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

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CHAPTER 3. Single locus affects embryonic segment polarity and multiple aspects of an adult evolutionary novelty

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The characterization of the molecular changes that underlie the origin and diversification of morphological novelties is a key challenge in evolutionary developmental biology. The evolution of such traits is thought to rely largely on co-option of a toolkit of conserved developmental genes that typically perform multiple functions. Mutations that affect both a universal developmental process and the formation of a novelty might shed light onto the genetics of traits not represented in model systems. Here we describe three pleiotropic mutations with large effects on a novel trait, butterfly eyespots, and on a conserved stage of embryogenesis, segment polarity. We show that three mutations affecting eyespot size and/or colour composition in *Bicyclus anynana* butterflies occurred in the same locus, and that two of them are embryonic recessive lethal. Using surgical manipulations and analysis of gene expression patterns in developing wings, we demonstrate that the effects on eyespot morphology are due to changes in the epidermal response component of eyespot induction. Our analysis of morphology and of gene expression in mutant embryos shows that they have a typical segment polarity phenotype, consistent with the mutant locus encoding a negative regulator of Wnt/Wg signalling. Continued work on the fine mapping of this gene will reveal its molecular identity and mode of action and will increase our understanding of the evolution of butterfly colour patterns in particular, and the origin and diversification of adaptive novel traits in general.

INTRODUCTION

Phenotypic variation is a universal feature of living organisms and the raw material for evolution by natural selection. Understanding its genetic and developmental bases is a central theme in evolutionary developmental biology. One of the major tasks of evo-devo research is to characterize the loci and individual mutations that contribute to phenotypic differences in adaptive traits, both within and between species (see Stern 2000). Analysis of laboratory mutants that mimic naturally occurring phenotypic variation can suggest candidate genes. Although this approach is biased and can be misleading (examples in Stern 2000), it might still be useful in non-model organisms with limited genomic resources. The candidate gene approach has already been successfully applied to studies of several polymorphic traits in animals and plants (see Haag & True 2001), including natural variation in coloration (*e.g.* the gene *Agouti*, identified in a laboratory mouse mutant, has been linked to melanism in several species, see Kingsley *et al.* 2009).

Studies of animal coloration have provided numerous examples of adaptive morphological evolution, including industrial melanism in the peppered moth (Majerus 1998) and Mullerian mimicry in *Heliconius* butterflies (Joron & Mallet 1998). Remarkably diverse wing patterns of butterflies and moths are known to play a role in many ecological contexts, including thermoregulation, predator avoidance and mate choice (examples in Monteiro & Prudic 2010). Although such ecological functions have been studied in detail, the genetic and developmental mechanisms responsible for the dramatic variation in wing patterns are still poorly understood. Studies of colour pattern formation focused mainly on eyespots – concentric rings of contrasting colours that show striking intra- and interspecific diversity in number, shape, size and colour composition (examples is Nijhout 1991). The tropical Nymphalid *Bicyclus anynana* has been recently established as an ‘eyespot evo-devo’ model (Brakefield, Beldade & Zwaan 2009).

Classical surgical manipulations of presumptive eyespots in pupal wings revealed that eyespot centres, called foci, act as organizers of pattern formation (Nijhout 1980; French & Brakefield 1995). The focal cells produce one or several diffusible morphogens in early pupal wings (Monteiro *et al.* 2001), and the neighbouring epidermal cells respond to these signals in a concentration-dependent manner and produce a particular pigment shortly before adult eclosion. Several conserved genes and signalling pathways, known to function in insect wing development, have been implicated in different stages of eyespot formation. For instance, members of the Hedgehog, Notch, Wingless and TGF- β pathways are expressed in or around eyespot foci (Keys *et al.* 1999; Reed & Serfas 2004; Monteiro *et al.* 2006), and the transcription factor *Distal-*

less has been shown to contribute to inter-individual variation in eyespot size (Beldade, Brakefield & Long 2002). Unfortunately, an expression-based approach is limited by the availability of cross-reactive antibodies and/or sequence information of butterfly genes, and it also provides little information on the function of implicated genes in eyespot formation and variation. Studies of laboratory mutants with large effect on eyespot morphology can be used to dissect the developmental mechanisms of eyespot formation and to identify candidate loci for inter- and intraspecific variation in eyespot morphology.

Captive populations of *B. anynana* harbour different types of genetic variation affecting eyespot patterns, including standing quantitative variation and alleles of large phenotypic effect (see Chapter 1). Of special interest are pleiotropic mutations that affect eyespots, a lineage-specific trait not represented in classical models, and the relatively well-conserved process of embryonic development (see Chapter 2 for an example). Comparison of embryonic mutant phenotype with mutants described in insect models can suggest which genes and/or pathways are altered by the mutant alleles. Here, we describe a single locus three alleles of which affect different aspects of eyespot morphology and disturb embryonic segment polarity in *B. anynana*. Analysis of embryonic phenotypes and of expression patterns of segment polarity genes suggest that a negative regulator of the Wnt/Wg signalling pathway is involved in formation of a butterfly wing patterns.

MATERIAL AND METHODS

Biological material

All butterfly stocks were reared in standard laboratory conditions at 27°C (*cf.* Brakefield, Beldade & Zwaan 2009). The mutant stocks Bigeye (BE) and Frodo (Fr) were each set up from single individuals isolated from different laboratory populations in 1994 and 2007, respectively. The Spread (Spr) stock was founded from an individual isolated from the BE stock in 2003. All mutant stocks have been maintained with selection in favour of the mutant phenotype in each generation and, when necessary, were outcrossed to the laboratory outbred ‘wild-type’ (WT) stock to avoid inbreeding depression.

Different crosses were set up to determine the mode of inheritance of the mutant alleles (Table 1). To test whether the mutations behave as embryonic recessive lethal alleles, butterflies from each mutant stock were crossed to individuals of the same phenotype and to WT stock butterflies. Crosses between butterflies of different mutant phenotypes were performed to determine whether the mutations occurred in the same locus (complementation tests). Hatched larvae were reared through to adulthood, and the eclosed butterflies were frozen and

scored for their eyespot phenotype. 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 under the light microscope.

For scanning electron microscopy (SEM) studies, fixed embryos were rinsed in 1xPBS and mounted on specimen holders, air-dried, coated with gold and viewed in a JEOL JSM 6400 scanning electron microscope. For the analyses of embryonic morphology and patterns of gene expression, at least 50 embryos were examined for each mutant line per developmental stage (*cf.* Broadie, Bate & Tublitz 1991). *In situ* hybridization and immunohistochemistry of embryos were performed as described in Chapter 2. Stainings with anti-En 4F11 (Patel *et al.* 1989) [dilution 1:25], anti-Dll (Panganiban *et al.* 1995) [1:200] and anti-Sal (de Celis, Barrio & Kafatos 1999) [1:500] antibodies on pupal wings were performed as in Brakefield, Beldade & Zwaan (2009). Anti-mouse Alexa Fluor 488 and anti-rabbit Texas Red (Molecular Probes) were used as secondary antibodies [1:200].

Induction of ectopic eyespots

Damage was applied to induce ectopic eyespot formation in Spr (N = 70) and WT (N = 50) pupae. Pupation times were scored by means of time-lapse photography with 30 minute intervals. The dorsal surface of left pupal forewings was damaged at 12 – 18 hours post pupation, within the time period when ectopic eyespots are produced most frequently (Brakefield & French 1995). Wings were pierced with a finely sharpened tungsten needle (World Precision Instruments, Cat. No. 501317) at a site in the third wing cell (next to the wing cell that bears the small anterior eyespot), approximately halfway between the wing margin and the normal location of the eyespots. Operated pupae were returned to 27 °C. Adults were then frozen soon after emergence, and their wings were photographed with a Leica DC 200 digital camera attached to a Leica MZ 125 microscope.

Statistical analysis

The Chi-square (χ^2) homogeneity and goodness-of-fit tests were used to compare observed proportions of the aberrant embryos and the mutant adults to those expected if BE, Fr and Spr phenotypes are determined by recessive embryonic lethal alleles with dominant effect on eyespots (*i.e.* 1/4 of the embryos die, 2/3 of the adults have mutant phenotype). Unfertilized eggs, identified as those lacking large blastoderm cells, were excluded from the analysis, and the observed frequencies of aberrant embryos and of WT and mutant adults were tested against those expected ratios. First, we tested whether the ratios varied significantly among

families (χ^2 homogeneity test). If this was not the case, the numbers were pooled over all families belonging to the same cross type and mutant phenotype; these overall frequencies were tested against the expected ratios using χ^2 goodness-of-fit test (d.f. = 1). If the χ^2 homogeneity test revealed significant variation among families, the frequencies of aberrant embryos and mutant adults were tested against the expected values for each of these family separately (d.f. = 1). The frequencies of aberrant embryos in complementation test crosses between different mutants were tested against 1/4 (expected frequency of aberrant embryos if mutant alleles do not complement) in a similar way.

RESULTS AND DISCUSSION

Eyespot mutations are embryonic recessive lethal alleles at a single locus

Three spontaneous mutants with disturbed eyespot size and/or colour composition have been isolated from laboratory populations of *B. anynana*. Relative to WT butterflies, BE mutants have dramatically enlarged eyespots (Brakefield *et al.* 1996; Beldade, French & Brakefield 2008), Fr have eyespots of normal size with a broader outer golden ring, and Spr have very large eyespots with gold scales almost completely replacing the black scales (Fig. 1a,b).

The failure to establish pure-breeding stocks of these mutants suggested embryonic lethality, similar to that described for Goldeneye (see Chapter 2). We compared proportions of mutant embryos and adults in experimental crosses to those expected if BE, Fr and Spr phenotypes were caused by embryonic recessive lethal alleles with a dominant effect on eyespot pattern. In this situation, crosses between individuals of the same mutant phenotype will result in 1/4 of the offspring dying during embryogenesis, and 2/3 of the adults having aberrant eyespots. On the other hand, crosses between mutant and WT butterflies will yield in 1/2 of the adults with aberrant eyespots and no embryonic lethality. The results of experimental crosses, including statistical analysis, are summarized in Table 1. As expected, crosses between mutant individuals of the same phenotype resulted in 1:2 segregation of WT and mutant adults (33.6 and 66.4%, respectively, in a total of 1720 adult progeny from 39 families), while crosses between each mutant and WT butterflies resulted in 1:1 segregation (47.4 and 52.6%, in 2113 adult offspring from 28 families). Consistent with BE, Fr and Spr phenotypes being due to recessive lethal alleles, approximately one fourth of the progeny of crosses between same-phenotype mutants died during embryogenesis (25.3% of 5526 progeny in 39 families), while no aberrant embryos were found in crosses to WT butterflies.

Cross	# fam.	# progeny	Aberrant embryos			# adult progeny					
			% [SD]	χ^2_{HOM} [P]	χ^2_{GF} [P]	WT	BE	Fr	Spr	χ^2_{HOM} [P]	χ^2_{GF} [P]
BE x BE	16	2945	24.0 [2.9]	12.89 [0.61]	1.68 [0.20]	250	521	0	0	3.70 [0.99]	0.28 [0.60]
Fr x Fr	10	1156	27.4 [3.1]	4.98 [0.84]	3.61 [0.06]	175	0	334	0	3.38 [0.95]	0.25 [0.62]
Spr x Spr	13	1416	26.4 [4.3]	8.97 [0.71]	1.51 [0.22]	0	153 ^a	0	287	4.60 [0.97]	0.41 [0.52]
BE ² x BE ²	8	1488	22.9 [2.2]	4.03 [0.78]	3.44 [0.06]	151	278	0	0	13.99 [0.05]	0.67 [0.41]
BE ³ x BE ³	4	667	3.1 [2.3]	10.78 [0.03]	--	0	393	0	0	--	--
BE x WT	14	2459	0	--	--	499	549	0	0	18.58 [0.14]	2.39 [0.12]
Fr x WT	5	659	0	--	--	227	0	249	0	0.69 [0.95]	1.02 [0.31]
Spr x WT	9	1272	0	--	--	275	314 ^b	0	0	4.68 [0.86]	2.59 [0.11]
BE ³ x WT	11	--	--	--	--	1486	0	0	0	--	--
BE x Fr	9	1671	24.4 [4.1]	10.24 [0.25]	0.31 [0.58]	185	185	164	0	--	--
BE x Spr	24	2974	23.6 [4.8]	27.26 [0.25]	3.05 [0.08]	408	480	0	412	--	--
Fr x Spr	5	954	23.7 [4.0]	5.12 [0.41]	0.88 [0.35]	153	129	0	146 ^c	--	--
BE ³ x BE	7	1092	1.0 [4.0]	90.98 [0.00]	--	356	0	0	310	--	--

^a BE³, ^b BE², ^c eyespots smaller than in *Spr* (see Fig. 2c)

Table 1. Segregation of aberrant embryos and adults in experimental crosses

The ratios of aberrant embryos and adults were tested for variation among families within each cross with χ^2 test for homogeneity (χ^2_{HOM} with d.f. = $N_{\text{fam}} - 1$). When ratios were not significantly different across families, the numbers of progeny were pooled and tested against the expected ratios with χ^2 goodness-of-fit test (χ^2_{GF} with d.f. = 1). Otherwise, the ratios were tested against the expected values in each family separately (d.f. = 1). Standard deviation (SD) and *P* values (P) are given in square brackets.

The aberrant embryos in the BE $\times$ BE, Fr $\times$ Fr, or Spr $\times$ Spr crosses showed severe and very similar morphological defects (Fig. 1c), suggesting that all three mutations are alleles of the same locus. This was confirmed by complementation tests: crosses between all three mutants yielded embryonic lethality in $\sim 1/4$ of the offspring (23.9%, N=5599, 38 families), with identical morphological aberrations as those found in the offspring of crosses between mutants of the same phenotype. In contrast, no embryonic lethality or aberrant morphology was observed in 1756 progeny of 13 crosses between BE and Goldeneye individuals. This shows that these two mutations occurred in different genes, which is also consistent with the fact that the embryonic phenotype caused by the *Goldeneye* allele (*i.e.* disturbed blastokinesis, see Chapter 2) is different from the embryonic effects produced by the alleles underlying the BE, Fr or Spr phenotypes.

Different BE phenotypes and variation in lethality of underlying alleles

Dramatic effects on eyespot morphology in BE, Fr and Spr mutants seem to be caused by different dominant alleles at the same locus, each disturbing embryonic development in homozygotes. However, the inheritance mode of the Spr phenotype appears to be more complex. The effect of the underlying allele on eyespot *colour composition* but not *size* is recessive, since offspring from Spr $\times$ WT crosses either have ‘wild-type’ eyespots or large eyespots with ‘normal’ colour scheme (46.7 and 53.3%, respectively, in 1272 adult offspring from nine families; Table 1). The latter phenotype is indistinguishable from that of BE individuals and is hereafter referred to as BE² (Fig. 2a). A similar phenotype, hereafter called BE³, is found in the ‘non-Spr’ progeny from Spr $\times$ Spr crosses (34.8% of 440 adults in 13 families; Fig. 2a). Single-pair crosses were set up to determine whether enlarged eyespots in BE individuals and in the offspring of Spr $\times$ WT (BE² individuals) and Spr $\times$ Spr crosses (BE³ individuals) were caused by the same or by different alleles (Table 1, Fig. 2b).

Similarly to BE $\times$ BE, crosses between two BE² individuals yielded embryonic lethality in approximately one fourth of the progeny (22.9% of 1488 progeny in eight families) and enlarged eyespots in two thirds of the adults (64.8% of 429 adult offspring). These results are consistent with BE and BE² phenotypes being due to the same embryonic lethal allele with a dominant effect on eyespot size. In contrast, only a small fraction of embryos from crosses between two BE³ individuals died before hatching and showed typical morphological defects (Table 1). This ratio was significantly lower than the expected 25% (χ^2 test of homogeneity among four families = 10.78, $P < 0.05$; χ^2 goodness-of-fit for each family = 53.05, 22.00, 29.37, 42.29, all with $P < 0.05$) and varied between 0 and 5.3%, perhaps due to modifier loci or incomplete penetrance. No embryonic

lethality was observed in five of the seven crosses between BE³ and BE individuals. The fraction of aberrant embryos in the other two families (10.8 and 1.1%) was significantly lower than the 25% expected if the BE³ and BE phenotypes were produced by the same lethal allele (χ^2 test for homogeneity among seven families = 90.98; χ^2 goodness-of-fit in two families = 20.79 and 7.55; $P < 0.01$). These results suggest that the mutation underlying the BE³ phenotype is a different allele. It has a mildly deleterious effect on embryogenesis (*i.e.* low incidence of embryonic mortality) and a recessive effect on eyespot size, since all offspring of the BE³ $\times$ WT crosses (*i.e.* BE³ heterozygotes) have ‘wild-type’ eyespots (Table 1). A novel eyespot phenotype was observed in a quarter of the offspring of crosses between Fr and Spr butterflies. Eyespots of these individuals were smaller than in the Spr mutant (compare Fig. 2c and Fig. 1a), but their colour composition was affected in a similar way.

On the basis of the segregation of eyespot and embryonic mutant phenotypes in the experimental crosses, we propose that the three mutations occurred in the *BE/Fr/Spr* (*BFS*) locus, and that two of them are embryonic recessive lethal. We suggest that different combinations of the wild-type (*BFS*⁺) and the three mutant alleles underlie the observed eyespot and embryonic phenotypes (Fig. 3). The BE/BE² phenotype (each obtained in a different cross; see Fig. 2a) is due to a single copy of the recessive lethal *BFS*^B allele, the identical BE³ phenotype is caused by the recessive nonlethal *BFS*^a allele, and the Spr phenotype, by a combination of both. The Fr phenotype is produced by a single copy of another recessive lethal allele, *BFS*^C. In this model, alleles *BFS*^a and *BFS*^C each carry a mutation at a single site, while the *BFS*^B allele carries two, one shared with *BFS*^a. This is consistent with the fact that the Spr mutant (genotype *BFS*^a/*BFS*^B) was originally isolated from the BE stock (see Materials & Methods), and it explains the data from all our crosses, including the more complex inheritance we described for Spr, and the different “enlarged eyespots” phenotypes we characterized (see previous section). Specifically, (a) crosses between two Spr individuals segregate for Spr and BE³ phenotypes (and not for Spr and WT, as would be the case if the Spr phenotype was due to a single, independent mutation at the *BFS* locus), and (b) the Spr phenotype is lost in progeny from Spr $\times$ WT crosses (showing that it is recessive), but recovered in progeny from BE³ $\times$ BE crosses (showing that BE individuals carry the relevant mutation).

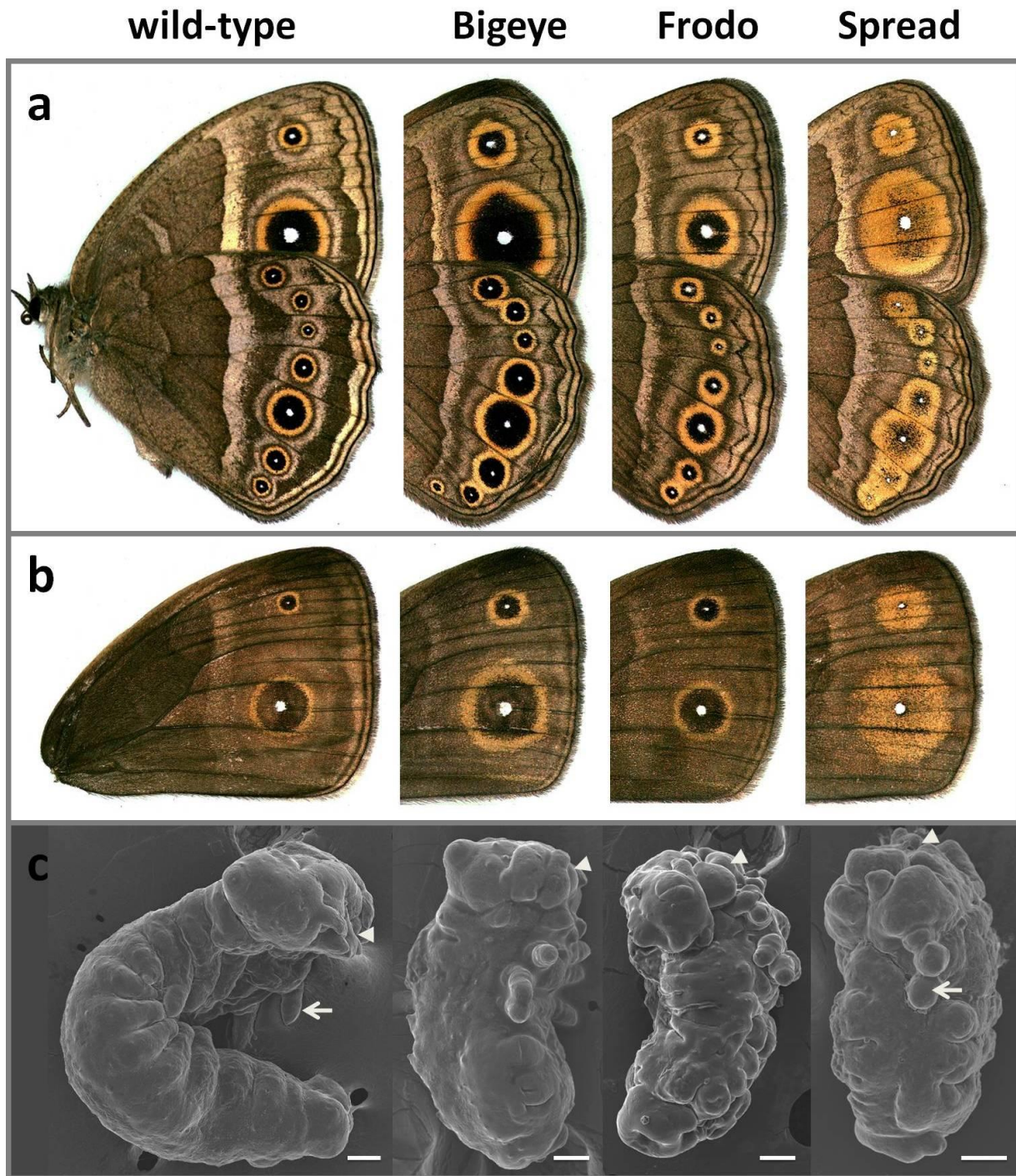


Figure 1. *B. anynana* mutants with altered eyespots and disturbed embryonic development. **a.** Representative images of the ventral surface of wings in WT and mutant females. **b.** Photos of dorsal forewing surface of the same individuals. **c.** Representative scanning electron microscopy images of WT and mutant embryos at ~ 60% DT (lateral view, anterior is up and dorsal is to the left; scale bar is 100 μ m). At this developmental stage, all embryos are still alive (Spr embryos die at 70% DT, while BE and Fr die at about 90% DT) and do not differ consistently in morphology, although Spr embryos seem more compacted (multiple individuals were observed for each mutant). Arrows point to thoracic legs, arrowheads indicate mouthparts.

Figure 2 (page 54). Phenotypes of embryos and adults in crosses between mutants.

a. Butterflies with enlarged eyespots are found in the BE stock and in the progeny of different Spr crosses. **b.** Aberrant embryos are found in different proportions when butterflies with different BE phenotypes are crossed. **c.** A novel phenotype obtained in Fr x Spr crosses, with eyespots smaller than in Spr (compare to Fig. 1a).

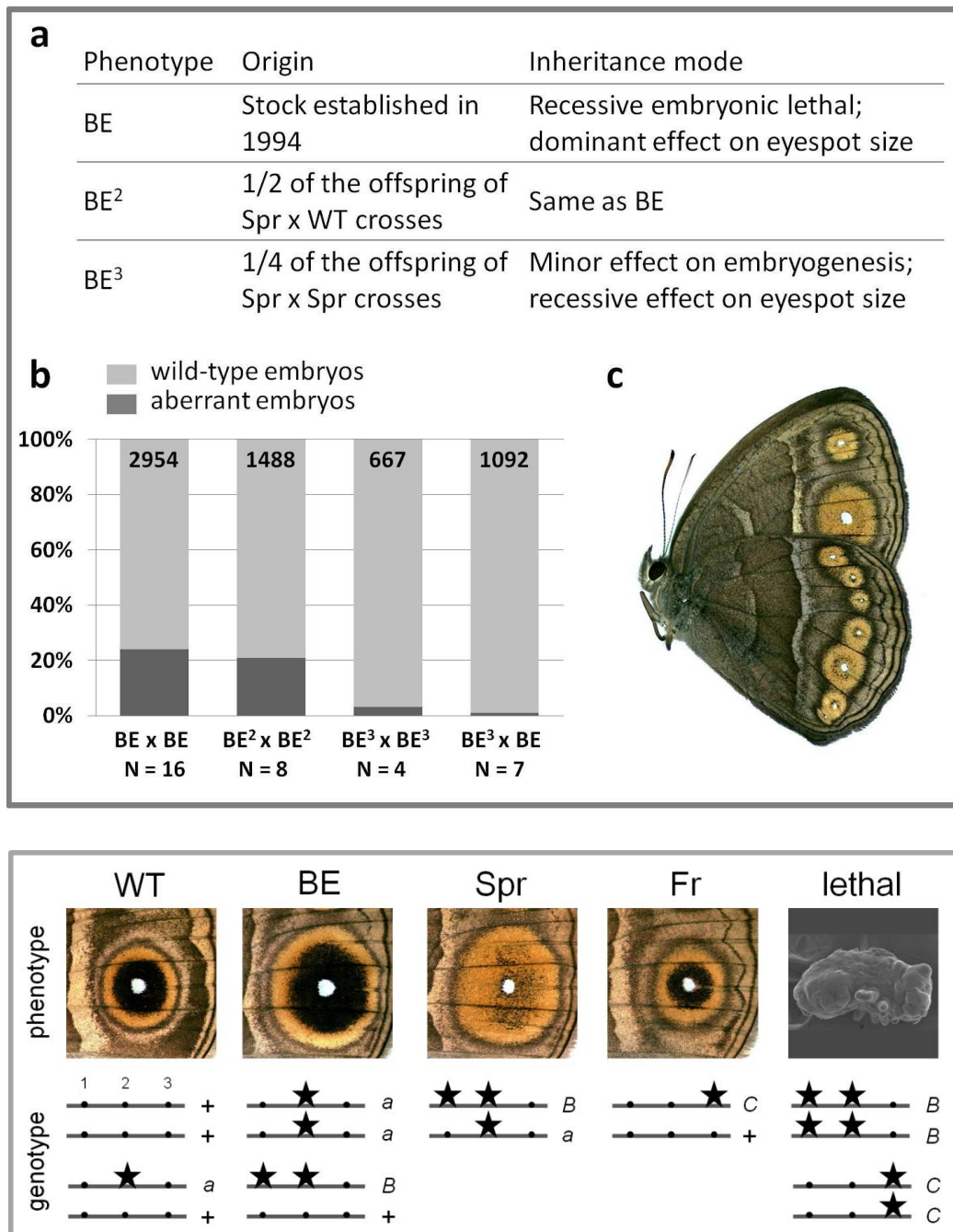


Figure 3. Model for the embryonic and eyespot effects of the *BFS* locus.

For each mutant phenotype, possible genotypes are shown with lines representing the locus, dots corresponding to different sites therein, mutations indicated with stars (the order and the distance between sites is arbitrary), and labels +, a, B and C representing the wild-type and three mutant alleles. Mutations at these three sites, isolated or in combination, define different alleles which can explain all our data. A single copy of the *BFS^a* allele has no obvious effect on eyespot morphology (the WT phenotype), but two copies produce enlarged eyespots which have ‘normal’ colour composition (the BE³ phenotype; phenotypically indistinguishable from BE/ BE²). Mutations at sites 1 and 2 together make up the *BFS^B* allele; it has a dominant effect on eyespot size (the BE/ BE² phenotype), and, in combination with the *BFS^a* allele, affects colour composition (the Spr phenotype). This explains the recessive colour composition aspect of Spr inheritance and the presence of BE³ individuals (*BFS^a* homozygotes) in crosses between two Spr individuals (Table 1), and is consistent with Spr having been isolated from the BE stock (see Materials and Methods). A mutation at a third site in this locus (corresponding to the *BFS^C* allele) affects eyespot ring boundaries (the Fr phenotype). The alleles *BFS^B* and *BFS^C* are embryonic recessive lethal and display a segment polarity phenotype (see below).

Analysis of aberrant embryos reveals defects in segment polarity

Compared to WT embryos of the same age, homozygotes for each of the lethal alleles displayed severe and similar abnormalities. They were much shorter and thicker than WT embryos (Fig. 1c). The typical three thoracic and ten abdominal segments were all present, as was clear from the number of thoracic and abdominal appendages, but the segments were compressed and their borders poorly defined. Dorsal closure was not completed in ca. 30% of the embryos. The thoracic legs and mouthparts (arrows and arrowheads in Fig. 1c) were short; some embryos were missing one leg while one of the remaining legs was branched. We observed variation in timing of death among mutants. Typically, embryos from Spr $\times$ Spr crosses died before the stage when bristles appear (~70% of developmental time, DT), and looked more compacted, while mutant embryos from crosses between BE or Fr butterflies were fully sclerotized and had melanized head capsules, and died at approximately 90% DT. The severe shortening of segments and poorly defined segment boundaries observed in all mutant embryos suggest that the mutations affect the structure of each segment, rather than their establishment, possibly by interfering with the normal function of segment polarity genes.

Analysis of expression patterns of key segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) revealed substantial differences between WT (see also Chapter 2) and mutant embryos. In early embryos (Fig. 4a), *en* was expressed in the posterior compartment of each segment, as is typical for insects (Patel *et al.* 1989). No aberrations in morphology or *en* expression were detected in 85 embryos dissected at 12-13% DT, revealing no differences among mutant and WT embryos. At 15% DT, the differences became obvious with the En protein detected not only in the posterior compartment, as in WT, but also in the anterior cells of each segment in mutant embryos (Fig. 4b). Defects in segmentation became even more apparent at 25% DT, when mutant embryos appeared shorter due to compacted segments, and the En stripes were almost twice as broad as those of WT (Fig. 4c). At 40% DT, mutant embryos looked almost spherical, with En clearly visible in a posterior and an anterior stripe within each segment, thoracic appendages, and abdominal prolegs (Fig. 4d). Differences in *wg* expression were also very clear at 25-40% DT. In WT, *wg* mRNA was detected in each segment in stripes positioned just anterior to the En domain, while in mutant embryos two stripes of *wg* were observed in each segment, with the additional stripe positioned posterior to the expanded En domain (Fig. 4e).

These results show that the lethal alleles at the *BFS* locus do not affect the specification of segment number or the establishment of *en* and *wg* expression domains in embryonic segments, but rather disturb their correct maintenance. Comparison of morphology and *en/wg* expression patterns of mutant *B. anynana*

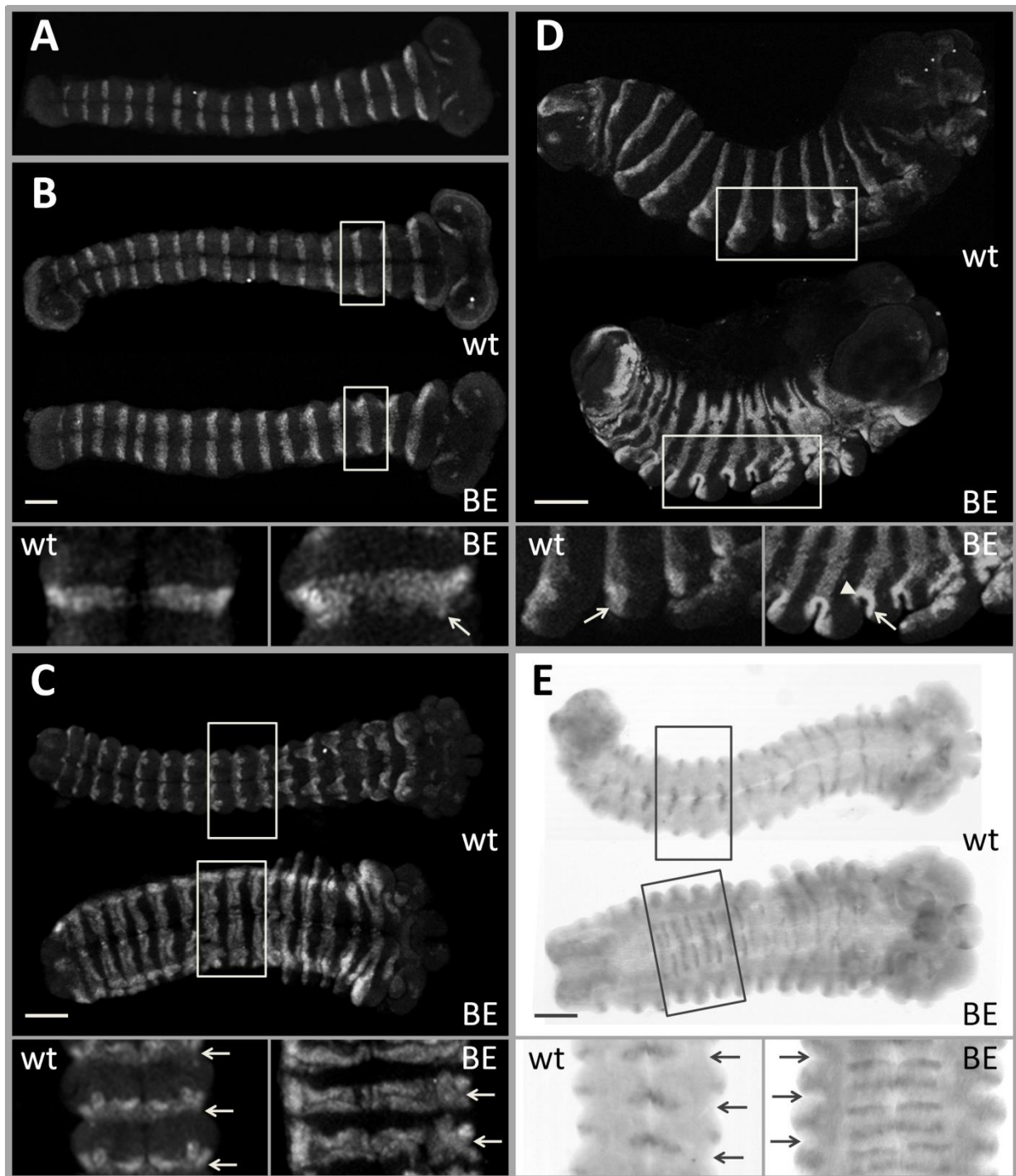


Figure 4. Expression of segment polarity genes in embryos.

a. Ventral view of an embryo from the BE stock at 12% DT; *en* is expressed in posterior compartments. **b.** Ventral view of a WT and a BE embryo at 15% DT; *En* is also detected in some anterior cells of the segments (arrow) in BE embryos. **c.** At 25% DT, BE embryos appear shorter than WT, with *En* present in the anterior and posterior cells of each segment (arrows indicate segment borders). **d.** Lateral view of a WT and a BE embryo at 40% DT. Arrows point to posterior cells of the second abdominal segment, expressing *en*. In BE embryos, *En* is also detected in anterior cells of each segment (arrowhead). **e.** *wg* expression in embryos at 25% DT (arrows indicate segment borders). In WT, *wg* mRNA is detected in a single stripe per segment, while extra *wg* stripes are present in segments of a BE embryo. Anterior is to the right; scale bar is 100 μ m. Mutant embryos from Spr or Fr stocks show identical patterns of *en* and *wg* expression.

embryos with phenotypes of *Drosophila melanogaster* segmentation mutants showed that all three mutations produce a typical segment polarity phenotype (e.g. Ingham 1991), ‘replacing’ the anterior part of each segment by a mirror-image duplication of the posterior part. Loss-of-function mutations in fruit fly genes *zeste-white 3* (Siegfried, Chou & Perrimon 1992), *naked cuticle* (Zeng *et al.* 2000), *axin* (Hamada *et al.* 1999) and *APC2* (McCartney *et al.* 1999), and an experimental knockdown of the gene *Bili* (Kategaya *et al.* 2009) produce very similar phenotypes. Interestingly, all these proteins are negative regulators of the highly conserved Wnt/Wg signalling pathway.

Epidermal response properties are affected in the Spr mutant

The formation of eyespots in developing pupal wings involves the production and diffusion of a morphogen from the cells at the center (stage called ‘focal signalling’) and the response of the surrounding epidermal cells (Nijhout 1980; French & Brakefield 1995). Different levels of the signal induce expression of different regulatory genes, e.g., *Distal-less (Dll)* and *spalt (sal)* in the inner disc, and *en* in the outer ring (Brunetti *et al.* 2001), which then directly or indirectly control biosynthesis of different pigments. Changes in the focal signal (e.g. morphogen concentration, stability or diffusion coefficient) have been shown to explain most of the variation in eyespot size between artificial selection lines of *B. anynana*, while the sensitivity levels of the epidermal cell to a particular morphogen concentration determine eyespot colour composition (Monteiro, Brakefield & French 1997; Allen *et al.* 2008) and, to a lesser extent, eyespot size (Monteiro, Brakefield & French 1994; Beldade, French & Brakefield 2008). Genetic correlations between eyespot size and colour composition are typically low (Monteiro, Brakefield & French 1997; Beldade & Brakefield 2003), suggesting that different sets of genes determine these two aspects of eyespot morphology, and their underlying components of pattern induction.

Damage applied to the developing wing in early pupa typically induces the formation of ectopic patterns (Brakefield & French 1995), and can be used to probe variation in epidermal response sensitivities (see Allen *et al.* 2008). Damage-induced ectopic eyespots were produced in more than 60% of cauterized WT and Spr pupae (32 of 50 for WT, and 56 of 70 for Spr), and typically resembled the native eyespots on the same wing surface (Fig. 5a). Damage-induced eyespots consisted of a black inner disc and an outer golden ring in WT, but were almost entirely made up of gold scales in Spr. These results show that the ability of the entire wing epidermis to respond to eyespot-inducing signals, such as damage, is altered in Spr mutants. We also show that the expression patterns of ‘response’ eyespot ring genes were altered in Spr wings: *en* was detected in almost all cells of the eyespot field (Fig. 5b), while the numbers of

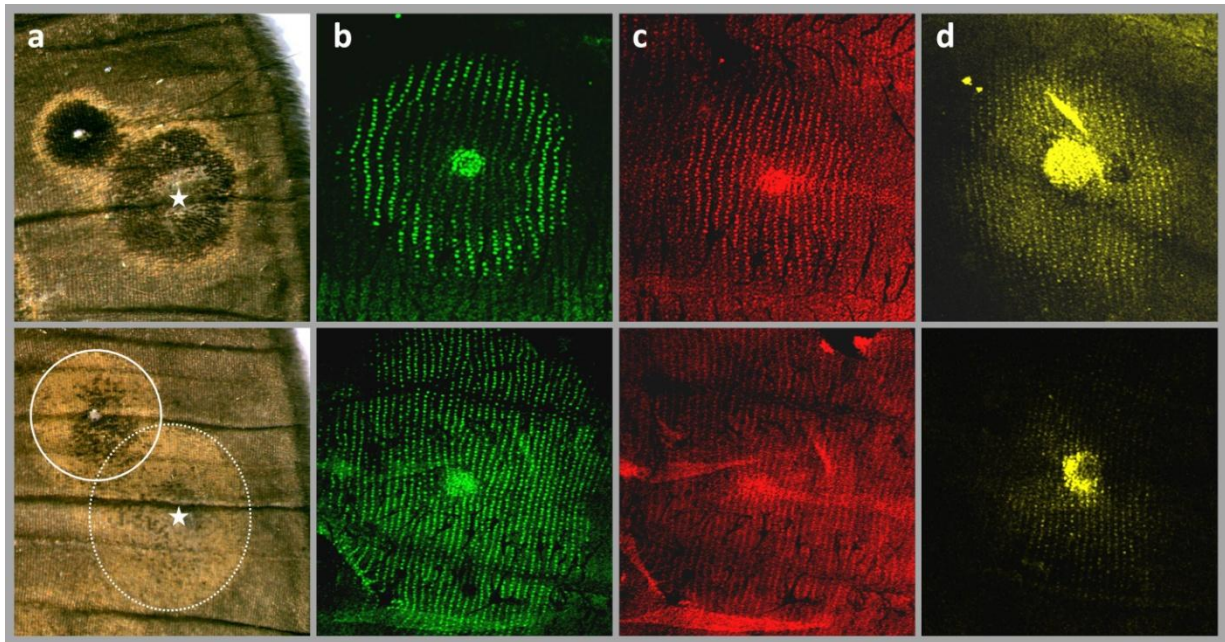


Figure 5. Ectopic eyespots and expression of eyespot patterning genes.

Eyespots of WT (top) and Spr individuals (bottom) differ in colour scheme in adults, and in gene expression patterns in developing pupal wings. **a.** Anterior distal part of dorsal adult forewings showing native and damage-induced eyespots formed around wound sites (stars). The colour composition of the ectopic eyespots resembles that of the native eyespots and reveals differences in the response properties of wing epidermal tissue between WT and Spr butterflies (solid and dashed rings outline the Spr native and the ectopic eyespots, respectively). These differences are reflected in the expression patterns of **b. en**, **c. Dll** and **d. sal** in 16- to 18-hr old pupal wings. Spr mutants were chosen for these experiments because they exhibit the most severe, and thus most noticeable, effects on eyespot phenotype, including damage-induced ectopic eyespots.

scale-building cells expressing *Dll* and *sal* were strongly reduced (Fig. 5c,d). Taken together, our results are consistent with an effect of the target gene downstream of the eyespot-inducing focal signalling, but upstream of the response patterning genes.

The Wnt/Wg signalling pathway and butterfly eyespot formation

The similarities between the *B. anynana* mutant embryonic phenotype and fruit fly *zeste-white 3*, *naked cuticle*, *axin* and *APC2* mutants or *Bili* knockdown embryos suggest that our butterfly *BFS* locus might be one of these segment polarity genes, and therefore implicate Wnt/Wg signalling in eyespot formation. This highly conserved and extensively studied pathway plays a crucial role in embryonic development and is important for numerous physiological processes during adult animal life; abnormalities in Wnt signalling cause various human diseases, including cancer (reviewed in Logan & Nusse 2004; van Amerongen & Nusse 2009; McDonald, Tamai & He 2009).

The canonical Wg signalling occurs when the secreted Wg ligand binds to its membrane receptors Frizzled and LRP/Arrow on the signal-receiving cells

(Bhanot *et al.* 1996; Wehrli *et al.* 2000), causing activation of Dishevelled and ultimately resulting in stabilization of Armadillo (Arm, insect β -catenin homologue). In the absence of Wg, a protein complex consisting of Axin, Zeste-white 3 and APC phosphorylates Arm and thereby promotes its degradation (Aberle *et al.* 1997; Kimelman & Xu 2006). When Wg signal is received, Dishevelled inhibits this so-called "Arm/ β -catenin destruction complex" by recruiting Axin to the cell membrane, ultimately leading to the release of stabilized Arm (Cliffe *et al.* 2003; Tamai *et al.* 2004). The latter then enters the nucleus and promotes tissue-specific expression of target genes (*e.g.* Thompson 2004; Mosimann, Hausmann & Basler 2009). The so-called 'inducible inhibitors' of Wg pathway, Naked cuticle and Bili, regulate Wg activity by determining how efficiently Wg signal stabilizes Arm. Naked cuticle does so by binding to Dishevelled (Rousset *et al.* 2001), while Bili regulates the recruitment of Axin to the membrane (Kategaya *et al.* 2009). It is not surprising that, when Naked cuticle, Bili or one of the "destruction complex" proteins does not function properly, Arm will not be sufficiently inactivated and will regulate the transcription of its target genes, including *en* (Siegfried, Chou & Perrimon 1992), even in the absence of Wg signal. In fly embryos lacking one of these proteins, nuclear Arm levels are upregulated, and its target gene *en* is expressed ectopically in the anterior cells of each segment (Fig. 3a). This, in turn and via Hedgehog signalling (Forbes *et al.* 1993; DiNardo *et al.* 1994), triggers ectopic expression of *wg* in the adjacent cells, resulting in double *wg* stripes in each segment of an embryo.

Earlier studies of spatial expression patterns in *B. anynana* suggested Wg as an eyespot-inducing morphogen (Monteiro *et al.* 2006), but functional tests are still missing. Our analysis of pleiotropic mutations affecting eyespot pattern and embryonic segment polarity supports the idea that Wg signalling is involved in eyespot development. However, the Wg pathway appears to be regulated at multiple levels (van Amerongen & Nusse 2009; McDonald, Tamai & He 2009), and mutations in genes other than those discussed above might produce a similar embryonic phenotype. For example, recent RNAi screens in *Drosophila* identified over 200 potential negative regulators of Wg signalling, including genes not previously linked to this pathway, genes with no previously assigned functions (DasGupta *et al.* 2005), and microRNAs (Silver *et al.* 2007; Kennell *et al.* 2008). Moreover, a new component of the " β -catenin destruction complex", WTX, has been recently identified in mammals (Major *et al.* 2007), but no homologue has yet been found in insects. This raises the possibility that novel players of this conserved pathway may evolve in a lineage-specific way. The evolution of such lineage-restricted genes could play a part in the origin of morphological novelties (Khalturin *et al.* 2009). The *BFS* locus might represent

one such gene. Further analyses will focus on genetic mapping of this locus via the candidate gene approach and a more unbiased genome-wide search (see Chapter 4).

The results of this study suggest that the *BFS* locus acts upstream of *En*, *Dll* and *Sal* transcription factors during the ‘signalling/response’ stage of eyespot development. Since *Wg* is known to upregulate *en* and *Dll* transcription in insect embryos and imaginal wing discs, respectively (Siegfried, Chou & Perrimon 1992; Neumann & Cohen 1997), and is expressed in eyespot foci in pupal wings of *B. anynana* butterflies (Monteiro *et al.* 2006 and Chapter 5 of this thesis), it may act as eyespot focal signal and induce circular domains of *Dll* and *en* expression that correspond to black and gold eyespot rings. In this case, epidermal cells that are close to the focus would receive high amounts of the *Wg* signal and express *Dll*, while in cells that are relatively further away *en* would be upregulated instead. Hence, mutations in a negative regulator of *Wg* pathway are likely to interfere with this process by altering the levels of nuclear Arm in the epidermal cells and thus changing their sensitivities to focal signal. As a result, the regulation of *en* and *Dll* expression will be modified as well, leading to alterations in the distribution of black and gold scales. The striking feature of the *BFS* locus is the diversity of phenotypic effects of different alleles, which seemingly act by altering the sensitivity levels of the epidermal cells to the focal signal, and yet produce changes in different features of eyespot morphology.

CONCLUDING REMARKS

We described three pleiotropic mutations in *B. anynana* butterflies which affect the morphology of eyespots, a lepidopteran novelty, and embryogenesis, a relatively conserved developmental process. These mutations disturb eyespot size and/or colour composition in the heterozygotes and embryonic segment polarity in the homozygotes. Complementation tests revealed that all three mutations are alleles of the same locus, and that one of these alleles has a minor deleterious effect on embryogenesis, while increasing eyespot size in the homozygotes. Non-lethal alleles at this locus might thus exist and contribute to naturally occurring variation in eyespot morphology within and across species. Analysis of gene expression in the developing pupal wings of the *Spr* mutant suggested that this locus acts upstream of *En*, *Dll* and *Sal* during the ‘response to focal signal’ stage of eyespot development. Furthermore, comparison of the defects in mutant embryo morphology and in *en/wg* expression patterns with *D. melanogaster* mutants suggested that this locus encodes a negative regulator of the Wnt/*Wg* signalling pathway.

Although studies of lab mutants represent a valuable approach for dissecting developmental mechanisms of morphological traits, they do not necessarily provide a true picture of evolutionarily relevant genetic variation (Stern 2000). The extent to which mutations of large effect identified in the laboratory are relevant for intra- and interspecific variation in natural populations remains an important issue (Haag & True 2001). In this study, comparative analysis of three *B. anynana* mutations implicated the Wg pathway and suggested a number of candidate genes for the ‘eyespot size/colour’ locus. It remains to be explored whether variation at the *BFS* locus contributes to naturally occurring variation in eyespot morphology. Identification of this locus and analysis of its contribution to eyespot variation will shed light on its role in the evolution of butterfly eyespots. This may be revealed by fine-scale mapping of lines artificially selected for either changed eyespot size or colour composition which have yielded novel eyespot phenotypes not associated with dramatic loss of viability (Beldade, Brakefield & Long 2002; Allen *et al.* 2008).

Another important issue in evolutionary developmental biology is the extent to which variation in conserved developmental pleiotropic genes can contribute to phenotypic variation in morphological traits. Recent studies suggest that evolutionarily relevant mutations tend to accumulate in a particular set of genes, often in *cis*-regulatory gene regions (Stern & Orgogozo 2009). The wing patterns of some butterflies seem to be controlled by such ‘hotspot’ genes, *e.g.* in the swallowtail *Papilio dardanus* and in different *Heliconius* species a limited number of loci appear to produce all the observed diversity (Papa, Martin & Reed 2008). Interestingly, the H-locus of *P. dardanus* has been linked to the genomic region that contains conserved transcription factor-encoding genes *en* and *invected* (Clark *et al.* 2008), while polymorphisms in the developmental gene *Dll* have been associated with variation in eyespot size in the laboratory populations of *B. anynana* (Beldade, Brakefield & Long 2002). Genetic mapping of the *BFS* locus, associated to the Wg pathway in this study, is likely to reveal whether this ‘eyespot size/colour’ gene encodes a conserved, or a butterfly-specific protein.

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