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

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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;...
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 Peal 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 aristaless (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 plectrotypic B. anynana eyespot mutants could be useful for identifying genes and pathways involved in eyespot formation.
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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, $\chi^2_{2} = 1.12$). 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 (*Galis 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.
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 vein 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.
hindwing in figure 2 accompanies an extra eyespot (compare with wild-type (Phil. Trans. R. Soc. B).

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 3c) 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.

We thank Sean Carroll and Nipam Patel for the antibodies they kindly provided, Michael Akam for fruitful discussion and suggestions during the first stage of this work, Cerisse Allen for critical comments on this manuscript, Andrew Peel and one anonymous referee for their thoughtful reviews, Arnaud Martin for help with

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NOTICE OF CORRECTION

Citations to figure 2 are now presented in the correct form. 3 March 2008