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

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CHAPTER 2. 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, or evo-devo. Comparative analysis of the genetic and developmental bases of such traits is, however, difficult when they have no obvious homologues 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. We discuss experimental data exploring genetic commonalities between eyespot patterning and three different conserved developmental processes: embryonic development, wing vein formation, and wound healing. Analysis of such well-described processes in the context of eyespot development holds great promise of furthering our understanding of these highly diverse morphological traits specific to the lineage of the Lepidoptera.

INTRODUCTION

How novel morphological traits arise and diversify is one of the most exciting questions in evo-devo. The genetic and developmental mechanisms that underlie such innovations are, however, poorly understood. Analysis of the mechanistic basis of novel traits is challenging when they are not represented in model organisms, and the comparative method, so successful in evo-devo, is then difficult to apply. The co-option of existing genes and genetic pathways in the evolution of novelties offers the opportunity to overcome this limitation.

Studies in butterflies and moths provide some spectacular examples of pathways that are shared across insects and have been co-opted in the evolution of colour patterns. The remarkably diverse lepidopteran wing patterns are built up from a mosaic of thousands of flattened pigmented scales produced by wing epidermal cells. The parallels between the genetic cascades studied in *Drosophila melanogaster* and involved in butterfly wing development have been documented in relation to scale formation (*e.g.* Achaete-Scute Complex and Notch in fruit fly bristles and in butterfly scales; Galant *et al*. 1998; Reed 2004) and coloration (*e.g.* vermilion in fruitfly eyes and butterfly wings; Reed & Nagy 2005), and in relation to specific pattern elements (*e.g.* the Hedgehog and Wingless pathways in fruitfly wings and butterfly eyespots; Keys *et al*. 1999; Monteiro *et al*. 2006). Such co-option of genetic pathways offers the potential to use extensive knowledge gathered from work on classical model organisms to dissect the formation of butterfly-specific colour patterns.

Butterfly eyespots as an example of an evolutionary novelty

The study of butterfly eyespots, characteristic pattern elements composed of concentric rings of different colours, has started to reveal how novel patterns have arisen and diversified in the Lepidoptera. Eyespots probably evolved from primitive spots through the recruitment and modification of conserved developmental genes and pathways, acquisition of signaling 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 (*e.g.* Costanzo & Monteiro 2007; Olofsson *et al*. 2010), as is the spectacular variation in eyespot morphology across species. Variation in eyespot number, position, shape, size, or colour composition is found not only across species and among individuals of one same species, but often also between different wing surfaces of the same individual butterfly (Nijhout 1991). 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,

to the biochemical networks involved in pigment production (Beldade & Brakefield 2002).

Models of eyespot formation involve the production and diffusion of one or more signaling 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; Dilão & 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 around the transplanted tissue (Nijhout 1980; French & Brakefield 1995). The molecular identity of the signal, however, is not known but both Wingless (Wg) and Decapentaplegic (Dpp) have been proposed as candidate morphogens (Monteiro *et al*. 2006). A number of genes have also been implicated in the determination of eyespot centres and colour rings, including members of the Hedgehog pathway (Keys *et al*.1999), the receptor-encoding gene *Notch* (Reed & Serfas 2004), and the transcription factor-encoding genes *Distal-less* (*Dll*), *engrailed* (*en*) and *spalt* (*sal*) (Carroll *et al*. 1994; Brakefield *et al*. 1996; Brunetti *et al*. 2001). Despite the fact that expression patterns of these and other genes have been associated with eyespot development, we still know little about the interactions between them (see Marcus & Evans 2008), about how they regulate pigment synthesis (see Koch *et al*. 2000), or about the extent to which they contribute to variation in eyespot morphology (Beldade, Brakefield & Long 2002).

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 ecologically-relevant phenotypic variation, with emphasis on wing patterns (Beldade, Brakefield & Long 2005). 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. Moreover, recently developed genomic resources (Beldade, McMillan & Papanicolaou 2008; Beldade *et al.* 2009) and transgenics techniques (Ramos & Monteiro 2007) can now be applied to analysing the phenotypically divergent mutant stocks and selection lines (Brakefield, Beldade & Zwaan 2009) 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 represented by butterfly eyespots.

First, we report on our study of spontaneous pleiotropic mutations isolated in *B. anynana* that affect not only eyespot morphology, but also other, more

conserved developmental processes, such as embryogenesis or wing vein development. We discuss how comparative analysis of these mutants within the context of current knowledge from model organisms provides an opportunity to dissect the genetic mechanisms involved in eyespot formation and variation. Then, we discuss experimental evidence for the similarities between eyespot patterning and wound repair, another process that is highly conserved and well-studied in model organisms. A detailed analysis of such genetic networks in the context of eyespot formation will be invaluable for our understanding of the evolutionary origin and diversification of butterfly eyespots.

MATERIAL AND METHODS

Experimental animals

All butterfly stocks were reared in standard laboratory conditions at 27^{o}C and 70% relative humidity, with larvae raised on maize plants and adults fed on sliced banana (*cf.* Brakefield, Beldade & Zwaan 2009). The mutant stocks Goldeneye, veinless, Cyclops and extra veins were started from single individuals isolated from different laboratory populations and have been maintained with selection in favour of the mutant phenotype in each generation. To avoid inbreeding depression, all mutant stock were regularly outcrossed to the laboratory outbred 'wild-type' (WT) stock.

Goldeneye crosses

To test if the *Goldeneye* (*GE*) mutation behaves as an embryonic recessive lethal allele, 14 single-pair crosses between GE butterflies were set up. Eggs from individual pairs were collected for analysis. Unhatched eggs were counted, dechorionated in 50% bleach solution for one minute, rinsed with water and fixed in 4% formaldehyde solution in 1x phosphate-buffered saline (PBS). Embryos were dissected and observed under the light microscope, and any aberrations in their appearance were documented. Hatched larvae were counted, and for six out of 14 experimental families, reared to adulthood. Eclosed butterflies were then frozen and scored for their eyespot phenotype.

To examine segregation patterns of aberrant embryos and mutant adults we used the Chi-square (χ^2) homogeneity and goodness-of-fit tests. According to our test hypothesis, *GE* is a recessive embryonic lethal allele (*i.e.* expected frequency of aberrant embryos is 1/4) with dominant effect on eyespot phenotype (*i.e.* expected frequency of GE adults is 2/3). Unfertilized eggs were excluded from the analysis, and the observed frequencies of aberrant embryos, and of WT and GE adults were tested against these expected ratios. First, we tested whether the ratios varied significantly among families (homogeneity test). If this was not the

case, the numbers were pooled over all families and these summed frequencies were tested against the expected ratios with goodness-of-fit test (d.f. = 1).

Embryo morphology and gene expression patterns

Eggs were collected for one hour periods, transferred into Petri dishes, and kept in a climate room. At different times after egg-laying, eggs were dechorionated in 50% bleach solution for one minute, rinsed with water, fixed in 10% formaldehyde in 1xPBS / 50 mM EGTA solution for 30 minutes, gradually dehydrated in increasing concentrations of methanol and stored at -20^{o}C until use. Fixed embryos were gradually rehydrated in 1xPBS, dissected under the microscope and staged according to the system developed for *Manduca sexta* (Broadie, Bate & Tublitz 1991).

In situ hybridization with *wg* anti-sense and sense (control) probes were performed at 55 ^{o}C for 48 hours following the protocol described in Brakefield, Beldade & Zwaan (2009). The 315 bp fragment of *B. anynana wg* gene (AY218276) was amplified from embryonic cDNA with primers 5'-GTCATGATGCCCAATACCG and 5'-GCAGTTGCATCGTTCCACTA and cloned into pCRII®-TOPO dual-promoter vector using the TOPO TA cloning kit (Invitrogen). Plasmids were isolated with QIAprep Spin Miniprep Kit (QIAGEN) and used as template for PCR reactions with vector primers M13F and M13R. The amplified products were cleaned with Wizard SV Gel and PCR Clean-Up System (Promega) and used for SP6 or T7 transcription. Sense and antisense digoxygenin-labeled riboprobes were synthesized using SP6 and T7 RNA polymerases and DIG RNA labeling mix (Roche Applied Science). The probes were run on an 1.2% agarose gel and measured with NanoDrop spectrophotometer (Thermo Scientific) to verify their quality and concentration. Stained embryos were mounted on slides and photographed with a Leica DC 200 digital camera attached to a Leica MZ 125 microscope.

Antibody stainings on embryos were performed using a modified version of the protocol described in Brakefield, Beldade & Zwaan (2009). After two hours of blocking in PBT (5 mg/ml of bovine serum albumine in 1xPBS), embryos were incubated with primary antibodies overnight at 4^{o}C. The following antibodies were used: monoclonal mouse antibodies DP311 (Davis, D'Alessio & Patel 2005) and anti-En 4F11 (Patel *et al*. 1989) [dilution 1:25], anti-Ubx/Abd-A FP6.87 (Kelsh *et al*. 1994) [1:5], and a polyclonal rabbit anti-Dll antibody (Panganiban *et al.* 1995) [1:200]. Embryos were washed 10 times in 1xPBS with 0.1% Triton X-100 and incubated for two hours at 4^{o}C in 1:200 dilution of the secondary antibodies: anti-mouse Alexa Fluor 488 and anti-rabbit Texas Red (Molecular Probes). After 10 washes in 1xPBS, embryos were incubated in 100% glycerol for one hour and mounted on slides. Images were collected on a BioRad MRC 1024 ES laser scanning confocal microscope.

Surgical manipulations

Pupation times were scored by means of time-lapse photography with 30 minute intervals using a digital camera. Left and right forewings of 183 female pupae were pierced with a fine tungsten needle (World Precision Instruments) at two sites, between the native eyespots but closer to the wing margin (Fig. 4). Cauteries were done under a dissection microscope at 12 hours post pupation (hpp), within the time period when ectopic eyespots are produced most often (Brakefield & French 1995). Forewings of 110 operated pupae were dissected and used for double stainings with anti-En and anti-Dll (left wings) or anti-En and anti-Spalt (right wings), performed as described in Brakefield, Beldade & Zwaan (2009). Dissections were done at six time points, varying from 18 to 35 hpp (*i.e.* 6 – 23 hours after damage induction), with 3.5 hour intervals between each time point. Between 11 and 32 individuals were dissected at each time point. Control stainings were performed using combinations of secondary antibodies only. The remaining 73 operated animals eclosed and were used to estimate the fraction of successfully induced ectopic eyespots.

Grafts were performed on the dorsal surface of left forewings in 3.5 - 4 hr old pupae, *cf*. Brakefield, Beldade & Zwaan (2009). A square of cuticle together with underlying epidermis containing focal cells of the large posterior eyespot was cut and moved from the wild-type pupae into a more anterior position on pupae of the veinless mutant. Adults were frozen soon after emergence, and the successful grafts, *i.e.* those for which the grafted tissue had healed properly, were photographed with a Leica DC 200 digital camera attached to a Leica MZ 125 microscope.

RESULTS AND DISCUSSION

1. EMBRYONIC LETHAL MUTATIONS AND EYESPOT DEVELOPMENT

A number of mutant stocks is maintained in our laboratory, each segregating for an allele that has a dramatic effect on eyespot morphology in the heterozygotes and that is embryonic lethal in the homozygous state (see Chapters 1 and 3). The mechanisms of early embryonic development are very well studied in the dipteran *D. melanogaster* and are becoming increasingly better understood in representatives of other insect orders, such as the coleopteran *Tribolium castaneum* (Schröder *et al*. 2008), the hemipteran *Oncopeltus fasciatus* (*e.g.* Panfilio *et al*. 2006), the hymenopteran *Nasonia vitripennis* (*e.g.* Lynch *et al.* 2006; Pultz *et al.* 2005), and the lepidopterans *Bombyx mori* (Nagy 1995) and *M. sexta* (Kraft & Jackle 1994). To the extent that the genetic mechanisms of embryogenesis are conserved across insects (reviewed in Peel, Chipman & Akam

2005 and Damen 2007), a comparison of disturbed embryonic development in *B. anynana* eyespot mutants with studies of insect model species may help identify pathways and/or specific genes involved in eyespot formation and variation.

Embryonic development in *B. anynana*

Embryonic development in wild-type *B. anynana* (Fig. 1) is similar to that described for other Lepidoptera (Nagy 1995; Broadie, Bate & Tublitz 1991). At 27^{o}C, embryogenesis lasts approximately 4 days, so that one hour corresponds to 1% of embryonic developmental time (DT). Segmentation of embryonic germ band is evident at 15% DT, and at 20% DT thoracic appendages become visible, followed by differentiation of abdominal appendages a couple of hours later. Dorsal closure and blastokinesis, the characteristic movement of the embryo within the egg that results in its reversal from a ventral to a dorsal flexion, are completed by 50% DT. During the second half of embryogenesis, red pigmentation appears in the eyes, segmentation of the appendages is completed, and cuticular sclerotization and pigmentation progress until hatching.

We analysed the patterns of expression of several conserved developmental genes in wild-type embryos. In a similar way to early embryos of *Drosophila* and *Schistocerca americana* (Davis, D'Alessio & Patel 2005), the DP311 antibody in *B. anynana* detects patterns that are consistent with the expected expression pattern 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* (Fig. 1a,b). Also, resembling their counterparts in *Drosophila* and a number of lepidopterans (Patel *et al*. 1989; Panganiban *et al.* 1995; Zheng *et al.* 1999), the products of the segment polarity genes *wg* and *en* are detected in a reiterated fashion in all embryonic segments (Fig. 1c,d), whereas the transcription factors Dll and Ultrabithorax/Abdominal-A (Ubx/Abd-A) are detected in the tips of the appendages (Fig. 1d) and in the abdominal segments (Fig. 1e), respectively. The conservation of some aspects of embryonic development (namely, segment patterning by segment polarity and Hox genes, and limb patterning by *Dll*), 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.

Embryonic lethality in homozygous Goldeneye mutants

One of the mutations showing lethality in homozygotes, *GE*, has been previously described as a dominant autosomal allele (Brunetti *et al.* 2001). It disturbs eyespot colour composition in the heterozygotes: the majority of scales that typically form the black inner ring of the eyespots in WT butterflies are replaced

by gold-coloured scales characteristic of the outer ring (Fig. 2a,b). The expression pattern of *en* in the pupal wing discs is also altered and closely corresponds to the changes in the adult scale coloration (Fig. 2c; see also Brunetti *et al.* 2001).

To investigate the effect of the *GE* mutation on embryonic development, we analyzed segregation of embryonic lethality and adult eyespot morphology in the offspring of 14 crosses between GE individuals. We found that overall, one quarter of the embryos (465 out of 1901), presumably those homozygous for the *GE* allele, died before hatching and displayed severe abnormalities. The frequency of aberrant embryos was not significantly heterogeneous among families (χ^2 = 14.36, d.f.= 13, P = 0.3489), and not significantly different from the expected 25% (χ^2 = 0.295, d.f.= 1, P = 0.5870). The GE:WT ratio in adults was also not heterogeneous among families (χ^2 = 4.25, d.f. = 5, P = 0.5140), and was close to the expected 2:1 (233 *GE* and 153 WT). However, the observed numbers of GE individuals were significantly lower than expected (χ^2 = 6.9, d.f.= 1, P = 0.0086), suggesting that GE heterozygotes might suffer from lower viability during larval or pupal developmental stages. Embryonic defects in GE homozygotes were detected at the stage of blastokinesis. We found that this embryonic movement does not occur in homozygous GE embryos, which subsequently become shorter and thicker than their wild-type siblings and die at about 60% DT (Fig. 2d).

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, Nagata & Suzuki 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 GE phenotype. Examples include the insect Hox3 orthologue *zerrknullt* which plays a role in the processes of katatrepsis in *O. fasciatus* (Panfilio *et al.* 2006) and *T. castaneum* (van der Zee, Berns & Roth 2005), and integrin and laminin genes, mutations in which disrupt germ band retraction in *Drosophila* (Schock & Perrimon 2002). Although mutant phenotypes described for these genes show no morphological resemblance to the GE embryonic phenotype, these loci might provide a valuable starting point for exploring the genetic basis of the *GE* mutation.

Three other spontaneous mutants isolated in our *B. anynana* laboratory population appear to disturb development during the segmented germband stage

(see Chapter 3), which, unlike blastokinesis, is highly conserved among arthropods (*e.g.* Farzana and Brown 2008). Furthermore, the genes and developmental pathways that regulate it have been studied in great detail in model organisms (Galis, van Dooren & Metz 2002). Thus, comparison of disturbed segmentation in these eyespot mutants with the phenotypes of segmentation mutants in model systems might reveal many more details about butterfly eyespot formation.

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. Recent studies extending the analysis of insect embryonic development outside *D. melanogaster* (see Peel, Chipman & Akam 2005; Damen 2007; Peel 2008) have shown that, while some aspects of embryonic development are indeed highly 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; Peel 2008). Still, whatever the embryonic stage affected in any pleiotropic eyespot mutant may be, because direct comparison of disturbed eyespot phenotypes with "eyespot mutants" in model species is impossible (model insects do not have eyespots!), comparative analysis of embryonic phenotypes in such mutants remains a valuable first approach. If it appears that the specific embryonic stage affected by a mutation is one showing great divergence across species, or if none of the candidate genes generated by this strategy will show an association with the mutant phenotype, a more unbiased, genome-wide search for the genetic factors involved in eyespot formation will be necessary (*e.g.* gene mapping; see Beldade *et al*. 2009).

2. WING VENATION AND EYESPOT FORMATION

Models of wing pattern establishment often involve an active role of wing veins and the wing margin (see Nijhout 1991), but their precise function in colour pattern formation on butterfly wings is not well understood. Whilst 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 wing veins and margin act as sources of diffusible molecules involved in the determination of the eyespot focal organizer (Nijhout 1991; Marcus & Evans 2008). Wg and Dpp have been proposed as candidate diffusible signals, based on

their role as long-range signaling molecules in *Drosophila* wing discs (McMillan, Monteiro & Kapan 2002; Marcus & Evans 2008). 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.

Parallels between fruitfly and butterfly vein development

The mechanisms of vein patterning in *Drosophila* are fairly well understood (reviewed in Blair 2007), and the role of veins in the distribution of melanin precursors in newly eclosed fruitflies has established a functional relationship between venation and pigmentation (True *et al.* 1999). As is often the case for work in non-model systems, this knowledge is a valuable starting point for our understanding of the mechanisms behind 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 fruitfly. Developing wing discs are divided into anterior-posterior and dorsal-ventral compartments by the expression of the genes *en* and *apterous*, respectively, and proximal-distal patterning is presumably regulated by *Dll* and *wg* (Carroll *et al.* 1994). The signaling pathways that are involved in the positioning and differentiation of longitudinal and cross veins in *Drosophila* (reviewed in Marcus 2001, and in Crozatier, Glize & Vincent 2004) might also be conserved between Diptera and Lepidoptera (De Celis & Diaz-Benjumea 2003). Detailed testing of the functional role of homologs of known *Drosophila* vein patterning genes during butterfly wing development will be crucial to our detailed knowledge of vein establishment and their role in forming pattern on butterfly wings.

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 (Fig. 3). In individuals bearing mutation *extra veins* the addition of a cross-vein in a variable position in the distal part of fore- and/or hindwings often leads to formation of an extra eyespot (Fig. 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 (Fig. 3b); in butterflies homozygous for *veinless* allele all veins appear to be at least partially vestigial and eyespots are strongly reduced on the

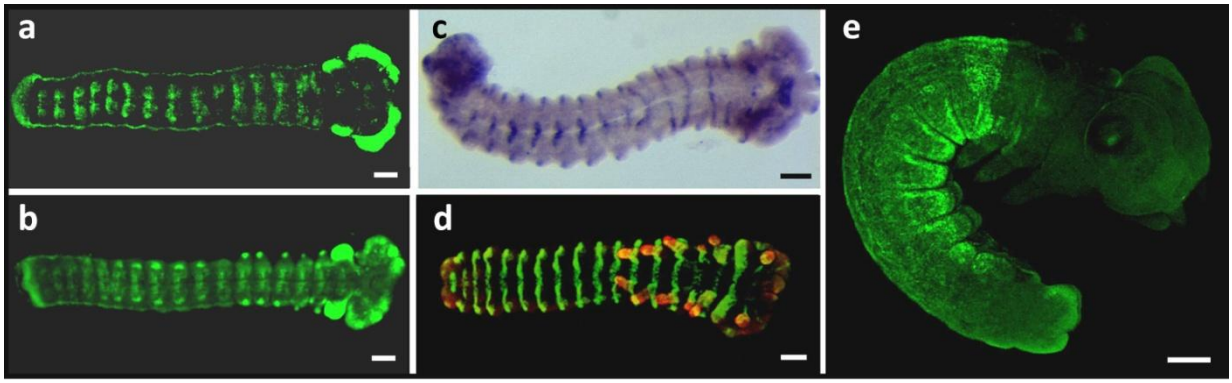


Figure 1. Expression patterns of developmental genes in *B. anynana* embryos.
a. At 15% DT, DP311 antibody detects the segment polarity protein Gooseberry in each segment, and probably the homeobox protein Rx in the head (arrow). **b.** At 20% DT, the same antibody also detects a pattern in the tips of limb primordia (arrow), likely to be Aristaless. **c.** At 25% DT, *wg* mRNA is detected in a segmentally-reiterated fashion. **d.** At 30% DT the proteins En and Dll are detected in the posterior segment compartments, and in the tips of the appendages, respectively. **e.** The antibody FP6.87 detects Ultrabithorax and Abdominal-A in the abdominal segments at 50% DT. Ventral view in a-d, lateral view in e; scale bar is 100 µm.

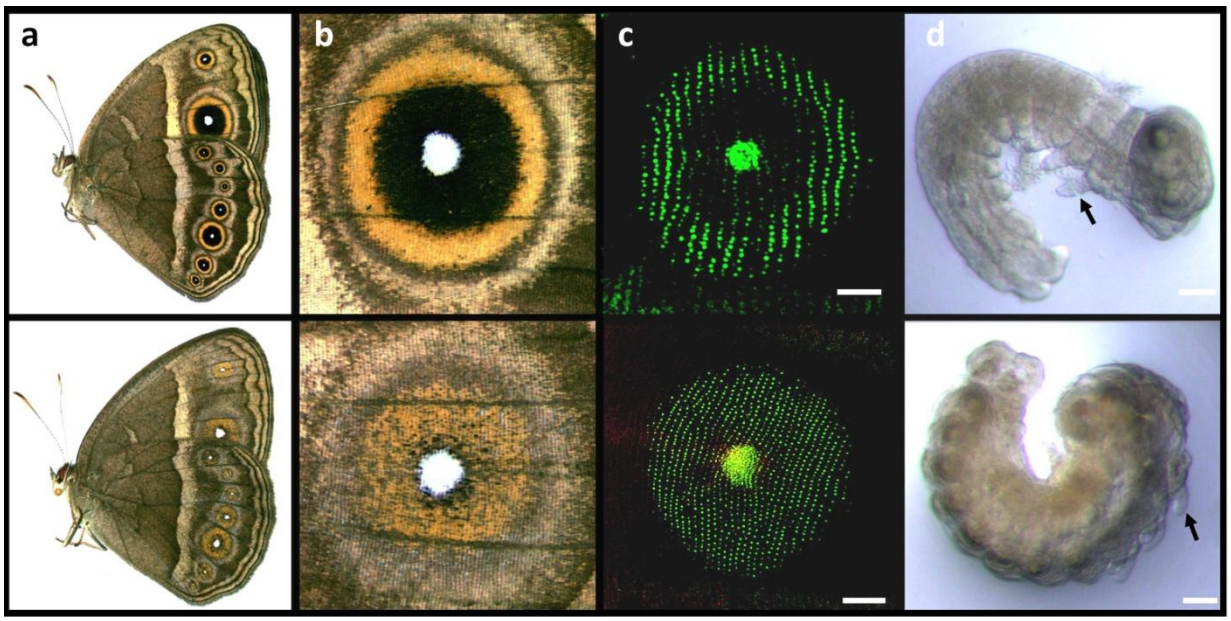


Figure 2. Wild-type *B. anynana* (top panel) and GE mutant (bottom panel).
a. Ventral view of a female showing serially repeated eyespots along the margins of the fore- and hindwings. **b.** Enlargement of the posterior forewing eyespot on the ventral surface. **c.** Expression pattern of *en* in the developing pupal wing corresponds to the distribution of gold-coloured scales in the adult eyespots (scale bar = 200 µm). **d.** Wild-type embryo after blastokinesis at 50% DT, and embryo homozygous for the *GE* allele that failed to undergo blastokinesis (scale bar = 100 µm); arrows point to the first thoracic leg.

Figure 4 (page 36). Expression of *en* in pupal wings after damage.
a. Schematic representation of *B. anynana* wing. The sites of damage are indicated by stars in all photographs. **b.** Example of an operated wing with two ectopic eyespots (white square corresponds to the area of the wing depicted in **d**). **c.** Typical result of immunostainings – no upregulation of any of the three examined transcription factors was observed around damage site in 218 operated wings (here a wing stained with anti-En antibody is shown). **d.** In all examined wings *en* was upregulated in scale-building cells of native (N) eyespots, while in only two wings this protein was also detected in presumptive ectopic eyespots (E). **e.** An area of the wing epidermis (at 32 hpp) showing such upregulation of *en* around wound site.

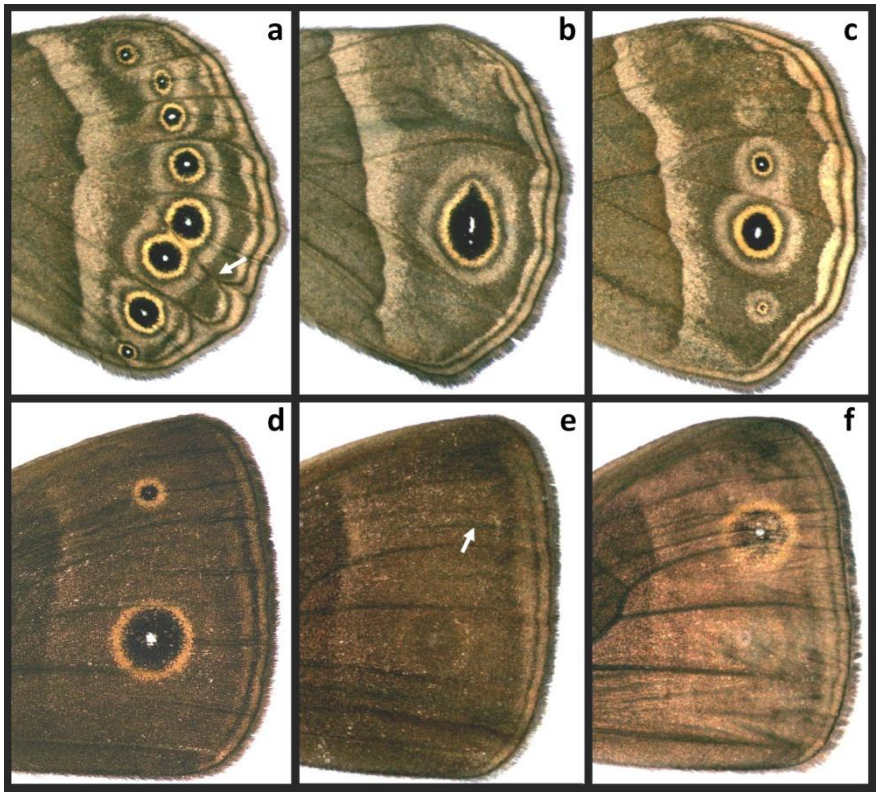
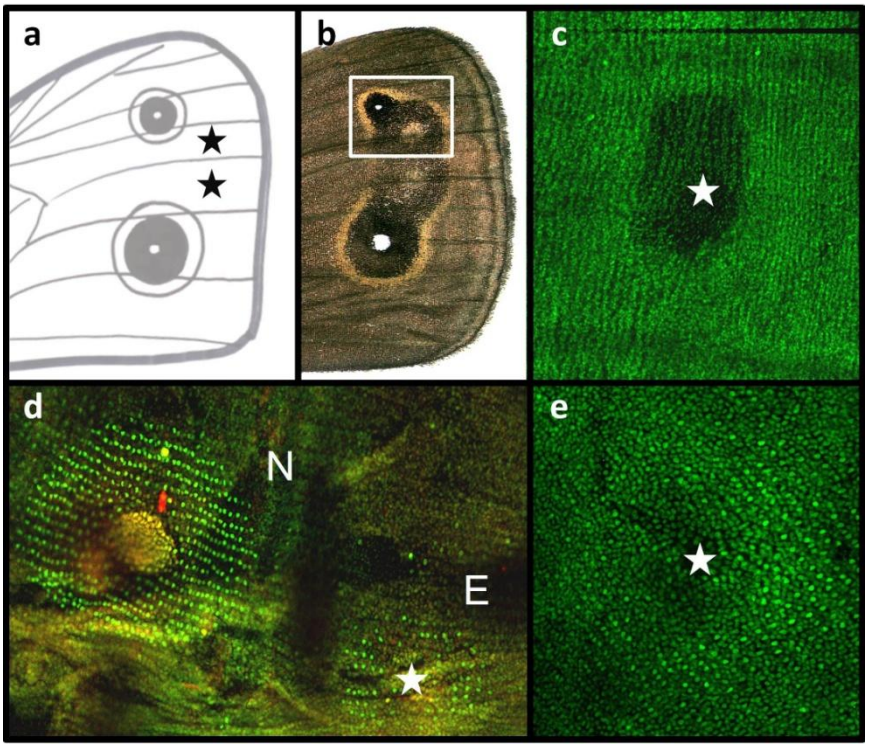


Figure 3. Venation mutants of *B. anynana*.
a. In this individual homozygous for *extra veins* mutation, the additional vein (arrow) accompanies an extra eyespot (compare to wild-type hindwing in figure 2a). **b.** *Cyclops* mutation causes partial loss of veins and the fusion of some eyespots and loss of the others. **c.** *veinless* mutation results in vestigial venation and reduction of ventral eyespots. **d.** Dorsal surface of a wildtype 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 (**d**) pupa into a veinless host 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**) and (**f**) are the eyespots present on the ventral wing surface. Ventral surface of hindwings on top panels; dorsal surface of forewings on the bottom.



ventral wing surface (Fig. 3c), and usually absent dorsally (Fig. 3e). This differential effect on dorsal and ventral eyespots, which is also seen in phenotypic plasticity in response to rearing conditions (Brakefield, Kesbeke & Koch 1998), might result from differences in timing in the onset of eyespot determination between the two wing surfaces.

Surgical manipulations in the veinless mutant

In relation to the signal–response model of eyespot formation explained above, absence of eyespots on the dorsal surface of the forewing in veinless mutants (Fig. 3e) can be caused either by the lack of focal signal or by the inability of epidermal cells to respond to that signal. We have investigated these alternatives by transplanting the signaling focus of the large dorsal forewing eyespot from early wild-type pupae into the forewing of veinless pupae (Fig. 3d). This manipulation consistently resulted in production of a well-defined ectopic eyespot (Fig. 3f) in the otherwise eyespot-less wing of veinless butterflies (Fig. 3e). These results show 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, but the molecular mechanisms of this relationship are yet to be explored.

3. WOUND HEALING AND EYESPOT FORMATION

The ability to repair wounded tissue is a fundamental property of all multi-cellular organisms, and a key topic of current research (see Gurtner *et al.* 2008). Here, we first review evidence suggesting that some components of this process are involved in eyespot formation. We then discuss the results of our own study of gene expression in damage-induced eyespots.

Damage-induced eyespot formation in *B. anynana*

Local damage to pupal wing tissue has long been known to disturb colour patterns in many lepidopterans, and has been used to study the mechanisms of pattern formation in butterflies and moths (*e.g.* Kühn & von Englehardt 1933; Nijhout 1980). In eyespot-bearing butterfly species, for example, early damage with a fine needle applied to the presumptive eyespot foci in pupae can completely eliminate eyespots in the adults (Nijhout 1980; French & Brakefield 1992). Also, damage to other locations of the pupal wing epidermis can result in the formation of an ectopic eyespot around the wound site (French & Brakefield 1995). In *B. anynana*, rings of black and/or gold, typically rather poorly-defined

and more assymetrical relative to those of the native eyespots, are found around the healed wound (Fig. 4b).

Interestingly, this type of damage produces eyespots only on the distal area of the wing, whereas the proximal wing tissue seems to be unable to respond to eyespot-inducing signals by producing yellow or black pigments (French & Brakefield 1995). Ectopic eyespots are formed only if damage is applied during a narrow time window (Brakefield & French 1995), which more or less corresponds to the period when 'colour-ring genes', *Dll, en* and *sal,* are upregulated in the presumptive eyespot area (Brunetti *et al*. 2001). The mechanisms by which the genes and pathways of the damage response machinery might contribute to the formation of ectopic eyespots are still unclear, but insights from studies in model organisms could provide some clues.

Genetic mechanisms of wound repair

Comparative studies on the genetic and cellular mechanisms of wound repair and regeneration in representatives of various animal phyla have suggested their evolutionary conservation (see Woolley & Martin 2000). For example, some steps of the wound healing process are regulated by the same transcription factor, Grainyhead, in flies and mice (Mace, Pearson & McGinnis 2005; Ting *et al*. 2005). Also, wound healing seems to recapitulate some aspects of embryonic morphogenesis, such as dorsal closure in flies and eyelid fusion in mice, raising the possibility of co-option of genetic pathways active during embryogenesis into wound repair processes during adult life (see Martin & Parkhurst 2004).

The mechanism by which damage results in the formation of ectopic eyespots is an intriguing question. It is known that candidate genes for eyespot signalling also perform wound healing-related functions. Studies in fly larvae, for example, have shown that wounds act as sources of short- and long-range signalling molecules and activate downstream pathways in a gradient-like manner, as has been proposed for eyespot development. In *Drosophila*, the Jun N-terminal kinase (JNK) pathway is activated in a gradient centred around the wound (Galko & Krasnow 2004) and upregulates the transcription factor AP-1 which, in turn, leads to induction of Dpp, a TGF-beta family member and one of the candidate eyespot-inducing signals (Monteiro *et al*. 2006). Some evidence also exists for the involvement of the Wnt proteins in wound repair in mammals (*e.g.* Okuse *et al*. 2005). Moreover, their insect homolog, Wg, is a candidate morphogen in eyespot formation (Monteiro *et al*. 2006).

Commonalities between native and damage-induced eyespots in *B. anynana*

To investigate whether the same genetic mechanisms that underlie wound healing are involved in damage-induced eyespot formation in butterflies, we performed immunostainings to detect the expression of "eyespot genes" *en*, *Dll* and *sal*

around damage sites in developing pupal wings. Ectopic patterns were produced in 85% (124/146) of control wings, but a high degree of variation in size and colour composition of ectopics was observed between and within individuals. Only 19% of all ectopics consisted of a gold outer ring and a black inner disc (Fig. 4b); the majority of damage sites were surrounded by just patches of gold or black scales. We found that all three genes were upregulated in the native eyespots in 220 wings of operated pupae (Fig. 4d). However, only En was detected in some scale-building cells of presumptive ectopic eyespots, in two wings examined at 16.5 and 20 hours after damage infliction, respectively (Fig. 4d,e). Thus, the vast majority of wings did not show any signs of upregulation of any of these genes in the epidermal cells surrounding the wound (Fig. 4c).

Our results do not implicate Dll and Sal in formation of ectopic eyespots, but do not exclude such a role for En, despite the fact that its expression was detected so rarely around damage sites. The two cases observed of En in scale-building cells surrounding wounds are unlikely to represent artefacts, given that we never observed aspecific binding of the secondary antibodies to wounded epidermis. However, based on these results it is very difficult to conclude whether ectopic and native eyespots share (some of) the underlying signaling pathways. Our findings to some extent contradict those of Monteiro *et al*. (2006) who found that all three genes were expressed in scale-building cells around the wound sites (although they do not mention the proportion of positive results). Monteiro and colleagues proposed that the formation of both native and ectopic eyespots relies on at least some identical molecules. Their, and our data, however, do not distinguish between the possibility that the rings of expression of the tested eyespot genes around wound sites are induced by candidate eyespot morphogens Wg and Dpp, or by some other, as yet unidentified, signals produced by wounded cells. In the future, a more unbiased approach (*e.g.* expression profiling of damaged vs. undamaged wings) could be more effective in identifying genetic pathways involved in response to damage, which have potentially been co-opted in eyespot formation.

CONCLUDING REMARKS

Here we discussed new approaches to study the genetic and developmental mechanisms underlying formation of butterfly eyespots, based on the commonalities with conserved developmental processes such as wound healing, embryonic development, or vein patterning. We described several spontaneous mutations which affect eyespots and either embryogenesis or wing vein development, both relatively well-conserved. The extent to which mutations 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; Stern & Orgogozo 2009). Whilst it seems unlikely that pleiotropic mutations with negative effects on other traits (*e.g.* embryonic lethality in GE, or fragile wings in mutants with vestigial venation) will contribute to variation in natural populations, it is possible that the same loci harbour other, less deleterious alleles, relevant to variation in eyespot patterns. Future work will focus on identification of mutant loci, and will explore whether these mutations contribute to eyespot variation in natural populations, within and across species.

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