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Evo-devo of novel traits: the genetic basis of butterfly colour patterns

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CHAPTER 1. General introduction, thesis outline and discussion

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1. GENERAL INTRODUCTION

The origin and diversification of novel morphological traits, such as flowers, bird feathers, insect wings, and beetle horns, is a subject that has always fascinated biologists and laymen alike, and is now a major research theme in evolutionary developmental biology, or evo-devo (Müller 2007). The fact that no unambiguous definition of novelty exists does not make them easy to study (see Moczek 2008; Pigliucci 2008). While some authors suggest that only a structure for which no homologue can be found in the ancestral species nor in the same organism can be considered a morphological novelty (*e.g.* Müller & Wagner 1991), others emphasize its ecological importance and define novelty as a trait that performs a new function within the ecology of a lineage (*e.g.* Pigliucci 2008), or even opens up new adaptive zones (*e.g.* Mayr 1960). The ecological and evolutionary factors that promote the diversification of novelties have been studied for a long time (*e.g.* Stebbins 1970), but the genetic and developmental mechanisms underlying these traits have become the focus of research only more recently (Wagner & Lynch 2009).

Among the different mechanisms that have been proposed to explain the origin of morphological novelties, the co-option, or recruitment, of pre-existing features into performing novel functions has received a great deal of attention (*e.g.* True & Carroll 2002; Sanetra *et al.* 2005). This phenomenon seems to be prevalent and includes the co-option of tissues and organs, as well as of single genes and whole developmental pathways, often with modification of components therein. Avian feathers, for example, have evolved from primitive feather-like epithelial outgrowths used for thermoregulation and/or camouflage in non-avian dinosaurs (Prum 1999), and insect wings and spider spinnerets have both derived from the respiratory organs of the common arthropod ancestor (Damen *et al.* 2002). The development of beetle horns involves the arthropod limb patterning gene *Distal-less* (Moczek & Rose 2009), and the Wnt signalling pathway has been implicated in the evolution of the turtle shell (Kuraku *et al.*

2005). Although the redeployment of pre-existing genes and gene networks seems to be the major driving force in morphological evolution, recent studies, facilitated by the advance of genomics, suggested that taxonomically-restricted genes may also play a significant role in the origin of novel traits (Khalturin *et al.* 2009). The relative importance of new and conserved developmental genes, and the particular molecular changes that produce novelty-specific gene networks, must be studied in a broad range of taxa in order to understand general principles about how novel morphologies arise and diversify.

The Lepidoptera (butterflies and moths) provide several examples of adaptive innovations. Colourful scales that cover lepidopteran wings, as well as the specific pattern elements formed by their two-dimensional arrangement, are evolutionary novelties that have permitted wing surfaces to attain new functions in thermoregulation and visual communication. The developmental and genetic bases of these lineage-specific traits are, however, poorly resolved. The aim of this study was to gain a better understanding of the genetic mechanisms underlying development and evolution of butterfly wing patterns. Specifically, we focused on the genetic pathways involved in formation of colour pattern elements called eyespots. In this chapter, we first introduce the study system and give a brief overview of the current knowledge of eyespot evo-devo. Next, we provide a summary of the scientific chapters and discuss the results of this work and some ideas for future research.

EVO-DEVO OF BUTTERFLY WING PATTERNS

The beautiful colour patterns decorating butterfly wings have been and continue to be inspirational for studies of a variety of biological issues, ranging from systematics and evolution to developmental genetics and biochemistry of pigmentation. The amazing diversity of these patterns, together with knowledge on their adaptive value and underlying developmental basis, make them a favourite system in the field of evo-devo (Nijhout 1991; Beldade & Brakefield 2002; McMillan, Monteiro & Kapan 2002; Joron *et al.* 2006; Parchem, Perry & Patel 2007; Wittkopp & Beldade 2009).

Wing pattern diversity is amazing and the majority of the more than 17,000 species of butterflies can be recognized based on their wing patterns. Dramatic variation has also been documented within species – between geographical populations and seasonal forms, between males and females, and between the dorsal and ventral surfaces of one same wing (examples in Nijhout 1991). Despite the dazzling panoply of colourful spots, bands, and stripes, most butterfly wing patterns can be recognized as derivations of a basic "nymphalid groundplan", which has been very useful to identify pattern element homologies within and across species (Nijhout 1991; 2001). In this groundplan representation,

different types of pattern elements, such as eyespots, chevrons and bands, are organized in parallel series; individual elements are repeated along the anterior-posterior wing axis within so-called wing cells, *i.e.* wing compartments bordered by veins. The independent development of individual pattern elements has presumably facilitated the diversification of butterfly wing patterns (see Nijhout 1994; 2001). The adaptive significance of wing patterns and the underlying genetic basis have long been the focus of studies in ecology and evolution and have provided important insights into how variation in natural populations is shaped by natural and sexual selection. In the last couple of decades, this focus has extended to the detailed analysis of the developmental processes and pathways involved in pattern formation and diversification.

Ecology and evo-devo of butterfly wing patterns

Lepidopterans include many textbook examples of adaptive colour polymorphisms ranging from industrial melanism in the peppered moth *Biston betularia* (Majerus 1998), to mimicry in swallowtails (*Papilio*) and passion-vine (*Heliconius*) butterflies (Joron & Mallet 1998), to seasonal colour pattern changes or polyphenisms in a number of species (Brakefield & French 1999). The ecological significance of natural variation in butterfly wing patterns has been associated with predator avoidance (*e.g.* Langham 2004; Olofsson *et al.* 2010), sexual selection (*e.g.* Robertson & Monteiro 2005), and thermal regulation (*e.g.* Watt 1968, 1969; Kingsolver 1985).

Contrasting with studies of the ecology and basic genetics of wing colour patterns, analyses of the developmental and molecular genetics of pattern formation have become topics of intensive research interest relatively more recently. Evo-devo studies in butterflies have focused on a few target species, and used a diversity of approaches to provide what are largely complementary insights about the developmental and genetic mechanisms behind the formation of different aspects of wing patterns. For example, studies in *Papilio* butterflies provided a detailed analysis of the biochemical pathways of pigment production and their relationship to polymorphism in overall coloration (Koch, Behnecke & French-Constant 2000). Linkage analysis in *Heliconius* has characterized the genetics underlying variation in wing patterns composed of large patches of different colours (Joron *et al.* 2006; Baxter *et al.* 2009). Analysis of developing wings of *Junonia coenia* and *Bicyclus anynana* have unravelled most of the information about the developmental processes and genetic pathways implicated in the formation of eyespots, *i.e.* concentric rings of different colours (reviewed in Nijhout 1991; Beldade & Brakefield 2002; McMillan, Monteiro & Kapan 2002). These pattern elements are thought to resemble vertebrate eyes and to function in deflection or intimidation of predators (reviewed in Stevens 2005).

Evo-devo of eyespot patterns in *Bicyclus anynana*

Efforts by Paul Brakefield two decades ago established the tropical nymphalid *B. anynana* as a laboratory system (Brakefield, Beldade & Zwaan 2009). These butterflies are small enough that large populations can be reared and analyzed, and large enough that manipulating single individuals and specific tissues is no technical challenge. Their eyespots are a valuable system for an integrated study of the evolutionary and developmental processes that shape morphological variation. Eyespots of *B. anynana* function in predator avoidance (Lyytinen *et al.* 2004; Brakefield & Frankino 2009) and mate choice (Robertson & Monteiro 2005; Costanzo & Monteiro 2007). Morphology of these pattern elements varies greatly within and across ca. 80 *Bicyclus* species (Beldade, Brakefield & Long 2005; Brakefield & Roskam 2006) and can be studied within the framework of morphological (Condamin 1973) and molecular (Monteiro & Pierce 2001) phylogenies of the genus. Eyespots are amenable to a detailed analysis of the underlying developmental mechanisms, ranging from cellular interactions and the genetic pathways involved in pattern specification, to biochemistry of pigment production (Fig. 1). Moreover, laboratory populations of *B. anynana* harbour genetic polymorphisms for different aspects of eyespot phenotypes and provide ideal material to analyze how variation in genotypes is translated into phenotypic variation, via development. Importantly, experimental tractability, including growing genomic resources and transgenic tools (Ramos & Monteiro 2007; Beldade, McMillan & Papanicolaou 2008; Beldade *et al.* 2009), can be combined with knowledge of ecology and natural variation for this species. All these enable the full integration of analyses at the population-, organismal- and molecular-levels.

DEVELOPMENTAL GENETICS OF EYESPOT FORMATION

Of all pattern elements that can be recognized in butterfly wings, eyespots are undeniably those whose underlying development is best understood. Surgical manipulations of pupal wings in *J. coenia* by Fred Nijhout established developmental models of eyespot formation (Nijhout 1978; 1980), and work in the lab of Sean Carroll on expression patterns of candidate genes in *J. coenia* and *B. anynana* started to explore the genetic pathways involved in this process (Carroll *et al.* 1994; Brakefield *et al.* 1996; Fig. 1a,b).

Butterfly wings are formed by two epidermal membranes nourished and supported by veins. Wing development starts during the first larval instar; the wings greatly increase in size and develop a venation system in the final larval stage, and scale maturation and pigment deposition occur in late pupal wings shortly before adult eclosion. Colour patterns on each wing surface are formed by the arrangement of partially-overlapping, monochromatic scales on a single cell

layer. This two-dimensional nature simplifies modeling of the underlying developmental interactions that occur in late larval and early pupal wings and are known to determine scale maturation and pigmentation long before any coloration is visible.

Models of eyespot formation: signal and response mechanism

Surgical manipulations of presumptive eyespots in pupal forewings are facilitated by cuticular landmarks that enable the localization of eyespot centres called foci. Such manipulations have established that these foci have “eyespot-organizing” properties. Transplanting an eyespot focus into an eyespot-less position on the wing typically results in the formation of an ectopic eyespot at the host site, whereas damaging foci during the sensitive early pupal period typically reduces or completely eliminates the corresponding eyespots in adult wings (Nijhout 1980; French & Brakefield 1992; 1995). Results such as these led to the proposal of developmental models whereby the focus acts as an organizer by signalling to the neighbouring cells via the production of a diffusible morphogen (Fig. 1c). Diffusion of this signal away from the focus presumably forms a concentration gradient in the wing epidermis; the surrounding cells respond to the signal concentration in a threshold-like fashion and become fated to produce a particular pigment. Alternative models of eyespot formation have also been proposed whereby foci degrade, rather than produce, the organizing signal (French & Brakefield 1992), or additional morphogen sources appear in concentric eyespot rings (Dilão & Sainhas 2004).

Genes implicated in eyespot formation

Studies of gene expression patterns in larval and pupal wings of *B. anynana* and *J. coenia* implicated a number of genes in eyespot development. These studies targeted candidate pathways involved in the development of insect wings and extensively studied in *Drosophila melanogaster*. Genes involved in wing compartmentalization, *e.g.* *engrailed* (*en*) and *Distal-less* (*Dll*), perform similar functions in flies and butterflies and have also been redeployed to regulate different stages of eyespot formation (Carroll *et al.* 1994; Brakefield *et al.* 1996; Keys *et al.* 1999; Brunetti *et al.* 2001; Fig. 2).

Establishment of the location of eyespot foci takes place during the final larval instar (Fig. 1a). To date, the earliest known event associated with this process is the upregulation of the gene *Notch* (*N*), first in the inter-vein midline and subsequently in the future focal cells (Reed & Serfas 2004). Expression of *N* is followed by the activation of *Dll*, and slightly later by the upregulation of *en* and *spalt* (*sal*, Reed, Chen & Nijhout 2007). Simultaneously, expression of genes from the Hedgehog (Hh) signalling pathway is activated in (*patched* and *cubitus*

interruptus) or around (*hh*) eyespot foci (Keys *et al.* 1999). Later, the Ecdysone Receptor (EcR) protein, presumably upregulated by Dll, appears in the focal cells (Koch *et al.* 2003). Exactly how these genes regulate each other and the downstream pathways leading to focal signalling and pigment synthesis is not known, but models have been proposed to describe this process based on the knowledge of the genetic interactions in *D. melanogaster* (Evans & Marcus 2006; Marcus & Evans 2008). The idea that these genes act in the determination of eyespot foci is strengthened by data on their expression patterns in *B. anynana* laboratory populations with altered eyespot morphology (discussed in the next section).

Even though models for eyespot formation involving focal signalling and epidermal response were proposed several decades ago, little is known about the molecular nature of these components. The proteins Wingless (Wg) and pSmad, the signal transducer in the Decapentaplegic (Dpp) pathway, were detected in the cells of the presumptive eyespot fields in early pupal wings of *B. anynana* (Monteiro *et al.* 2006). This suggested that both Wg and Dpp can be eyespot-inducing signals, but functional analysis is necessary to confirm these results. Putative targets of these signals include genes that are upregulated in the cells of the future eyespot rings in early pupal wings. In *B. anynana*, for example, *en* is expressed in the area that corresponds to the outer (golden) ring of the adult eyespot (Fig. 1b), and *Dll* and *sal* in the inner (black) ring. The transcription factors encoded by these genes are presumably involved in the interpretation of focal signal(s) and activation of pigment biosynthesis pathways as their expression domains perfectly correlate with adult colour rings, both in the ‘wild type’ *B. anynana* as well as in mutants with disturbed colour composition, such as Goldeneye (Brunetti *et al.* 2001). The same transcription factors are expressed in concentric rings in the eyespot fields of other butterfly species, but in different relative spatial domains that correlate with divergent adult eyespot colour schemes (Brunetti *et al.* 2001).

Pigment production and scale maturation in late pupal wings

Most studies have focused on the cellular interactions and genetic pathways during the early stages of eyespot development. Much less is known about the downstream processes, whereby scale-forming cells interpret prepattern information to produce specific pigments late in pupal development. Four major classes of pigments have been characterized in butterfly wing scales: melanins (red-brown to black), ommochromes and pterins (yellow to red), and flavonoids (white to red and blue). The timing of scale maturation correlates with pigment production, with dark, melanized scales typically maturing last in most butterfly species (Nijhout 1991; Reed & Nagy 2005). In *B. anynana* eyespots, for example, the cells at the white center of the eyespot are the first to mature, followed by the

yellow cells of the outer ring, and finally the black cells of the middle ring (Koch *et al.* 2000; Fig. 1d-f).

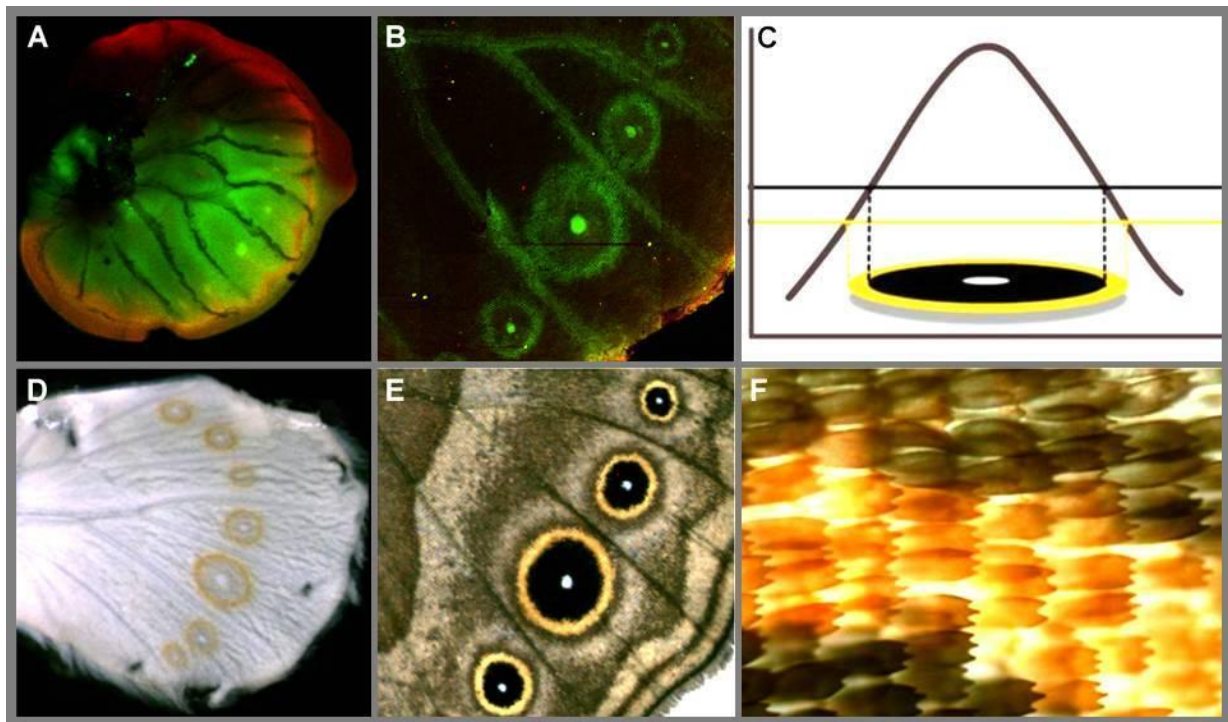


Figure 1. Eyespot development in *B. anynana*.

A. Eyespot formation starts during the last larval instar, when future eyespot centres are established, presumably by the action of genes such as *Dll* (red) and *en* (green). *Dll* is also detected in the wing margin, and *En* throughout the posterior compartment, as is characteristic of insect wing development. **B.** The same genes continue to be co-expressed in eyespot foci in the early pupal wings, and their protein products are also detected in the cells that form the inner (*Dll*) and the outer (*En*) rings. **C.** The formation of the colour rings presumably relies on a signal-response mechanism whereby focal cells produce a signalling molecule that diffuses away and forms a concentration gradient (curve) to which the neighbouring cells respond in a threshold-like fashion (horizontal lines). **D.** The epidermal cells become fated to produce a particular pigment shortly before adult eclosion. This late pupal hindwing shows mature scales in the white center, and an outer golden ring. The black inner ring and brown background scales will mature later. **E.** The adult wing pattern is composed of serially-repeated eyespots; shown here is a section of an adult hindwing with four eyespots. **F.** Colour patterns are formed by the arrangement of scales, each bearing one particular (black, yellow or brown) pigment.

Recent studies have analyzed expression levels of several pigmentation genes in association with wing sections of different colours in *Vanessa cardui* and *Heliconius* butterflies (Reed & Nagy 2005; Reed, McMillan & Nagy 2008; Ferguson & Jiggins 2009). Some of these genes (*e.g.*, *vermillion*, *henna* and *ruby*) are also expressed in *B. anynana* developing wings (Beldade, Brakefield & Long 2005) and might be involved in making the different colour rings of the eyespot. Pigmentation mutants isolated in the laboratory (*e.g.* *Band*, Beldade *et al.* 2009) are invaluable to study the expression of these and other candidate genes and explore their relationship to different aspects of coloration.

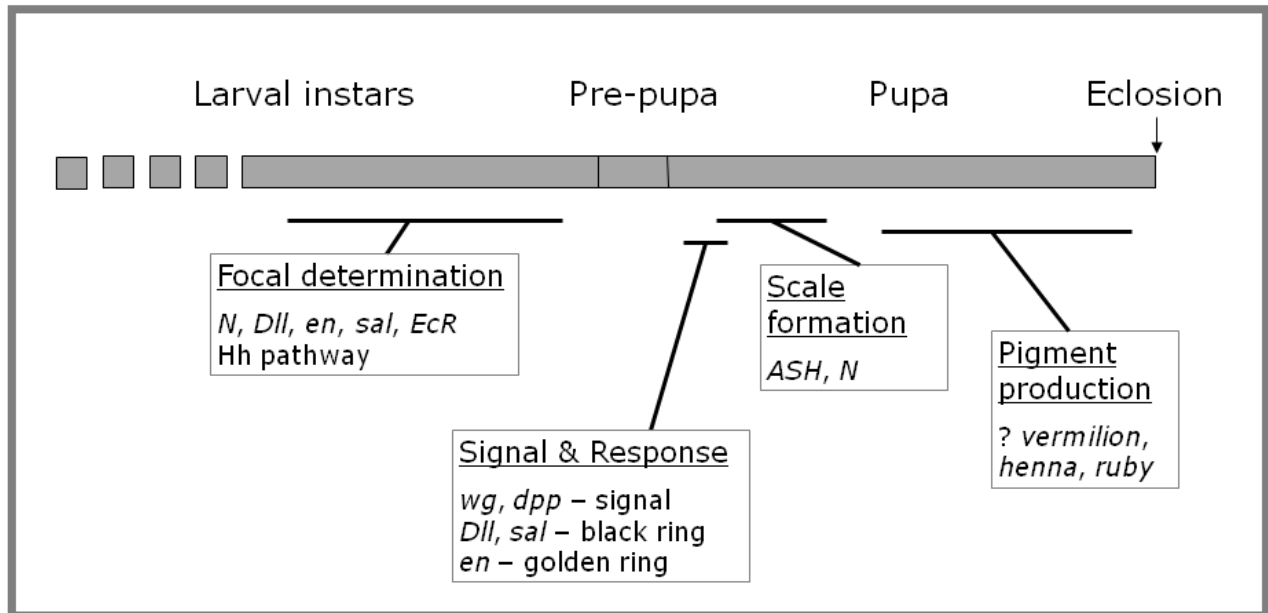


Figure 2. Genes associated with different stages of eyespot formation.

The bar represents the consecutive stages in *B. anynana* development and the lines underneath it refer to those stages of eyespot formation which have been examined so far. Genes whose expression has been detected in association with wing pattern development are listed in the boxes (see text for references). Data for the stages of focal determination, signal-response interactions, and scale maturation were mostly gathered from studies in *B. anynana* and *J. coenia*. Data on pigmentation genes, on the contrary, have focused on species that do not have eyespots, e.g. *Heliconius*. The role of these genes in eyespot formation still needs to be investigated.

VARIATION IN EYESPOT MORPHOLOGY

The experiments summarized above identified a number of pathways and processes involved in eyespot formation. The extent to which specific components of these pathways (*i.e.* individual genes) and of these processes (*e.g.* focal signal strength and epidermal response thresholds) can, and do, contribute to variation in eyespot morphology addresses a key issue in evo-devo research. Laboratory populations of *B. anynana* with distinct wing pattern phenotypes have been used to analyze the genetic and developmental basis of variation in eyespot patterns.

Eyespot variation in laboratory population of *B. anynana*

Lab populations of *B. anynana* harbour different types of genetic variation affecting eyespot patterns (Brakefield, Beldade & Zwaan 2009). Artificial selection experiments have explored heritable variation segregating in the laboratory stock, and produced gradual and progressive changes in traits such as eyespot size, colour composition and shape (McMillan, Monteiro & Kapan 2002; Beldade, Brakefield & Long 2005). Also, spontaneous mutations with large effect on those same aspects of eyespot morphology have been isolated and are maintained in stable stocks (*e.g.* Brakefield *et al.* 1996; Brunetti *et al.* 2001). The

extent to which the loci carrying alleles of large effect are the same as those harboring alleles of more subtle effect that contribute to segregating, quantitative variation in laboratory and natural populations is not known (*e.g.* Wittkopp *et al.* 2009). Mutant stocks and selection lines, however, offer the opportunity to characterize the genetic architecture of wing patterns and to identify additional developmental pathways and processes involved in colour pattern formation and variation. Work on laboratory populations of *B. anynana* has already enabled characterization of variation across consecutive stages of eyespot formation, from expression of pre-patterning genes in late larval wings, to the signal-response components in early pupal wings, to scale maturation and pigment deposition in late pupae.

A number of mutant stocks and selection lines show altered patterns of eyespot-associated gene expression already in larval or pupal wings. For example, there is a perfect match between the gain/loss of eyespots in the Spotty and Missing mutants, and the gain/loss of expression of focal marker genes *N*, *Dll* and *en* in their larval wings (Brakefield *et al.* 1996; Monteiro *et al.* 2003; Reed & Serfas 2004; Monteiro *et al.* 2007). The appearance of the areas of gene expression resembles eyespot shape and size. For instance, elongated areas of *Dll* expression match elongated eyespot foci in the Cyclops mutant (Brakefield *et al.* 1996), and individuals from artificial selection lines with reduced or enlarged eyespots show quantitative differences in the expression of *Dll* and *en* (Beldade, Brakefield & Long 2005). Other mutations alter expression patterns of these transcription factors in pupal wings (*e.g.* *Goldeneye*, Brunetti *et al.* 2001), suggesting that these alleles are involved in the signal/response stage of eyespot formation. Transplants of the eyespot-inducing centers between pupae from selection lines with divergent eyespot morphologies identified variation both in the strength of the focal signal, and in the threshold levels of epidermal response. Quantitative variation in eyespot size seems to be largely due to variation in focal signal (Monteiro, Brakefield & French 1994), whereas the properties of the epidermis explain differences in colour composition (Monteiro, Brakefield & French 1997; Allen *et al.* 2008), but also contribute to those in size (Beldade, French & Brakefield 2008). Nothing is known, however, about the way such differences in signal/response components influence scale maturation and pigment production. Because rates of scale maturation correlate with scale colour, heterochronic changes in scale development, described in *B. anynana* and *Papilio glaucus* mutants, have been proposed as a mechanism for generating variation in butterfly wing patterns (Koch *et al.* 2000).

Results such as these suggest that a number of candidate pathways harbour allelic variation which contributes to phenotypic variation in eyespot morphology. But differences in expression patterns *per se* do not identify the loci that are

responsible for differences in adult phenotype. Indeed, variation in expression of any specific gene can be due to allelic variation at that locus or to variation in another gene involved in regulating the expression of the first gene. The extent to which these factors contribute to variation in gene expression is an area of active research in relation to morphological diversification (see Carroll 2005, 2008; Hoekstra & Coyne 2007; Stern & Orgogozo 2008).

Identification of genes responsible for wing pattern variation

Whereas genome-wide mapping techniques have become increasingly powerful, it remains a challenge to move from mapped genetic regions to identifying single loci responsible for phenotypic variation. This is especially true for non-classical model organisms which do not have fully sequenced genomes or very rich information on patterns of DNA sequence polymorphisms. Candidate genes play an important role in the quest for identifying which loci contribute to variation in all sorts of phenotypes, including colour patterns in butterfly wings. Close linkage between candidate genes and wing colour pattern variation has been reported for different types of pattern elements in several species (Kronforst *et al.* 2006; Clark *et al.* 2008). Work on *B. anynana* has shown that candidate genes from pathways implicated in eyespot development, *e.g.* *Dll*, can contribute to inter-individual variation in eyespot size (Beldade, Brakefield & Long 2002). However, the same gene, whose pattern of expression also changes in association with variation in eyespot number, did not show close linkage with the locus *Missing*, which affects that aspect of eyespot morphology (Monteiro *et al.* 2007). Genes upstream of *Dll* must thus be responsible for the *Missing* phenotype.

Genes within the developmental pathways shown to be involved in eyespot formation are clearly good candidates for contributing to variation in eyespot patterns. However, direct tests of this contribution require sequence and polymorphism information. This type of information was very scarce until recently, when Expressed Sequence Tag (EST) projects boosted gene discovery in a few target butterfly species, including *B. anynana* (Beldade *et al.* 2006; Beldade, McMillan & Papanicolaou 2008; Papanicolaou *et al.* 2008). Comparative analyses showed high levels of synteny (*i.e.* the conservation of gene order) for a large number of orthologous loci in *B. anynana*, *Heliconius melpomene* and the silkworm *Bombyx mori* (Pringle *et al.* 2007; Beldade *et al.* 2009). Thus, future efforts in mapping genes responsible for butterfly wing pattern variation will be able to rely on linkage information available for other lepidopterans to facilitate moving from mapped genomic regions to a testable number of candidate genes.

DIVERSIFICATION OF EYESPOT PATTERNS

Much of the diversity in animal morphologies can be accounted for by two processes: the origin and modification of evolutionary novelties (*e.g.* the turtle shell and the bird feather), and the diversification of serially-repeated structures (*e.g.* vertebrate teeth and insect body segments). Butterfly wings capture both processes beautifully: their colour patterns are an evolutionary novelty, and are composed of different types of serially-repeated pattern elements which have diversified between repeats and across species. Butterfly wing patterns, and eyespots in particular, have already made important contributions to our understanding of key mechanisms behind morphological diversification.

The nymphalid groundplan is probably the best illustration of the modular nature of butterfly wing patterns. Independence between pattern elements of different series (*e.g.* eyespots and chevrons) and correlations between serially-repeated elements (*e.g.* two eyespots on the same wing surface) have been documented for several different species (Paulsen & Nijhout 1993; Nijhout 2003; Allen 2008). Moreover, the genes expressed in association with the eyespot field show a nearly identical pattern of expression in relation to all eyespots, and typically are not expressed in association with any other types of pattern elements (Fig. 1b,c). Whether the serially-repeated pattern elements appeared at once and then diversified, or whether individual repeats were added at different times, is still an open question (Monteiro 2008). However, the genetic and developmental mechanisms underlying the diversification of serially-homologous eyespots have started to be explored. Despite strong correlations between serially-repeated eyespots, artificial selection experiments in *B. anynana* have uncovered great flexibility for individual changes in eyespot morphology (Beldade, Koops & Brakefield 2002). Moreover, spontaneous mutations of large phenotypic effect, *e.g.* *Spotty* and *Missing*, have been shown to affect only sub-sets of eyespots (Monteiro *et al.* 2003; Beldade, French & Brakefield 2008). This compartmentalization of eyespot series led to the proposal of a genetic model in which each of the future eyespots is associated with eyespot-specific regulatory regions controlling the expression of a yet unidentified key ‘eyespot gene’ (McMillan, Monteiro & Kapan 2002; Monteiro *et al.* 2003). A very fine-scaled analysis of the regulatory sequences and functions of candidate eyespot genes will be needed to verify this model.

One of the touchstones of evo-devo is that the same genetic pathways are used in association with different developmental stages and tissues both within and across species. Butterfly wings offer several examples of gene co-option. The scales that cover lepidopteran wings, for example, are homologous to insect sensory bristles and have evolved through the recruitment of bristle-patterning genes of the Achaete-Scute Complex, followed by acquisition of target genes

responsible for typical scale morphology (Galant *et al.* 1998; Zhou *et al.* 2009). The pigments that colour the scales derive from the redeployment of several enzymes of the ommochrome synthesis pathway which are known to function in insect eye pigmentation (*e.g.* vermilion and cinnabar, Reed & Nagy 2005). The patterns made by the spatial arrangement of pigmented scales rely on genetic pathways involved in embryonic and wing development in butterflies and other insects (*e.g.* the Hh and Wg pathways, Keys *et al.* 1999; Monteiro *et al.* 2006). Analysis of these pathways in the context of eyespot development offers not only the possibility for translating knowledge from model systems into a deeper understanding of lineage-specific traits, but also the opportunity to study the evolution of key developmental pathways in the context of their involvement in novel functions.

2. OUTLINE OF THIS THESIS

The work on laboratory populations of *B. anynana* discussed above has provided important insights into the mechanisms of eyespot development and has implicated several genes and signalling pathways in this process. There are, however, still many gaps in our understanding of eyespot development and evolution. Identification of eyespot patterning genes has so far been biased towards candidate pathways involved in insect wing development. Although studies of spatial expression of these genes have provided correlational evidence for their role in eyespot formation, the functional relationships between gene expression patterns and the adult eyespot phenotypes have yet to be established. Some stages of eyespot development have received little to no attention, and we are still far from understanding how all the genes and processes we do know about interact with each other and are regulated by environmental factors. Moreover, most genes have been studied in a few species that belong to a single butterfly family of Nymphalidae, and it is unclear whether the same genetic pathways underlie eyespot formation and diversification in other butterflies.

This thesis explores different aspects of eyespot evo-devo, ranging from redeployment of conserved developmental genes in eyespot formation, to the genetic basis of variation in laboratory populations of *B. anynana*, to evolutionary diversification of eyespot gene networks in a variety of butterfly species. In order to better understand eyespot development, we applied different experimental approaches, including: a) a less biased search for candidate genes via analysis of pleiotropic mutations that affect eyespot formation and some other, relatively conserved developmental process, b) linkage mapping of one such candidate gene, c) analysis of expression patterns and function of conserved embryonic patterning genes in relation to eyespot development in *B. anynana*, and d) a comparative analysis of eyespot patterning gene network in a broad range of species. Below

we will give a brief overview of the five research chapters that constitute this thesis.

The genetic and developmental analysis of butterfly eyespots is challenging because these lineage-specific novelties are not represented in any model organism, and the comparative method is therefore difficult to apply. Co-option of genes/pathways involved in relatively more conserved processes offers the potential to overcome this problem by using knowledge of these processes obtained in model species. In **Chapter 2**, we proposed to use this approach in relation to *B. anynana* eyespots and suggested that new candidate genes/pathways for eyespot formation could be identified based on commonalities between this and more conserved processes, such as wound healing, embryonic development, or wing vein patterning. We reviewed evidence suggesting that some components of wound repair mechanism might be involved in eyespot formation, and that native and damage-induced eyespots share the same signalling pathways. However, our own study of expression of eyespot patterning genes in and around damaged epidermal tissue did not provide a clear support for this hypothesis. Furthermore, we described several spontaneous mutations which affect eyespots and either embryogenesis or wing vein development. The effects of three mutant alleles on wing venation and eyespot formation strongly suggested that the latter depends on normal development of veins and tracheae. The comparative analysis of embryonic development in the recessive lethal mutant Goldeneye provided evidence that this allele disturbs the process of embryonic movement called blastokinesis. Despite the fact that the specific genetic regulation of blastokinesis is poorly understood even in model organisms, such analysis provided a valuable starting point for exploring the genetic basis of this mutation.

In **Chapter 3**, we followed up the idea of the comparative analysis of embryonic lethal mutations and investigated three other pleiotropic alleles: *Bigeye*, *Frodo* and *Spread*. We found that they affect eyespot size and/or colour composition in heterozygotes and severely disturb embryonic segment polarity in homozygotes. Complementation tests revealed that all three mutations are alleles of the same locus, and that one of these alleles has a minor deleterious effect on embryogenesis while increasing eyespot size in the homozygotes. Non-lethal alleles at this locus might thus exist and contribute to naturally occurring variation in eyespot morphology within and across species. Analysis of gene expression in pupal wings suggested that this locus acts upstream of *En*, *Dll* and *Sal* during the ‘response to focal signal’ stage of eyespot development. Furthermore, comparison of the defects in mutant embryo morphology and expression patterns of segment polarity genes *en* and *wg* with those described in mutants of *D. melanogaster* suggested that this locus encodes a negative regulator of the Wnt/Wg signalling pathway. In the fruit fly, downregulation of five members of this pathway produces alterations in morphology and *en/wg*

expression patterns very similar to those found in mutant *B. anynana* embryos. Although these five genes were considered good candidates for the mutant locus, the possibility that other genes regulate the Wnt/Wg signalling in embryos and eyespots of *B. anynana* could not be excluded.

The attempts to identify the locus and mutations which are responsible for the Bigeye, Frodo and Spread phenotypes are described in **Chapter 4**. These efforts were greatly facilitated by the recently developed genomic resources for *B. anynana*, such as the EST and Bacterial Artificial Chromosome (BAC) libraries (Beldade *et al.* 2006; 2008) and a gene-based linkage map (Beldade *et al.* 2009). In the study that is not part of this thesis (Beldade *et al.* 2009), the mutant locus was coarsely mapped to linkage group (LG) 17. Hence, all candidate genes that were not assigned to this chromosome were excluded. The implicated LG showed high levels of synteny with the orthologous genomic region of *B. mori*, the reference lepidopteran with the fully sequenced and annotated genome. This allowed us to identify candidate genes for the mutant locus using the silkworm genome sequence (<http://silkworm.genomics.org.cn/>). Such analysis revealed that the orthologous chromosome contains two genes associated with the Wnt/Wg signalling pathway, *Axin* and *doubletime*. Both genes were, however, excluded by linkage analysis. We then developed additional markers on LG17 and narrowed down the chromosomal interval associated with the three mutations to ~140 kb. Markers in this interval were used to screen the BAC-clone based genomic library of *B. anynana*. One BAC clone was sequenced, and linkage analysis with newly developed markers further refined the position of the mutant locus to a ~34 kb interval. Analysis of this genomic region revealed that it does not contain any known genes related to Wnt/Wg signalling or any pigment biosynthesis pathway. Instead, it includes several predicted open reading frames which show no homology to any known gene in any other organism. Identification of the actual locus and of mutations therein that cause the Bigeye, Frodo and Spread phenotypes is in progress.

The work described in the earlier chapters focused on co-option of conserved pathways in eyespot evolution. **Chapter 5**, too, is dedicated to the study of expression patterns of key players that belong to fundamental insect embryo patterning pathways, potentially redeployed in different stages of eyespot formation in nymphalid butterflies. Here, we examined patterns of *wg* expression in pupal wings to investigate its role as a focal signal, proposed by Monteiro *et al.* (2006) and also indicated by the comparative analysis of pleiotropic mutations reported in Chapter 3. The results of this study were consistent with Wg being an eyespot morphogen, but also suggested some unexpected aspects of its regulation via anti-sense mRNA transcripts. Next, we investigated whether the Hh signalling pathway, implicated in eyespot focus determination in *J. coenia* (Keys *et al.* 1999), was performing a similar role in *B.*

anymana larval wings. We found that *hh* and its receptor-encoding gene *patched* were not expressed in eyespot foci (a result very different from that in *J. coenia*!), and that this pathway is probably not involved in focus determination in our model butterfly. Unexpectedly, we found that the Hox gene *Antennapedia* (*Antp*) was expressed in *B. anymana* eyespot foci in last larval instar wings, and that its expression perfectly correlated with focal shape in the Cyclops mutant. This gene was upregulated in discrete focal patterns before other eyespot genes, which are always expressed in ‘non-eyespot’ areas such as the wing margin and intervein bands. This suggested that *Antp* might be the first key regulator of eyespot focus determination. Functional analysis of candidate genes expressed in suggestive patterns during eyespot formation is a critical step in the study of wing pattern evolution. Hence, we attempted to manipulate the function of two genes, *Antp* and *Dll*, by inhibiting the translation process via RNAi or morpholino injections. Unfortunately, these knock-down experiments failed to establish a relationship between observed expression patterns and the adult eyespot phenotype. Performing RNAi in butterflies appears to be not so straight-forward (Terenius *et al.* in review) and alternative approach should thus be applied to the analysis of candidate gene function in the future (*e.g.* transgenic techniques, see Ramos & Monteiro 2007).

The findings described above suggested that the genetic mechanisms underlying eyespot formation in *B. anymana* and *J. coenia* might have diversified substantially and emphasized the importance of comparative analysis of eyespot development in a broad range of butterfly species. This was the aim of the experiments reported in **Chapter 6**. Here, we focused on four genes implicated in eyespot focus determination in the lab model *B. anymana*, and examined their expression patterns in ten other species of butterfly families Nymphalidae and Papilionidae (Wahlberg *et al.* 2009). All butterflies examined have spots or eyespot-like wing patterns, characterized by divergent colour, size, number, and position on the wing. We aimed to investigate which components of the ‘eyespot gene network’, identified in *B. anymana*, were associated with these patterns during the early stages of eyespot formation. This study showed that (eye)spots in the more basal butterfly lineages (*i.e.* Danainae and Papilionidae) do not express same genes as eyespots of higher Nymphalidae (*i.e.* subfamilies Satyrinae and Nymphalinae). We suggest that these pattern elements have evolved independently but it remains to be investigated which mechanisms are responsible for colour pattern formation in danains and papilionids. We also found that the genetic mechanisms underlying eyespot patterning even in relatively closely related butterflies seem to differ. For example, upregulation of the Hox gene *Antp* was associated with eyespot centres in all Satyrinae, but in none of the Nymphalinae examined here (both subfamilies belong to the family Nymphalidae). Eyespots in these subfamilies could have arisen independently by

using at least some of the same genes, or could have originated in a common ancestor and diversified substantially in the underlying genetic machinery (these scenarios should be investigated in the future!). Variation within subfamilies was detected as well, *e.g.* not all genes upregulated in eyespot foci in *B. anynana* were found in other satyrines. This suggests that the mechanisms of eyespot development are flexible, and open to tinkering at the level of the loss and gain of regulating genes. It is, however, possible that at least some of the genes found to be expressed in eyespot foci are actually not involved in eyespot formation. Analysis of gene function in this context should always complement gene expression studies but was unfortunately ineffective here (see Chapter 5).

3. GENERAL CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

The goal of this study was to advance our understanding of the genetic and developmental mechanisms underlying the origin and diversification of butterfly eyespots. The experiments described here led to several interesting, and sometimes unexpected, observations and conclusions. Here, we discuss how these results can contribute to our understanding of the evolution of novel morphological traits and propose some ideas for future eyespot evo-devo work.

Since the discovery that specification of butterfly eyespot centres was associated with the expression of *Dll* (Carroll *et al.* 1994), several other transcription factors, receptors and signalling molecules have been detected in the eyespot field and are therefore likely to be linked to the process of pattern formation. The emerging picture of eyespot development tells us that these relatively simple two-dimensional patterns are produced by rather complex networks of genes, which interact during larval and pupal stages and ultimately regulate the biosynthesis of different pigments in the epidermal scale-building cells. Interestingly, all genes and signalling pathways implicated in eyespot formation to date are also involved in patterning of the insect wing, and were presumably co-opted to perform novel functions on the wings of some butterflies. One of the exciting findings described in Chapter 5 is that proteins that do not play a role in the development and patterning of insect wings (*e.g.* the Hox transcription factor *Antp*) may also have been recruited to perform a truly novel function in establishing butterfly eyespots. Clearly, this role for *Antp* and other genes implicated in eyespot formation needs to be confirmed by functional tests (*i.e.* gene knock-down and overexpression) but these techniques are not yet easily accessible in butterflies. Current attempts to inhibit gene function by RNAi in butterfly wings have so far booked little success (Chapter 5; Terenius *et al.* in review). Future research should therefore focus on development of gene manipulation techniques that could be relatively easily applied in *B. anynana* and other butterflies (see Ramos & Monteiro 2007; Monteiro & Prudic 2010).

Another exciting conclusion from this work is that not only conserved, but also putative lineage-specific genes can play a role in the formation of novel traits. Here we characterized several pleiotropic mutations that alter eyespots and some other developmental processes, *e.g.* blastokinesis or embryonic segment polarity (Chapters 2 and 3). Remarkably, although this analysis implicated the conserved Wnt/Wg signalling pathway in the regulation of eyespot size and colour composition (Chapter 3), fine mapping of one such mutant locus excluded all known components of this pathway (Chapter 4). This suggests that some unknown, potentially butterfly-specific gene can perform Wg-related functions in *B. anynana* eyespot and embryo development. Identification of this locus and mutations therein will require sequencing of the ~34 kb implicated genomic region in mutant and wild-type butterflies. Once all the genetic differences between these individuals are described for this region, it might become possible to identify those mutations that actually cause alterations in eyespot and embryonic phenotype. If it appears that mutations are in the coding sequence of one of the predicted genes (*e.g.* a frame shift or a premature stop codon), it would be necessary to perform some sort of functional analysis to confirm this. Alternatively, mutations might have occurred in a regulatory region of a gene outside the implicated genomic interval. In this case, analysis of expression levels of genes located on both sides of the mutant locus, both in embryos and in pupal wings, might indicate the potential locus under control of such regulatory element.

In this thesis we began to analyze the genetic basis of alleles with large effect on eyespot size and colour composition. Genetic dissection of mutant alleles which affect other aspects of wing patterning (*e.g.* eyespot number and shape, wing venation, overall pigmentation) will be invaluable to improve our understanding of pattern formation. Identification of these loci by linkage analysis is facilitated by the availability of the gene-based linkage map (Beldade *et al.* 2009), and, hopefully in the near future, of the genome sequence for *B. anynana*. Once such genes and mutations therein are identified, it will be important to test their contribution to variation in wing colour patterns in artificial selection lines with divergent phenotypes (*e.g.* Beldade, Brakefield, Long 2002), and, once it becomes feasible, in natural populations of *B. anynana*.

An important evo-devo issue that should be investigated in the future is how all these genes were co-opted in butterfly eyespot formation. What are the genetic changes responsible for recruitment of such genes in a novel developmental context? For instance, novel cis-regulatory regions could have evolved in the *Antp* locus in the butterfly lineage that includes *B. anynana* and other satyrines, which are characterized by upregulation of this gene in presumptive eyespot centres. We are currently screening the *B. anynana* BAC library to identify and sequence clones that contain the *Antp* locus. This will allow us to analyze non-coding, potentially conserved regulatory regions around

this locus. Information as this might be used to identify novel, causal genetic changes underlying co-option of *Antp* and other genes in butterfly eyespot evolution. Hopefully, in the near future, modern molecular techniques such as ‘enhancer trapping’ (e.g. Wilson *et al.* 1989) and ‘chromatin immunoprecipitation’ (e.g. Kuo & Allis 1999) will be available for our model butterfly and will be applied to study the evolution of regulatory sequences and protein-DNA interactions that drive redeployment of developmental toolkit genes into novel, lineage-specific functions. Even more exciting and unexpected conclusions were drawn by the comparative analysis of gene expression patterns in a broad range of butterfly species (Chapter 6). This analysis showed that genetic mechanisms underlying eyespot patterning differ considerably even in rather closely related butterflies. Therefore, it seems that the mechanisms of eyespot development are much more flexible than it was thought before, and that different components of eyespot patterning network can operate in different lineages, resulting in the diversity of these patterns that is ultimately shaped by the action of natural and/or sexual selection. It will be crucial to investigate which modifications of eyespot genetic and developmental pathways occurred in different lineages, and what are the ecological pressures that facilitated eyespot diversification.

The ultimate goal of evo-devo research of such novel traits as butterfly eyespots is to unravel the complete genetic networks underlying their development, from pre-patterning genes that establish the position of eyespot centres, to signalling molecules that define future colour rings, to transcription factors that regulate the effector genes which, in turn, produce different pigments. Once these developmental programs are disentangled in a few model species, it will be important to analyze which modifications of these programs produced the current diversity of eyespot patterns observed in the species-rich lineage of butterflies. Then it will be essential to go back to natural populations and integrate knowledge of ecological pressures, to understand how those mechanisms impact the evolutionary diversification of wing patterns.

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