

Conserved developmental processes and the formation of evolutionary novelties: examples from butterfly wings

Suzanne V. Saenko^{1,*}, Vernon French², Paul M. Brakefield¹
and Patrícia Beldade¹

¹*Section of Evolutionary Biology, Institute of Biology, Leiden University, Kaiserstraat 63, 2311 GP Leiden, The Netherlands*

²*Institute of Evolutionary Biology, University of Edinburgh, Ashworth Labs, EH9 3JT Edinburgh, UK*

The origin and diversification of evolutionary novelties—lineage-specific traits of new adaptive value—is one of the key issues in evolutionary developmental biology. However, comparative analysis of the genetic and developmental bases of such traits can be difficult when they have no obvious homologue in model organisms. The finding that the evolution of morphological novelties often involves the recruitment of pre-existing genes and/or gene networks offers the potential to overcome this challenge. Knowledge about shared developmental processes obtained from extensive studies in model organisms can then be used to understand the origin and diversification of lineage-specific structures. Here, we illustrate this approach in relation to eyespots on the wings of *Bicyclus anynana* butterflies. A number of spontaneous mutations isolated in the laboratory affect eyespots, lepidopteran-specific features, and also processes that are shared by most insects. We discuss how eyespot mutants with disturbed embryonic development may help elucidate the genetic pathways involved in eyespot formation, and how venation mutants with altered eyespot patterns might shed light on mechanisms of eyespot development.

Keywords: evolutionary novelties; butterfly eyespots; embryonic development; wing venation; *Bicyclus anynana* mutants

1. INTRODUCTION

One of the main objectives of evolutionary developmental biology (evo–devo) is to understand the mechanisms that underlie the generation and diversification of evolutionary novelties (Muller & Newman 2005), lineage-specific structures that permit new functions and open up new adaptive zones (Mayr 1960). However, the genetic and developmental analysis of such traits can be a challenge when they are not represented in model organisms, and the comparative method, so successful in evo–devo, is harder to apply.

(a) *Co-option of conserved developmental pathways in the evolution of novelties*

Among the different genetic mechanisms that have been proposed to explain the origin of novelties, the redeployment of pre-existing genes and developmental pathways, often with changes in the regulation of components therein, has received a great deal of attention (reviewed in True & Carroll 2002). For example, the highly conserved Wnt signalling pathway, involved in various developmental processes in vertebrates, has been implicated in the evolution of turtle shells (Kuraku *et al.* 2005), and the arthropod

limb patterning genes *Distal-less* and *aristaless* have been redeployed in the development of horns in a number of beetle species (Moczek & Nagy 2005). Studies in butterflies provide some spectacular examples of pathways that are shared across all insects, and extensively studied in the genetic model *Drosophila melanogaster*, which are co-opted in the development of wing scales. Formation and pigmentation of these lepidopteran-specific structures involve genes known from fruit fly sensory bristle development (Galant *et al.* 1998) and eye pigmentation (Beldade *et al.* 2005; Reed & Nagy 2005), respectively. This type of co-option of genetic pathways offers the potential to dissect the formation of lineage-specific traits by using accumulated knowledge of genetics and development gathered from work on classical model organisms.

(b) *Butterfly eyespots as an example of evolutionary novelty*

The study of butterfly eyespots, characteristic pattern elements composed of concentric rings of different colours, has started to shed light on how novel patterns have arisen and diversified in the Lepidoptera. Eyespots probably evolved from primitive, uniformly coloured spots through the recruitment and modification of conserved developmental genes and pathways, acquisition of signalling activity, and further diversification of colour schemes under the influence of natural selection (Brunetti *et al.* 2001;

* Author for correspondence (s.v.saenko@biology.leidenuniv.nl).

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Monteiro *et al.* 2006). Their ecological significance in predator avoidance and sexual selection is well documented (Stevens 2005; Costanzo & Monteiro 2007), as is the spectacular variation in eyespot morphology across species. Eyespot development is amenable to detailed characterization ranging from the genetic pathways involved in establishing the pattern, to the molecular and cellular interactions underlying pattern specification and to the biochemical networks involved in pigment production (reviewed in Beldade & Brakefield 2002).

Models of eyespot formation involve the production and diffusion of one or more signalling molecules from a central eyespot organizer, the focus, and the response of the surrounding epithelial cells to the signal(s) in a threshold-like fashion, culminating in pigment production (Nijhout 1980; Dilao & Sainhas 2004). The organizer properties of the focus are supported by experiments in early pupae where transplantation of the focal cells into a different position on the wing induces formation of an ectopic eyespot (Nijhout 1980; French & Brakefield 1995). The molecular identity of the signal, however, is not known, but both *Wingless* and *Decapentaplegic* have recently been proposed as candidate morphogens (Monteiro *et al.* 2006). Moreover, despite the fact that a number of genes including *Distal-less* and members of the Hedgehog signalling pathway have been implicated in eyespot development (Carroll *et al.* 1994; Brakefield *et al.* 1996; Keys *et al.* 1999), we know little about the interactions between them (Evans & Marcus 2006) or how they regulate pigment synthesis (Koch *et al.* 2000) or about the extent to which they contribute to phenotypic variation in eyespot morphology (Beldade *et al.* 2002).

(c) *Bicyclus anynana* as an emerging 'eyespot *evo-devo*' model

The tropical nymphalid butterfly *Bicyclus anynana* has been established as a laboratory system and used to study the reciprocal interactions between evolutionary and developmental processes underlying the formation of, and variation in, butterfly colour patterns (Beldade *et al.* 2005, 2007). This system allows us to combine knowledge of ecology (often minimal for classical genetic model species) with experimental tractability, all the way through to the study of the molecular underpinnings of variation in eyespot morphology. Moreover, recently developed genomic resources (Beldade *et al.* 2007) and gene expression manipulation techniques (Marcus *et al.* 2004; Ramos *et al.* 2006) can now be applied to analysing the phenotypically divergent mutant stocks and selection lines (Beldade *et al.* 2005) available in our laboratory. This type of integrated analysis holds much promise for deepening our knowledge about the origin and diversification of the lineage-specific morphologies such as butterfly eyespots.

Here, we report on analyses of a number of spontaneous mutations isolated in *B. anynana* which affect both eyespot morphology and some other, more conserved, developmental processes, such as embryogenesis or wing vein development. Analysis of these mutants within the context of what is known from model organisms provides an opportunity to dissect

the genetic mechanisms involved in eyespot formation and variation. We show how comparative analysis of disturbed embryonic development with mutants described in model insects might help identify genes involved in eyespot development and how mutations that affect wing venation can provide insights into the mechanisms of eyespot formation.

2. EMBRYONIC LETHAL MUTATIONS AND EYESPOT DEVELOPMENT

We currently maintain five stocks, each segregating for an allele that has a dramatic effect on eyespot morphology in heterozygotes and that is embryonic lethal in homozygous state. The mechanisms of early embryonic development are very well studied in the dipteran *D. melanogaster* and are becoming increasingly better understood in the representatives of other insect orders, such as the coleopteran *Tribolium castaneum* and the hemipteran *Oncopeltus fasciatus* (reviewed in Liu & Kaufman 2005), the hymenopteran *Nasonia vitripennis* (e.g. Pultz *et al.* 2005; Lynch *et al.* 2006) and in the lepidopterans *Bombyx mori* (Nagy 1995) and *Manduca sexta* (Kraft & Jackle 1994). To the extent that the genetic mechanisms of embryogenesis are conserved across insects (reviewed in Peel *et al.* 2005; Damen 2007), a comparison of disturbed embryonic development in *B. anynana* eyespot mutants with studies of insect model species may help identify signalling pathways and/or specific genes involved in eyespot formation and variation.

(a) Embryonic development in *B. anynana*

Embryonic development in wild-type *B. anynana* is similar to that described for other Lepidoptera (Nagy 1995). We analysed the patterns of expression of several conserved developmental genes in wild-type embryos staged according to the system developed for *M. sexta* (Broadie *et al.* 1991). In a way similar to early embryos of *Drosophila* and *Schistocerca americana* (Davis *et al.* 2005), the DP311 antibody in *B. anynana* detects patterns that are consistent with the expected expression of the segment polarity gene *gooseberry*, as well as the patterns in the head and in the tips of the appendages that may reflect expression of the homeobox genes, *Rx* and *aristal-less* (figure 1a,b). Also, resembling their counterparts in *Drosophila* and a number of lepidopterans (Patel *et al.* 1989; Panganiban *et al.* 1994; Zheng *et al.* 1999), the products of the segment polarity genes *wingless* and *engrailed* are detected in a reiterated fashion in all embryonic segments (figure 1c,d), whereas the transcription factors *Distal-less* and *Ultrabithorax/Abdominal-A* are detected in the tips of the appendages (figure 1d) and in the abdominal segments (figure 1e), respectively. The conservation of some aspects of embryonic development (namely, segment patterning by segment polarity and Hox genes, and limb patterning by *Distal-less*) as illustrated by these results suggests that the study of disrupted embryonic development in the pleiotropic *B. anynana* eyespot mutants could be useful for identifying genes and pathways involved in eyespot formation.

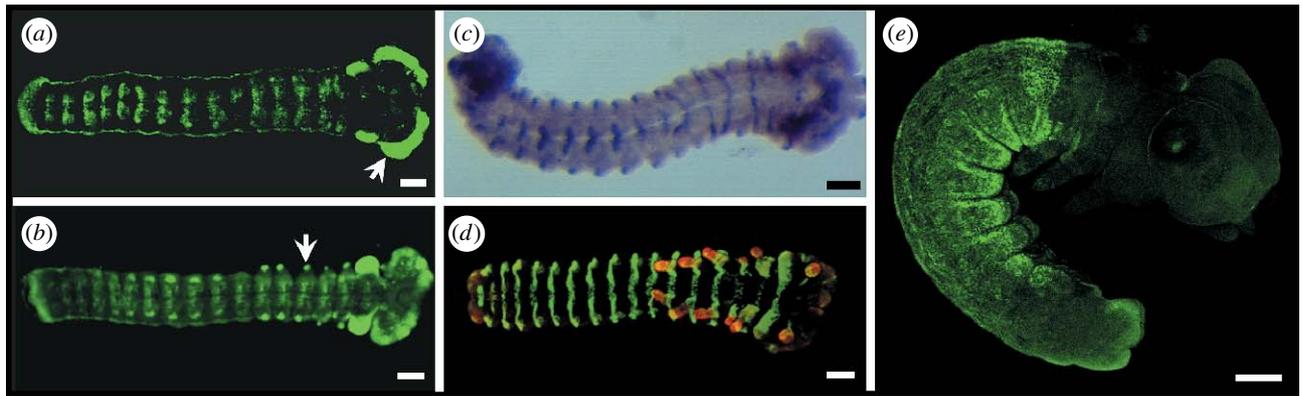


Figure 1. Expression patterns of developmental genes in *B. anynana* embryos (ventral view in (a–d); lateral view in (e), scale bar 0.1 cm). (a) At 15% developmental time (DT), DP311 antibody (Davis *et al.* 2005) detects the segment polarity protein Gooseberry in each embryonic segment, and probably the homeobox protein Rx in the head (arrow). (b) At 20% DT, the same antibody also detects a pattern in the tips of limb primordia (arrow) that is likely to be *Aristaless*. (c) At 25% DT, *wingless* mRNA is detected in a segmentally reiterated fashion. (d) At 30% DT the proteins Engrailed (green; anti-En antibody 4F11, Patel *et al.* 1989) and Distal-less (red; anti-Dll antibody, Panganiban *et al.* 1994) are detected in the posterior segment compartments and in the tips of the appendages, respectively. (e) The antibody FP6.87 (Kelsh *et al.* 1994) detects Ultrabithorax and Abdominal-A in the abdominal segments at 50% DT. Antibody staining was performed according to Patel *et al.* (1989). *In situ* hybridization was performed as described in Tautz & Pfeifle (1989), using digoxigenin-labelled riboprobe against a 315 bp fragment of the *B. anynana wingless* gene (AY218276) and carried out at 55°C for 48 hours. Control reaction with sense-strand probe produced no staining.

(b) Embryonic lethality in homozygous *Goldeneye* mutants

One of the mutations showing lethality in homozygotes, *Goldeneye*, has been previously described as a dominant autosomal allele (Brunetti *et al.* 2001). It disturbs eyespot colour composition in the heterozygotes—the scales that typically form the black inner ring of the eyespots in wild-type butterflies are replaced by gold-coloured scales characteristic of the outer ring (figure 2a,b,e,f). The expression pattern of *engrailed* in the pupal wings is also altered and closely corresponds to the changes in the adult scale coloration (figure 2c,g; see also Brunetti *et al.* 2001).

To investigate the effect of *Goldeneye* mutation on embryonic development, we analysed segregation of embryonic lethality and adult eyespot morphology in a number of individual families from crosses between *Goldeneye* individuals. All unhatched embryos from 14 families were dissected and their morphology was compared with that of wild-type embryos. We found that overall one quarter of the embryos, presumably those homozygous for the *Goldeneye* allele, died before hatching and displayed severe abnormalities (465 out of 1901; ratio not significantly heterogeneous among families, $\chi^2_{13} = 10.84$). The remaining 75% developed normally and all hatched larvae from 6 out of 14 experimental families were reared through to adulthood and scored for eyespot phenotype. Of a total of 386 eclosed adults, 233 had *Goldeneye* eyespots, consistent with heterozygosity for the mutant allele (2 GE : 1 WT ratio not significantly heterogeneous among families, $\chi^2_5 = 1.12$). Embryonic defects in *Goldeneye* homozygotes are detected at the stage of blastokinesis, the characteristic movement of the embryo within the egg which results in its reversal from a ventral to dorsal flexion. This stage is completed by 50% of developmental time (DT) in the wild-type. We found that blastokinesis does not occur in homozygous *Goldeneye* embryos which subsequently

become shorter and thicker and also lack bristles (figure 2d,h). Mutant embryos die at approximately 60% DT.

(c) Candidate genes for embryonic lethal mutations

A number of mutations that affect other aspects of embryonic morphology also seem to disturb blastokinesis (e.g. homeotic mutations at the E locus in *B. mori*; Ueno *et al.* 1995), but the specific genetic regulation of this process is poorly understood. Even though it is unclear how many genes control blastokinesis in butterflies and to what extent the processes of embryonic movements in Lepidoptera and other insects are regulated by similar mechanisms, mutations affecting embryonic movements in insects might provide clues about the genetic basis of the *Goldeneye* phenotype. Examples include the insect Hox3 orthologue *zen* which plays a role in the processes of katatrepsis in *O. fasciatus* (Panfilio *et al.* 2006) and *T. castaneum* (Van der Zee *et al.* 2005), and integrin and laminin genes mutations in which disrupt germ band retraction in *Drosophila* embryos (Schock & Perrimon 2002). Although described mutant phenotypes for these genes show no morphological resemblance to the *Goldeneye* embryonic phenotype, these genes might provide a valuable starting point for exploring the genetic basis of altered eyespot colour composition in *Goldeneye*.

We are currently investigating embryonic lethality in four other eyespot mutants, three of which appear to disturb development during the segmented germ band stage which, unlike blastokinesis, is highly conserved among arthropods, and the genes and developmental pathways that regulate it have been studied in great detail in model organisms (Galís *et al.* 2002). Comparison of disturbed segmentation in these eyespot mutants with the phenotypes of segmentation mutants in model systems is likely to reveal many more details about butterfly eyespot formation.

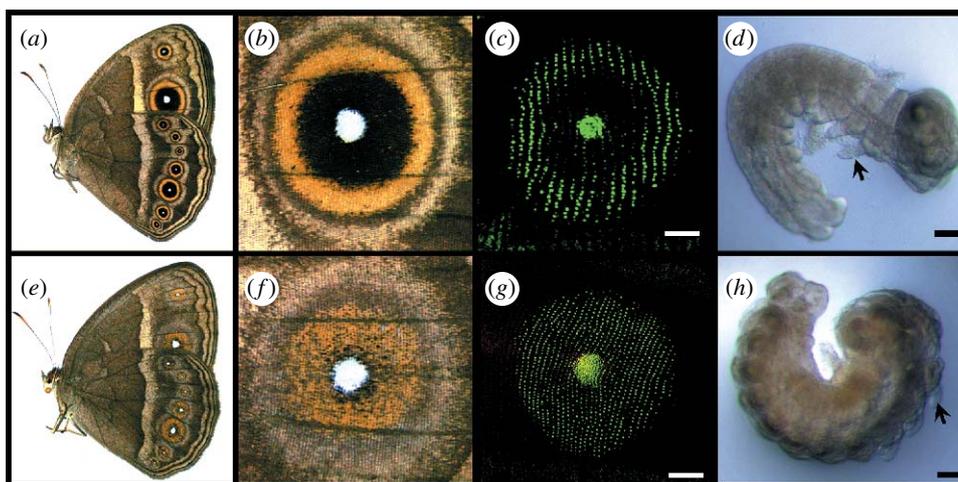


Figure 2. Wild-type *B. anynana* (a–d) and *Goldeneye* mutant (e–h). (a, e) Ventral view of one adult female showing serially repeated eyespots along the margins of the fore and hindwings. (b, f) Enlargement of the posterior eyespot on the ventral surface of the forewing. (c, g) Expression pattern of *engrailed* in the developing pupal wing corresponds to the distribution of gold-coloured scales in the adult eyespots (staining with anti-En 4F11 as described in Brunetti *et al.* (2001); bar = 0.02 cm). (d, h) Wild-type embryo after blastokinesis at 50% DT, and embryo homozygous for the *Goldeneye* allele that failed to undergo blastokinesis (bar = 0.1 cm); arrows point to the first thoracic leg.

(d) Conservation versus divergence in insect embryonic development

The strategy outlined above will be useful only to the extent that the genetic mechanisms of embryonic development are conserved across insect orders, enabling direct comparisons to be made with model organisms. Most knowledge about genetic mechanisms regulating insect embryonic development comes from extensive studies in *D. melanogaster* (see Peel *et al.* 2005). However, a recent focus on organisms from other insect orders is painting a different scenario (Damen 2007). While some aspects of embryonic development are indeed remarkably conserved (e.g. the functions of segment polarity and Hox genes), others appear to be unexpectedly diverged (e.g. the functions of gap and pair-rule genes; see Peel *et al.* 2005; Damen 2007). Yet, because direct comparison of disturbed eyespot phenotypes with eyespot mutants in model species is impossible, comparative analysis of mutations with pleiotropic effects is a valuable alternative strategy. If it appears that the specific embryonic stage affected by a mutation is one showing great divergence across species, this strategy will need to be complemented with a more unbiased, genome-wide search for the genetic factors involved in eyespot formation (e.g. gene mapping; see Beldade *et al.* 2002).

3. WING VENATION AND EYESPOT FORMATION

Models of wing pattern establishment often involve an active role of wing veins and the wing margin, but their precise function in colour pattern formation on butterfly wings is not well understood. While description of venation mutants in *Papilio* and *Heliconius* butterflies has provided evidence for the relationship between wing venation and patterns of colourful stripes and bands (Koch & Nijhout 2002; Reed & Gilbert 2004), the role of wing veins in eyespot formation remains untested. Models of eyespot formation have suggested that the wing veins and margin act as sources

of diffusible molecules involved in the determination of the eyespot focal organizer (Nijhout 1991; Evans & Marcus 2006). Wingless and Decapentaplegic have been proposed as candidate diffusible signals, based on their role as long-range signalling molecules in *Drosophila* wing discs (McMillan *et al.* 2002; Evans & Marcus 2006; Monteiro *et al.* 2006). A role of wing veins, as well as the nature or even the existence of the proposed diffusible signals, has not yet been shown experimentally.

(a) Parallels between fruit fly and butterfly vein development

The mechanisms of vein patterning in *Drosophila* have been extensively studied (reviewed in De Celis 2003; Crozatier *et al.* 2004), and the role of veins in the distribution of melanin precursors in newly eclosed fruit flies has established a functional relationship between venation and pigmentation (True *et al.* 1999). This knowledge will be crucial for our understanding of vein establishment and its role in pattern formation in butterfly wings. Unsurprisingly, positional specification in butterfly wing discs seems to be achieved in a manner very similar to that in the fruit fly. Developing wing discs are divided into anterior–posterior and dorsal–ventral compartments by the expression of the genes *engrailed* and *apterous*, respectively, and proximal–distal patterning is presumably regulated by *Distal-less* and *wingless* (Carroll *et al.* 1994). The signalling pathways that are involved in the positioning and differentiation of longitudinal and cross veins in *Drosophila* (reviewed in Marcus 2001; Crozatier *et al.* 2004) might also be conserved between the lineages of Diptera and Lepidoptera (De Celis & Diaz-Benjumea 2003). Detailed testing of the functional role of homologues of known *Drosophila* vein patterning genes during butterfly wing development will be crucial to our detailed knowledge of vein establishment and role in butterfly wings.

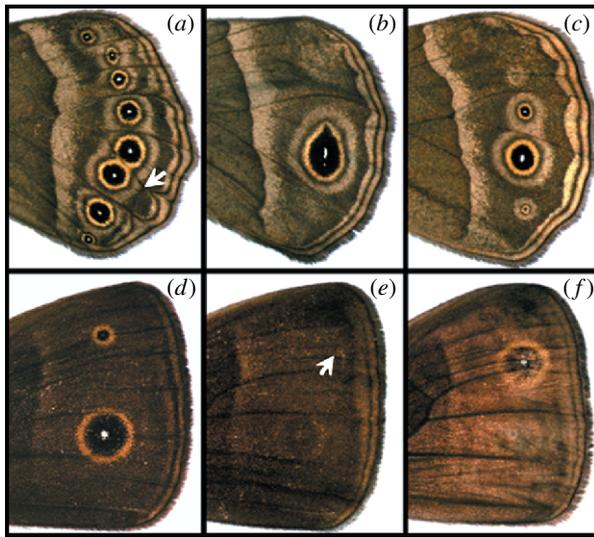


Figure 3. Venation mutants of *B. anynana* (ventral surface of hindwings on (a–c) and dorsal surface of forewings on (d–f)). (a) In this *extra veins* individual, the additional vein (arrow) accompanies an extra eyespot (compare with wild-type hindwing in figure 2a). (b) *Cyclops* mutation causes partial loss of veins and the fusion of some eyespots and loss of the others. (c) *veinless* mutation results in vestigial venation and reduction of ventral eyespots. (d) Dorsal surface of a wild-type forewing with the two characteristic eyespots, which are absent in *veinless* adults (e). Grafting of focal tissue from the larger eyespot of a wild-type pupa into a *veinless* host (cf. French & Brakefield 1995) in the position indicated by the arrow in (e) consistently produced ectopic eyespots in a *veinless* background (f). Note that faint patterns visible in (e, f) are the eyespots present on the ventral wing surface.

(b) Mutations affecting venation and eyespot pattern in *B. anynana*

Our observation that mutants of *B. anynana* with disturbed venation also have aberrations in their eyespot patterns very strongly suggests that eyespot formation depends on normal formation of veins and tracheae. Here we describe three spontaneous mutations with effects on vein and eyespot phenotypes (figure 3). In *extra veins* the addition of a cross vein in a variable position in the distal part of fore- and/or hindwings often leads to the formation of an extra eyespot (figure 3a). This presumably happens when the ectopic vein bisects an existing eyespot focus, or because the additional vein itself acts as an inducer of eyespot formation. In contrast, the mutations *Cyclops* (Brakefield et al. 1996) and *veinless* partially inhibit vein development in the distal part of the wing. In *Cyclops* adults, loss of several veins typically results in fusion of some eyespots and loss of others (figure 3b), while in *veinless*, all veins appear to be at least partially vestigial and eyespots are strongly reduced on the ventral wing surface (figure 3c), and usually absent dorsally (figure 3e). This differential effect on dorsal and ventral eyespots, which is also seen in phenotypic plasticity in response to rearing conditions (Brakefield et al. 1996), might result from differences in timing in the onset of eyespot determination between the two wing surfaces.

(c) Surgical manipulations in the *veinless* mutant

In relation to the signal–response model of eyespot formation explained previously, absence of eyespots on

the dorsal surface of the forewing in *veinless* mutants (figure 3e) can be caused either by a lack of focal signal or by the inability of epidermal cells to respond to that signal. We have investigated these alternatives by transplanting the signalling focus of the large dorsal forewing eyespot from early wild-type pupae into the forewing of *veinless* pupae (figure 3d; cf. French & Brakefield 1995). This manipulation consistently resulted in the production of a well-defined ectopic eyespot (figure 3f) in the otherwise eyespotless wing of *veinless* butterflies (figure 3e), showing that the *veinless* wing epithelium is fully competent to respond to the focal signal in a threshold-dependent manner and to synthesize the black and gold pigments that make up a typical eyespot. Our results suggest that the vestigial venation in *veinless* butterflies is associated with the impairment of determination of the eyespot focus and/or production of the focal signal. The molecular mechanisms of this relationship have yet to be explored. Further analysis will include the comparison of the disturbed vein phenotype of *B. anynana* mutants and well-characterized venation mutants in *D. melanogaster* to identify candidate genes and pathways for mutations in our butterfly.

4. CONCLUDING REMARKS

We reported on the analysis of a number of spontaneous mutants in *B. anynana* butterflies which affect eyespot patterning (a lepidopteran novelty) and other developmental processes that are conserved across insects (namely, embryogenesis or wing vein development). Analysis of these mutants in the context of the extensive genetic and developmental knowledge available for model systems holds promise for furthering our understanding of the origin and diversification of butterfly eyespots.

(a) Shared developmental processes and evolutionary novelties

Among the different genetic mechanisms that have been proposed to account for the origin of novel traits, it is the redeployment of existing pathways that is discussed here. The fact that some shared pathways are reutilized to produce novel structures (with more or less modification of the components therein) offers the potential for using the extensive knowledge of such pathways coming from model organisms, to understand structures present in other systems. Here, we have illustrated this approach using laboratory mutations in *B. anynana* with pleiotropic effects on eyespot patterns and either embryonic development or wing venation, both well studied in *D. melanogaster*. This approach can, in theory, be used to analyse a whole suite of novel traits in any insect species provided pleiotropic mutants have been identified and can be kept in the laboratory.

Wound healing is another example of a fundamental process that is likely to be shared by all animals and might have been co-opted in the evolution of eyespots. Damage of wing tissue in early pupae can lead to the formation of ectopic eyespots (Brakefield & French 1995), probably via the upregulation of expression of characteristic ‘eyespot genes’ (e.g. *Distal-less*, *engrailed*

and *spalt*) in scale-building cells around the wound site (Monteiro *et al.* 2006). Detailed analysis of such shared genetic networks in the context of eyespot formation will be invaluable for our understanding of the evolutionary diversification of butterfly eyespots.

(b) Mutations of large effect and morphological diversification

A related issue of great importance in evo–devo is that of the genetic and developmental mechanisms underlying phenotypic variation. In particular, the extent to which mutants of large effect identified in the laboratory are relevant for natural variation within and across species is a matter of debate (see Haag & True 2001). While it seems unlikely that recessive lethal alleles such as *Goldeneye* will contribute to eyespot variation in natural populations (unless there is a strong heterozygote advantage), it is possible that the same loci harbour other alleles, relevant for variation in eyespot patterns. Also, while mutations that eliminate wing veins and lead to rapid wing damage and, consequently, to reduction in flight ability (as in *Cyclops* and *veinless*) are unlikely to be favoured by natural selection, more localized changes in venation or vein additions (as in *extra veins*) might be relevant mechanisms for wing pattern evolution. Future work will explore the extent to which loci identified in laboratory eyespot mutants contribute to quantitative variation segregating in natural populations and potentially fixed across species.

We have illustrated how studies of *B. anynana* wing patterns and, in particular, of eyespot mutants, can shed light on some of the most exciting questions in evo–devo. Butterfly eyespots, like some other evolutionary novelties, have evolved largely via the redeployment of genetic circuitry involved in other, shared, developmental processes. The study of the latter and the comparison with model insects offer a new approach to studying the origin and diversification of lineage-specific structures.

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