (2008) Developmental plasticity and the evolution of parental effects. *Trends in Ecology & Evolution* **23**, 432-438.

West-Eberhard MJ (2005) Developmental plasticity and the origin of species differences. *Proceedings of the Natural Academy of Sciences USA* **102**, 6543-6549.

Willi Y, Van Buskirk J, Hoffmann AA (2006) Limits to the adaptive potential of small populations. *Annual Review of Ecology, Evolution & Systematics* **37**, 433-458.

CHAPTER 7. SUMMARY

It has become increasingly clear that a complete understanding of the effects of changing environments in natural populations requires knowledge of trends in environmental variables, of the species composition of communities, as well as of the biology of those species. Of extremely importance in the study of how organisms cope with changing environments is adaptive developmental plasticity, the ability of some genotypes to develop into distinct phenotypes depending on environmental conditions encountered during development. Adaptive phenotypic plasticity represents a fundamental component of evolutionary change and can represent an optimal solution to the challenges of an unpredictable environment (reviewed in CHAPTER 1). This process is regulated by changes in physiology, and has one of its most compelling examples in butterfly wing patterns that dramatically differ across seasons.

Adaptive developmental plasticity in butterfly wing patterns has been characterized in relation to its ecological and evolutionary relevance, and the recent development of molecular and genetic tools have opened the possibility to study extensively how environmental conditions during development lead to the production of alternative seasonal forms. We have focused on an emerging model in evolutionary and ecological genomics, *Bicyclus anynana* butterflies for which knowledge of the adaptive value of plasticity in natural populations can be complemented with an understanding of its underlying mechanisms. Butterflies which develop in one or the other season differ in ecological strategies reflected by wing pattern and life-history traits. The alternative seasonal phenotypes seen in natural populations of this species can be produced in the laboratory by rearing at different temperatures. The adaptive value of such alternative seasonal phenotypes and their relation to hormone cycles has been previously established. However, little is known about how the environmental cues modulate development to produce those phenotypes, and about the evolution of plasticity.

The main focus of this thesis was the analysis of the mechanisms underlying developmental plasticity, represented by temperature-regulated variation in adaptive butterfly wing color patterns and life-history traits. We tried to integrate the analysis of changes in hormone physiology, spatial patterns of gene expression, development and genetic-by-environment effects with the ecological and evolutionary analysis of phenotypes in different genetic backgrounds of *B. anynana*.

The effect of the environment on developmental outcome is typically mediated by hormonal signals which translate information about environmental cues to the developing tissues. In order to explore the physiological mechanism and start to explore the genetic mechanisms underlying developmental plasticity in CHAPTERS 2 AND 3 we manipulated ecdysteroid levels during pupal development, at different temperatures, to measure the effects on wing pattern and adult life-history traits. In CHAPTER 2, our results show for wing pattern that the effects of hormone manipulations depend on temperature and time point, and that different groups of cells within the same tissue have sensitivities and patterns of response that are distinct for the external environmental cue and for the internal hormonal signal. While patterns of significant response to temperature contrasted traits on autonomously-developing wings, significant response to hormone manipulations contrasted neighboring groups of cells with distinct color fates. We also show that this compartmentalization does not reflect compartmentalization of expression of hormone receptor. CHAPTER 3 shows that manipulating pupal ecdysteroid levels is sufficient to mimic in direction and magnitude the shifts in adult reproductive resource allocation normally induced by seasonal temperature. This allocation shift is accompanied by changes in ecologically relevant traits, including timing of reproduction, lifespan and starvation resistance. Together CHAPTER 2 AND 3 underscore the complexity of the interactions between environment and physiology in shaping the development of different body parts and in mediating reproductive investment decisions allowing organisms to cope with fluctuating environments.

Reaction norms are an important tool in the study of developmental plasticity and different genotypes can show different properties of reaction norms such as height or shape. In CHAPTER 4 we hypothesized that alleles that affect pigmentation also can affect plasticity therein. In order to investigate this hypothesis we characterizing thermal reaction norms for the eyespot color rings of four *B. anynana* genetic stocks. Our results provide evidence for GxE effects with different genetic stocks showing variation in the height, slope and shape of reaction norms. Genotypes with alleles affecting eyespot size and color were the most sensitive to variation in developmental temperature. These mutant alleles might contribute to genetic accommodation and the evolution of plasticity mediating the origin of novel adaptive phenotypes. However, this was true for only one of the wings suggesting organ-specific allelic effects. CHAPTER 4 in general

underscores the complexity of GxE interactions in the light of evolution of developmental plasticity.

Finally, in CHAPTER 5 we present our preliminary results and ongoing work in order to explore the GxE effects in *B. anynana* development. In order to achieve that we re-deriving lost artificial DRY and WET selected lines, characterizing thermal reaction norms for a wider range of temperatures that is usually explored in this species for wing pattern and life-history traits and, performed a qualitative analysis of wing background color. This could inform about the nature of GxE and allow us to investigate possible novel/extreme phenotypes and increased range of phenotypic variation that might result from exposure of cryptic genetic variation. Preliminary results show that artificial selection lines for wing patterns at intermediate temperatures resulted in genotypes with different reaction norms height and possibly also shape. The response to selection seems to have been most extreme for the WET line, with heighest reaction norms, while for the DRY line the reaction norms are lowest and flattest in comparison with the WT. Previous studies targeting *B. anynana* eyespot plasticity were able to change reaction norms height but not shape. Future directions include developing a detailed formal mathematical treatment of the influence of external environment on development to characterize shape of thermal reaction norms. We show that our artificial selection lines also differ in pupal development time and survivorship. Because there is no replication in the artificial selection experiment, we cannot tell whether this reflects genetic correlations with the wing pattern traits that were the direct targets of selection. These correlated responses to selection likely reflect genetic pleiotropy. For both sexes, both temperature and genotype factors had significantly affected development time. For survivorship, DRY, WET and WT genotypes show differences that depend on temperature, with higher levels of mortality at extreme temperatures. In CHAPTER 5 we also show differences in developmental plasticity for wing background color with three groups of pigments for low temperatures and for high temperatures four well distinct groups. We also found differences between Proximal and Distal sides not only in terms of wing color background but also at the width of eyespot color rings. Finally, our preliminary results also show that a possibly new orange color appears at extremely low temperatures mainly for the DRY artificial line. We do not know what causes these differences, but we suggested that the orange color might correspond to a pigment from a different type or to a modification of a product of the melanin biosynthesis pathway. In order to explore these preliminary results we would like to develop a general method

to quantify plasticity in wing background color possible to apply to most of the organisms.

In CHAPTER 6 we give a short summary of the main conclusions of this thesis and of possibilities for future work in this area. Concluding, the results presented in this thesis contribute to the general knowledge about the mechanism of adaptation to environmental variation and, in a broader perspective, they may also add to our understanding how species may adapt to climate change. We would like to be an example for future research in this area by demonstrating how exciting is combining different approaches in order to understand what is arguably one of the most fascinating abilities of biological systems - that of translate the environment into biological variation.

SAMENVATTING

Het is steeds duidelijker geworden dat een volledig inzicht in de effecten van veranderingen in de omgeving van natuurlijke populaties kennis vereist van trends in omgevingsfactoren, de soortsamenstelling van de gemeenschappen, alsmede van de biologie van deze soorten. Buitengewoon belangrijk bij de studie naar omgang van organismen met veranderingen in de omgeving is ontwikkelingsplasticiteit, het vermogen van sommige genotypen te ontwikkelen tot verschillende fenotypen afhankelijk van de omgevingsfactoren tijdens de ontwikkeling. Fenotypische plasticiteit is een fundamenteel onderdeel van evolutionaire verandering en kan een optimale oplossing zijn voor de uitdagingen van een onvoorspelbare omgeving (HOOFDSTUK 1). Dit proces wordt gereguleerd door veranderingen in de fysiologie, en heeft als een van de meest aansprekende voorbeelden vlindervleugelpatronen die drastisch verschillen per seizoen.

Ontwikkelingsplasticiteit in vlindervleugelpatronen werd gekarakteriseerd in relatie tot de ecologische en evolutionaire relevantie. De recente ontwikkelingen van moleculaire en genetische technieken geven de mogelijkheid om op grote schaal te bestuderen hoe omgevingsomstandigheden gedurende de ontwikkeling leiden tot de productie van alternatieve seizoensgebonden vormen. We hebben ons gericht op een model organisme in de evolutionaire en ecologische genomica (genomics), *Bicyclus anynana* een vlinder waarvan kennis van de adaptieve waarde van plasticiteit in natuurlijke populaties kan worden aangevuld met een goed inzicht in de onderliggende mechanismen. Vlinders welke ontwikkelen in het ene of andere seizoen verschillen in ecologische strategieën wat zich uit in vleugel patroon en levensgeschiedenis (life-history) eigenschappen. De verschillende seizoensgebonden fenotypes die voorkomen in natuurlijke populaties van deze soort kunnen in het laboratorium worden gekweekt door het grootbrengen bij verschillende temperaturen. De adaptieve waarde van dergelijke verschillende seizoensgebonden fenotypen en hun relatie tot hormooncycli was al van eerder onderzoek bekend. Er is echter weinig bekend over hoe de omgevingsfactoren ontwikkeling moduleren om die fenotypes te produceren en over de evolutie van plasticiteit.

De belangrijkste focus van dit proefschrift was de analyse van de mechanismen die ten grondslag liggen aan ontwikkelingsplasticiteit, temperatuur gereguleerde variatie in vlindervleugel kleurpatronen en levensgeschiedenis eigenschappen. We hebben

geprobeerd de analyse van veranderingen in Hormoonfysiologie ruimtelijke patronen van genexpressie, ontwikkeling en genetische x omgeving effecten(genetic-by-environment effects, GxE) van de ecologische en evolutionaire analyse van fenotypes in verschillende genetische achtergronden van *B. anynana* te integreren.

Het effect van de omgeving op de ontwikkelingsuitkomst wordt gemedieerd door hormonale signalen die informatie over omgevingssignalen vertalen naar de ontwikkelende weefsels. Om het fysiologische mechanisme te onderzoeken en te beginnen met het exploreren van de genetische mechanismen die ten grondslag liggen aan ontwikkelingsplasticiteit hebben we in HOOFDSTUK 2 EN 3 de niveaus van het steroïde hormoon Ecdyson gemanipuleerd tijdens het popstadium, bij verschillende temperaturen, om de effecten op de vleugelpatronen en volwassen levensgeschiedenis eigenschappen te meten. In HOOFDSTUK 2, laten onze resultaten voor de vleugelpatronen zien dat de effecten van hormoon manipulaties afhankelijk zijn van de temperatuur en tijdspunt, en dat verschillende groepen cellen binnen hetzelfde weefsel gevoeligheden en patronen van respons hebben die uitgesproken zijn voor de externe omgevingssignalen en voor de interne hormonale signalen. Terwijl patronen van significante respons op temperatuur contrasteren op autonoom ontwikkelende vleugels, significante respons op hormoon manipulaties contrasteren naburige groepen cellen met opvallende kleurovergangen. We tonen ook dat deze compartimentering niet overeen komt met compartimentering van expressie van hormoon receptor. HOOFDSTUK 3 laat zien dat het manipuleren van niveaus van het steroïde hormoon Ecdyson in het popstadium volstaat om de verschuiving in allocatie van de hoeveelheid beschikbare bron die word toegewezen aan voortplanting na te bootsen, normaliter geïnduceerd door temperatuurswisseling tussen de seizoenen. Deze allocatie verschuiving gaat gepaard met veranderingen in ecologisch relevante eigenschappen, waaronder de timing van reproductie, levensduur en uithoudingsvermogen bij voedselschaarste. HOOFDSTUK 2 EN 3 onderstrepen samen de complexiteit van de interacties tussen omgeving en fysiologie in vormgeven van de ontwikkeling van verschillende lichaamsdelen en het mediëren van reproductieve investeringsbesluiten welke organismen de mogelijkheid geven om te gaan met fluctuerende omgevingen.

Curves van de respons (reaction norms) zijn een belangrijk instrument in de studie van ontwikkelingsplasticiteit en verschillende genotypen kunnen verschillende eigenschappen van de curves van de respons laten zien zoals hoogte of vorm. In HOOFDSTUK 4 hebben we de hypothese gesteld dat allelen die invloed hebben op

pigmentatie ook invloed hebben op de plasticiteit daarin. Om deze hypothese te onderzoeken karakteriseren we thermische curves van de respons voor de oogvlek kleurringen van vier genetische *B. anynana* stocks. Onze resultaten leveren het bewijs voor "GxE" effecten waarbij de verschillende genetische stocks variatie laten zien in de hoogte, helling en de vorm van de curves van de respons. Genotypen met allelen die invloed hebben op oogvlek grootte en kleur waren het meest gevoelig voor variatie in temperatuur tijdens de ontwikkeling. Deze mutanten allelen kunnen mogelijk bijdragen tot genetische accommodatie en de evolutie van plasticiteit mediërend in het ontstaan van nieuwe adaptieve fenotypes. Echter, dit gold voor slechts één van de vleugels wat orgaan specifieke allel effecten suggereert. HOOFDSTUK 4 benadrukt de complexiteit van GxE interacties in het licht van de evolutie van ontwikkelingsplasticiteit.

Tenslotte presenteren we HOOFDSTUK 5 onze voorlopige resultaten en de lopende werkzaamheden om de GxE effecten in *B. anynana* ontwikkeling te onderzoeken. Om opnieuw voortkomen van verloren artificiële "DRY" en "WET" geselecteerde lijnen te bereiken, karakteriseren we de thermische curves van de respons voor vleugel patroon en levensgeschiedenis eigenschappen voor een breder scala aan temperaturen dan doorgaans wordt onderzocht in deze soort en voerden een kwalitatieve analyse van de vleugel achtergrond kleur uit. Dit zou ons in kennis kunnen stellen over de aard van GxE en ons de mogelijkheid geven om mogelijke nieuwe/extreme fenotypes en een groter range van fenotypische variatie te onderzoeken die zou kunnen voortvloeien uit de blootstelling van cryptische genetische variatie. Voorlopige resultaten laten zien dat artificiële selectie lijnen voor de vleugel patronen bij intermediaire temperaturen resulteerde in genotypen met verschillende curves van de respons in hoogte en mogelijk ook vorm. De respons op de selectie lijkt het meest extreme te zijn geweest voor de WET lijn, met hoogste curves van de respons, terwijl voor de DRY lijn de curves van de respons het laagste en vlakste zijn in vergelijking met het "WT". Bij eerdere studies naar *B. anynana* oogvlek plasticiteit konden curves van de respons in hoogte gewijzigd worden, maar niet vorm. Toekomstige richtingen omvatten het ontwikkelen van een gedetailleerd mathematische behandeling van de invloed van de externe omgeving op de ontwikkeling om thermische curves van de respons van vorm te karakteriseren. Wij tonen aan dat de selectielijnen ook verschillen in ontwikkelingstijd van de pop en in overleving. Omdat het selectie-experiment niet gerepliceerd is, is het niet vast te stellen of deze respons een gevolg is van genetische correlaties met de vleugelpatronen die het directe doelwit van selectie waren. De

gecorreleerde veranderingen in ontwikkelingstijd en overleving wijzen waarschijnlijk op genetische pleiotropie. Voor beide geslachten, hebben zowel de temperatuur als genotype factoren de ontwikkelingstijd significant beïnvloed. Voor overleving, vertonen DRY, WET en WT genotypen verschillen die afhankelijk zijn van de temperatuur, met hogere levels van sterfte bij extreme temperaturen. In HOOFDSTUK 5 laten we ook verschillen zien in ontwikkelingsplasticiteit voor vleugel achtergrondkleur, met drie groepen van pigmenten voor lage temperaturen en vier groepen van pigmenten voor hoge temperaturen. Tevens vonden we verschillen tussen proximale en distale zijden niet alleen in termen van vleugel achtergrond kleur, maar ook in de breedte van oogvlek kleurringen. Tot slot tonen onze voorlopige resultaten ook aan dat een mogelijk nieuwe kleur oranje verschijnt bij extreem lage temperaturen voornamelijk bij de DRY artificiële lijn. We weten niet wat deze verschillen veroorzaakt, maar we suggereren dat de oranje kleur zou kunnen corresponderen met een pigment van een ander type of een modificatie van een product van de melanine biosyntheseroute. Om deze voorlopige resultaten te onderzoeken willen we een algemene methode ontwikkelen om plasticiteit in vleugel achtergrondkleur te kwantificeren, welke kan worden toegepast bij de meeste organismen.

In HOOFDSTUK 6 geven we een korte samenvatting van de belangrijkste conclusies van dit proefschrift en van de mogelijkheden voor vervolgonderzoek op dit gebied. De in dit proefschrift gepresenteerde resultaten dragen bij aan de algemene kennis van het mechanisme van adaptatie aan variatie van de omgeving en, in een breder perspectief, ook aan ons begrip hoe soorten kunnen aanpassen aan klimaatverandering. We willen een voorbeeld zijn voor toekomstig onderzoek op dit gebied door te laten zien hoe boeiend het is verschillende benaderingswijzen te combineren om te begrijpen wat misschien wel een van de meest fascinerende capaciteiten is van biologische systemen - dat van het vertalen van het omgeving in biologische variatie.

SUMÁRIO

Tornou-se cada vez mais evidente que o conhecimento global dos efeitos das alterações das condições ambientais nas populações naturais requer o conhecimento dos efeitos tanto ao nível das variáveis ambientais, como das espécies que fazem parte das comunidades, tal como da biologia dessas mesmas espécies. De extrema importância no estudo de como os organismos respondem às alterações ambientais temos a plasticidade do desenvolvimento, que consiste na capacidade de alguns genótipos originarem diferentes fenótipos dependendo das condições ambientais que vivenciam durante o desenvolvimento. A plasticidade adaptativa do desenvolvimento representa uma componente fundamental da mudança evolutiva e pode ser uma óptima solução face aos desafios impostos pela imprevisibilidade das mudanças ambientais (revisto no CAPÍTULO 1). Este processo é regulado por mudanças fisiológicas e tem como um dos seus melhores exemplos os padrões das asas de borboletas que se alteram drásticamente entre estações do ano.

A plasticidade adaptativa do desenvolvimento nos padrões das asas das borboletas tem sido caracterizada em relação à sua relevância ecológica e evolutiva e o recente desenvolvimento de ferramentas moleculares e genéticas abriu a possibilidade para ser estudado intensivamente como as condições ambientais durante o desenvolvimento levam à produção de formas sazonais alternativas. O nosso focus foi um modelo biológico emergente em genomica evolutiva e ecológica, a borboleta *Bicyclus anynana*, para a qual o conhecimento do valor adaptativo da plasticidade em populações naturais pode ser complementado com a compreensão dos seus mecanismos. Borboletas desta espécie que se desenvolvem em diferentes estações diferem em estratégias ecológicas que se reflectem nos seus padrões de asas e em outros orgãos/funções relacionados com a sua estratégia de sobrevivência. Os alternativos fenótipos sazonais observados nas populações naturais desta espécie podem ser obtidos em laboratório ao crescer a differentes temperaturas. Já é conhecida a relação entre os ciclos hormonais e o valor adaptativo destes fenótipos alternativos. No entanto, pouco se conhece sobre como os factores ambientais moldam o desenvolvimento de forma a produzir esses fenótipos e sobre a evolução da plasticidade.

O principal objectivo desta tese foi analisar os mecanismos envolvidos na plasticidade do desenvolvimento representados pela variação, regulada pela temperatura, nos padrões adaptativos de cores das asas das borboletas e estratégias de

sobrevivência. Para tal, tentámos integrar a análise de modificações ao nível hormonal, com os padrões espaciais de expressão génica, com o desenvolvimento, com os efeitos da interação genética x ambiente, e com a análise ecológica e evolutiva dos fenótipos de diferentes genótipos de *B. anynana*.

O efeito do ambiente no resultado do desenvolvimento é tipicamente mediado por sinais hormonais que traduzem a informação sobre os factores ambientais para os tecidos em desenvolvimento. De forma a explorar os mecanismos fisiológicos e iniciar o estudo dos mecanismos genéticos envolvidos na plasticidade do desenvolvimento nos CAPÍTULOS 2 E 3 manipulámos os níveis de ecdisona, durante o período de desenvolvimento do estádio de pupa, a diferentes temperaturas, de forma a medir os resultados nos padrões de asas e nas estratégias de sobrevivência. No CAPÍTULO 2, os nossos resultados demonstram que os efeitos da manipulação hormonal para os padrões de asas dependem da temperatura e do tempo em que foi realizada a injecção hormonal, e que diferentes grupos de células do mesmo tecido têm diferentes sensibilidades e padrões de resposta face ao ambiente externo e ao sinal hormonal interno. Enquanto os padrões de resposta em relação à temperatura contrastam padrões em asas com desenvolvimento autónomo, a resposta à manipulação dos níveis hormonais contrasta grupos de células vizinhas com diferentes destinos ao nível de coloração. Também é demonstrado que esta compartimentalização de efeitos não se reflecte na compartimentalização da expressão génica do receptor hormonal. O CAPÍTULO 3 demonstra que a manipulação dos níveis de ecdisona durante a fase de pupa é suficiente para imitar, em direcção e magnitude, as alterações ao nível da alocação de recursos associados à reprodução normalmente induzidos somente pela temperatura. Esta alteração da alocação de recursos é acompanhada por alterações em características relevantes ao nível ecológico, incluíndo o período reprodutivo, na sobrevivência e na resistência à escassez de recursos. Em conjunto os CAPÍTULOS 2 E 3 dão relevância à complexidade das interacções entre ambiente e fisiologia que modelam o desenvolvimento de differentes partes do corpo e que ajudam a mediar as decisões ao nível do investimento reprodutivo, permitindo ao organismo responder às flutuações ambientais.

As curvas de desenvolvimento são uma ferramenta importante no estudo da plasticidade do desenvolvimento e diferentes genótipos podem mostar diferentes características para estas curvas de desenvolvimento tais como na altura ou na forma. No CAPÍTULO 4 foi levantada a hipótese de alelos que afectam a pigmentação também

poderem afectar consequentemente a plasticidade. De forma a investigar esta hipótese foram caracterizadas as curvas térmicas de desenvolvimento para os anéis coloridos dos ocelos de quatro genótipos diferentes de *B. anynana*. Os nossos resultados mostram evidência para efeitos "GxE" (genéticos e ambientais) com diferentes genótipos a mostrarem variação na altura, declive e forma das suas curvas de desenvolvimento. Genótipos com alelos que afectam o tamanho e cor dos ocelos foram os que demostraram maior sensibilidade à variação de temperatura. Estes alelos mutantes podem eventualmente contribuir para a acomodação genética e para a evolução da plasticidade interferindo na origem de novos fenótipos adaptativos. No entanto, estes resultados foram apenas observados para uma das asas sugerindo efeitos alélicos específicos ao nível de cada órgão. Em geral, o CAPÍTULO 4 sublinha a complexidade das interacções GxE à luz da evolução da plasticidade do desenvolvimento.

Finalmente, no CAPÍTULO 5 apresentamos os resultados preliminares e trabalho em progresso em relação ao aprofundamento dos efeitos GxE no desenvolvimento de *B. anynana*. De forma a conseguirmos esta análise foram re-derivadas antigas linhas de selecção artificial "DRY" e "WET" que tinham sido outrora perdidas, foram caracterizadas as curvas de desenvolvimento para uma maior amplitude de temperaturas do que é normalmente utilizado para esta espécie em termos de padrões de asas e estratégias de sobrevivência e, realizada uma análise qualitativa para caracterizar a cor das asas. Este tipo de análise pode dar a indicação sobre a natureza da interacção GxE e permitir investigar a existência de possíveis fenótipos novos/extremos, assim como o aumento do intervalo da variação fenotípica resultante da exposição à variação genética críptica. Resultados preliminares mostram que as linhas de selecção artificial para os padrões das asas a temperaturas intermédias resultaram em genótipos com diferentes curvas de desenvolvimento ao nível de altura e possivelmente da forma. A resposta à selecção parece ter sido mais acentuada para a linha WET, com uma curva de desenvolvimento mais elevada, enquanto a linha DRY mostra uma curva mais baixa e achatada comparativamente ao stock original "WT". Trabalhos anteriores com foco no estudo da plasticidade dos ocelos de *B. anynana* conseguiram alterar a altura das curvas de desenvolvimento mas não a forma das mesmas. As direcções futuras do nosso trabalho incluem a elaboração de uma fórmula matemática detalhada para o tratamento da influência das variáveis externas no desenvolvimento de *B. anynana* de maneira a caracterizar a forma das curvas térmicas de desenvolvimento. Mostramos que a selecção artificial ao nível dos padrões das asas também afecta outras características tais como o

tempo de desenvolvimento da fase de pupa e a sobrevivência. Devido a não existir replicação ao nível da selecção artificial, não é possível dizer se este resultado reflecte correlação genética entre estas características e os padrões das asas que são o alvo directo da selecção. Estas respostas correlacionadas resultantes da selecção possivelmente reflectem pleiotropia genética. Para ambos os sexos, os factores temperatura e genótipo afectam significativamente o tempo de desenvolvimento. Para a sobrevivência os genótipos DRY, WET e WT mostram diferenças que dependem da temperatura, com níveis mais elevados de mortalidade a temperaturas extremas. No CAPÍTULO 5 também mostramos diferenças na plasticidade do desenvolvimento da cor de fundo das asas com três grupos de pigmentos para temperaturas baixas e quatro grupos bem definidos de pigmentos para temperaturas altas. Também encontrámos diferenças entre as margens Proximal e Distal, não apenas em termos de cor de fundo da asa, mas também na largura dos anéis de cor dos ocelos. Finalmente, os nossos resultados preliminares também mostram que uma possível nova cor laranja surge a temperaturas baixas extremas principalmente para a linha artificial DRY. Não sabemos o que causa esta diferença, no entanto sugerimos que esta cor laranja possa corresponder a um pigmento de um tipo diferente ou a uma modificação do produto da via da biosíntese da melanina. De forma a explorar estes resultados preliminares gostariamos de desenvolver um método geral para quantificar a plasticidade na cor de fundo das asas de borboleta possível de aplicar à maioria dos organismos.

No CAPÍTULO 6 apresentamos um pequeno resumo das principais conclusões desta tese e de possíveis ideias para trabalhos futuros nesta área. Os resultados apresentados nesta tese contribuem para o conhecimento geral sobre os mecanismos de adaptação às variações ambientais e, numa perspectiva mais abrangente, podem também adicionar ao nosso conhecimento a forma como as espécies se podem adaptar às alterações climáticas. Gostariamos de consistir num exemplo para a investigação futura nesta área ao demonstrar o quão empolgante é combinar differentes abordagens de forma a compreender aquela que é possivelmente uma das mais fascinantes capacidades dos sistemas biológicos - a de tranformar o ambiente em variação biológica.

LIST OF PUBLICATIONS

Peer-reviewed publications

7) David S, **Mateus ARA**, Duarte EL, Albuquerque J, Portugal C, Sancho L, Lavinha J, Gonçalves G (2015) Determinants of the Sympatric Host-Pathogen Relationship in Tuberculosis. *PLoS ONE* **10**, e0140625. doi:10.1371/journal.pone.0140625.

6) **Mateus ARA**, Marques-Pita M[#], Oostra V[#], Lafuente E, Brakefield PM, Zwaan B, Beldade P (2014) Adaptive developmental plasticity: Compartmentalized responses to environmental cues and to corresponding internal signals provide phenotypic flexibility. *BMC Biology* **12**, 97.

5) Oostra V, **Mateus ARA**, Van der Burg K, Piessens T, Van Eijk M, Brakefield PM, Beldade P, Zwaan B (2014) Ecdysteroid hormones link the juvenile environment to alternative adult life histories in a seasonal insect. *American Naturalist* **184**, E79-92.

4) Serronha AM, **Mateus ARA**, Eaton F, Santos-Reis M, Grilo C (2012) Towards effective culvert design: monitoring seasonal use and behavior by Mediterranean mesocarnivores. *Environmental Monitoring and Assessment* **185**, 6235-6246.

3) Beldade P, **Mateus ARA**, Keller R (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology* **20**, 1347-1363.

2) **Mateus ARA**, Grilo C, Santos-Reis M (2011) Surveying drainage culvert use by carnivores: sampling design and cost-benefit analyses of track-pads vs. video-surveillance methods. *Environmental Monitoring and Assessment* **181**, 101-109.

1) Waser NM, Price MV, Alele P, Baranyovits A, Corcoran C, Djagoun CAMS, Nana ED, Jonker M, Koch H, Marialva MP, **Mateus ARA**, Musvuugwa T, Mwema M, Mwololo M, Nuttman C, Percival G, Rakotonoely H., Ramamonjisoa N, Veríssimo N, Voillemot M, Wala Z (2010) A preliminary early-season flower-visitation web for the Kirindy forest, Madagascar. *Journal of Pollination Ecology* **1**, 1-6.

[#] equal contribution

Manuscripts to be submitted

1) **Mateus ARA**, Beldade P. Thermal reaction norms for pigmentation mutants: G, T and GxT effects (CHAPTER 4).

2) **Mateus ARA**, Marques-Pita M, Alves F, Beldade P. Thermal pigmentation plasticity: shape of reaction norms and color analysis (CHAPTER 5).

CURRICULUM VITAE

Ana Rita Amaro Mateus was born on 18 May 1984 in *Lisboa*, Portugal. In 2002 Ana Rita finished her high-school studies in *Escola Secundária José Afonso* (*Loures*, Portugal) and started her university education in Biology at *Faculdade de Ciências da Universidade de Lisboa*. She did two years of general Biology studies and then chose to pursue for two more years Environmental Biology. By the time of the last semester of the last Bachelor´s year she started to work with Prof. Margarida Santos-Reis in the project "Transportation infrastructures effects on carnivores and mitigation measures of negative impacts" in collaboration with *BRISA - Auto-Estradas de Portugal S.A.* Because she was so fascinated with the impact that human infrastructures could have in the environment, she chose to continue in the same project to do her Master´s thesis entiteled "Highway drainage culverts: comparison of monitoring methods and evaluation of factors promoting its use by carnivores" that was defended in 2008 in *Faculdade de Ciências da Universidade de Lisboa*. Because just the environmental field was not enough for her, she decided to follow her research work on the impact of the environment in the genetic and evolutionary aspects of the biological organisms. She fell in love by *Bicyclus anynana* butterfly when she saw a TV programme with Dr. Patrícia Beldade talking about her research work and she understood that with this model she could combine all these ideas. Therefore, she met Dr. Patrícia Beldade and Prof. Paul Brakefield whom accepted her as a Ph.D. student and applied to FCT (*Fundação para a Ciência e a Tecnologia*) in order to obtain a Ph.D. grant with the title "Coping with fluctuating environments: temperature effects on genetic and physiological mechanisms of adaptive plasticity". Both supervisors were working in *Leiden University* (The Netherlands), so when Ana Rita awarded the FCT grant she moved to the Netherlands to start her research work. She attended to many courses such as: "Tropical forests ecosystems in Madagascar" certificate by Tropical Biology Association (TBA, *Cambridge*), for which she awarded a travel grant; "The R course - Generalised Linear Modelling" certificate by the Imperial College, *London*; or "MDARB10 - Microarray Data Analysis using R and Bioconductor" certificate by *Instituto Gulbenkian de Ciência* (IGC, *Lisboa*). She also gave several oral and poster presentations in countries including Canada, Poland, UK and Portugal. In addition, to help with her project costs, in 2009 in collaboration with Dr. Patrícia Beldade they applied and won a FCT research project grant with the title "Coping with changing

environments: genetic and physiological mechanisms of adaptive plasticity". Finally, during recent years she also dedicated her time to writing scientific papers with the results from her Master´s and Ph.D. thesis and, recently, she helped with Biostatistics for a paper related to the Genetic of Human Diseases department at *Instituto Ricardo Jorge* (*Lisboa*, Portugal).

https://www.researchgate.net/profile/Ana_Mateus2

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