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

Saenko, S.V.

CHAPTER 6. Butterfly wing pattern evolution: insights from comparative analysis of the eyespot developmental network

Suzanne V. Saenko, Paul M. Brakefield and Patrícia Beldade

The origins and diversification of evolutionary novelties is an important issue, and butterflies have already provided some insights into the mechanisms underlying formation of, and variation in, novel traits. Here we explore the developmental genetic regulation of butterfly (eye)spots, wing pattern elements that show remarkable diversity in size, colour, shape, and position on the wing. Studies in a few model species implicated the network of conserved embryonic and wing patterning genes in eyespot formation. We previously demonstrated that differences in some components of these network exist among *Bicyclus anynana* and *Junonia coenia*, and suggested that comparative analysis of gene expression in a broad range of species is required for a more complete understanding of wing pattern evolution. Here, we used antibodies against Antennapedia, Notch, Distal-less, and Spalt to investigate their role in development and diversification of colour patterns in Nymphalidae and Papilionidae. We showed that the more basal Danainae and Papilionidae do not express same eyespot genes as higher Nymphalidae. We also found that the genetic mechanisms underlying eyespot patterning even in relatively closely related butterflies seem to differ. For example, Antennapedia was detected in eyespot centres in only one of the two subfamilies of Nymphalidae. Our results demonstrate differential co-option of conserved developmental genes during the evolution of butterfly wing patterns and suggest that the mechanisms of eyespot development are flexible, and open to tinkering at the level of the loss and gain of regulating genes.

INTRODUCTION

Butterfly wing patterns are beautiful examples of a lineage-specific adaptive novelty (*e.g.* Stevens 2005). They are made up from a mosaic of partially overlapping pigmented scales, which themselves are a key innovation that presumably evolved through modification of insect sensory bristles (Galant *et al*. 1998). One of the best studied wing pattern elements in terms of the underlying genetic and developmental mechanisms is the eyespot. The evolutionary origin of eyespots is poorly understood, but it has been proposed that they belong to one of the three so-called 'symmetry systems', *i.e.* pigmented bands that run parallel to each other along wing margin in anterio-posterior direction (Nijhout 2001). These symmetry systems have presumably evolved in several steps from simple, undifferentiated spots present in the ancestor of butterflies and moths (Nijhout 1994). Compartmentalization of the wing surface by veins presumably resulted in uncoupling of eyespot development within each wing cell (an area bounded by veins) and facilitated eyespot diversification, resulting in their extraordinary diversity (Nijhout 1994). Eyespots are found in a range of butterfly families and vary in size, shape, colour composition and position on the wing across and within species, and even across different wing surfaces of the same individual (Nijhout 1991).

The genetic and developmental bases of eyespot formation have been analyzed in detail in only two nymphalid butterflies, *Bicyclus anynana* and *Junonia coenia* (reviewed in Beldade & Brakefield 2002; McMillan *et al*. 2002). Classical surgical manipulations of pupal wings showed that each eyespot is formed around a group of cells called a focus. Transplanting a focus into an eyespot-less position on the wing typically results in the formation of an ectopic eyespot at the host site, whereas damaging foci during the sensitive early pupal period reduces or completely eliminates the corresponding eyespots in adult wings (Nijhout 1980; French & Brakefield 1992, 1995). The cells of the focus presumably produce one or several signaling molecules that diffuse through the epidermis, and the neighbouring cells respond to these morphogens in a concentration-dependent manner by activating different pigment biosynthesis pathways and subsequently producing wing scales of different colours. Studies of expression patterns of key developmental genes in larval and pupal wing primordia suggested that eyespots have evolved through co-option of conserved genes and pathways involved in insect embryonic and wing patterning (reviewed in Beldade & Saenko 2009). These studies demonstrated that eyespot development begins at the end of the larval stage, when genes *Notch* (*N*), *Distal-less* (*Dll*), *spalt* (*sal*) and *engrailed* (*en*) become upregulated in positions of the wing epidermis corresponding to adult eyespot foci (Carroll *et al*. 1994; Brakefield *et al*. 1996; Reed & Serfas 2004; Reed, Chen & Nijhout 2007).

Signaling from these foci occurs during the early pupal stage and probably involves both Wingless and Decapentaplegic as morphogens (Monteiro *et al*. 2006; Chapter 5 of this thesis). Subsequently, *Dll*, *sal* and *en* become expressed in concentric domains that correspond to adult eyespot rings (Brunetti *et al*. 2001), possibly as a direct response to these signals.

Remarkably, the genes encoding the Hh ligand and its receptor Patched have been associated with eyespot focus determination in *J. coenia* (Keys *et al*. 1999), but not in *B. anynana* (Chapter 5 of this thesis). This suggests that the genetic mechanisms underlying nymphalid eyespot formation might have diversified substantially, whereas the general appearance of these patterns has remained largely unchanged. Alternatively, eyespots with similar morphology could have evolved independently, but happened to utilize in part the same group of genes for their formation. For instance, development of concentric colour rings in moths of the family Saturniidae appears to share at least two nymphalid eyespot proteins, Dll and En (Monteiro *et al*. 2006). A more complete understanding of eyespot evolution requires examination of gene expression patterns in a broad range of taxa, within and outside the nymphalid clade.

Here, we focused on genes implicated in eyespot center determination in the laboratory model *B. anynana*, and examined their expression in a variety of species that belong to two butterfly families, Nymphalidae and Papilionidae (Wahlberg *et al*. 2009). All species examined bear spots or eyespot-like patterns (Fig. 1), which differ considerably in colour, size, number, and position on the wing. We aimed to investigate which components of the 'eyespot gene network' as identified previously in *B. anynana* are associated with (eye)spot patterns in other butterflies during the earliest stage of eyespot formation: the determination of the focus. Several transcription factors and the Notch and Hedgehog signaling pathways have been implicated in this process in nymphalids, although functional studies have yet to be made. Here, we compared expression patterns of *Antp*, *N*, *Dll* and *sal* to determine some of the conserved and divergent components of the gene network involved in focal establishment. In addition, we started to explore the similarities in the cellular interactions underlying focal signaling and the epidermal response to it in several species of nymphalid butterflies by using surgical manipulations such as damage and transplantation of eyespot focal tissue during the early pupal stage.

MATERIAL AND METHODS

Biological material

Larvae were reared in climate rooms at 27^{o}C with 12L:12D cycle or at room temperature indoors and fed on maize (*Bicyclus anynana*), *Oplismenus*

(*Heteropsis iboina*) or *Poa* grasses (*Pararge aegeria* and *Melanargia galathea*), narrowleaf plantain (*Junonia coenia* and *Melithea cinxia*), nettles (*Aglais io*), banana leaves (*Caligo memnon*), milkweed (*Danaus plexippus*), stonecrop (*Parnassius apo*llo) and fennel plants (*Papilio machaon*). Antibody stainings were performed as described in Chapters 2 (embryos) and 5 (wings).

Surgical manipulations

Pupation times were scored by means of time-lapse photography with 15 minute intervals using a digital camera. Grafts on the dorsal surface of left forewings were performed between *B. anynana* and either *H. iboina* (N = 50), *P. aegeria* (N = 8) and *J. coenia* (N = 22) in 3.5 – 4.5 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 donor pupae into a more anterior-distal position on the host pupae. Additionally, eyespot foci on left pupal forewings of *H. iboina* (N = 49) were damaged at 3 - 6 hours after pupation with a fine tungsten needle (World Precision Instruments). Adults were frozen soon after emergence, and their wings were photographed with a Leica DC 200 digital camera attached to a Leica MZ 125 microscope.

RESULTS AND DISCUSSION

Upregulated levels of Antp are associated with eyespot centres in Satyrinae

The species-rich nymphalid subfamily Satyrinae comprises ~2400 described species (Peña *et al*. 2006) and includes such spectacular representatives as owl butterflies and large blue morphos, as well as the lab model for integrated studies of wing pattern evo-devo, *B. anynana* (Brakefield, Beldade & Zwaan 2009). The wings of many satyrines are decorated with eyespots, the morphology and function of which varies considerably across and within species. Hindwings of butterflies of the subclade Satyrini, which includes *B. anynana*, often bear a series of small marginal eyespots, consisting of a white pupil surrounded by black and yellow/orange rings (Fig. 2b) and used to deflect predators' attacks from the vital body parts (reviewed in Stevens 2005). Hindwings of the numerous representatives of the group Brassolini, on the other hand, bear conspicuous large eyespots without a clear pupil in the centre (*e.g. Caligo memnon*, Fig. 2b). These are located more centrally on the wing and presumably serve to startle predators (Stevens 2005). Expression patterns of genes presumably involved in development of these morphologically and functionally diverged colour patterns were studied in larval wing discs of five representatives of the subfamily (summarized in Fig. 2).

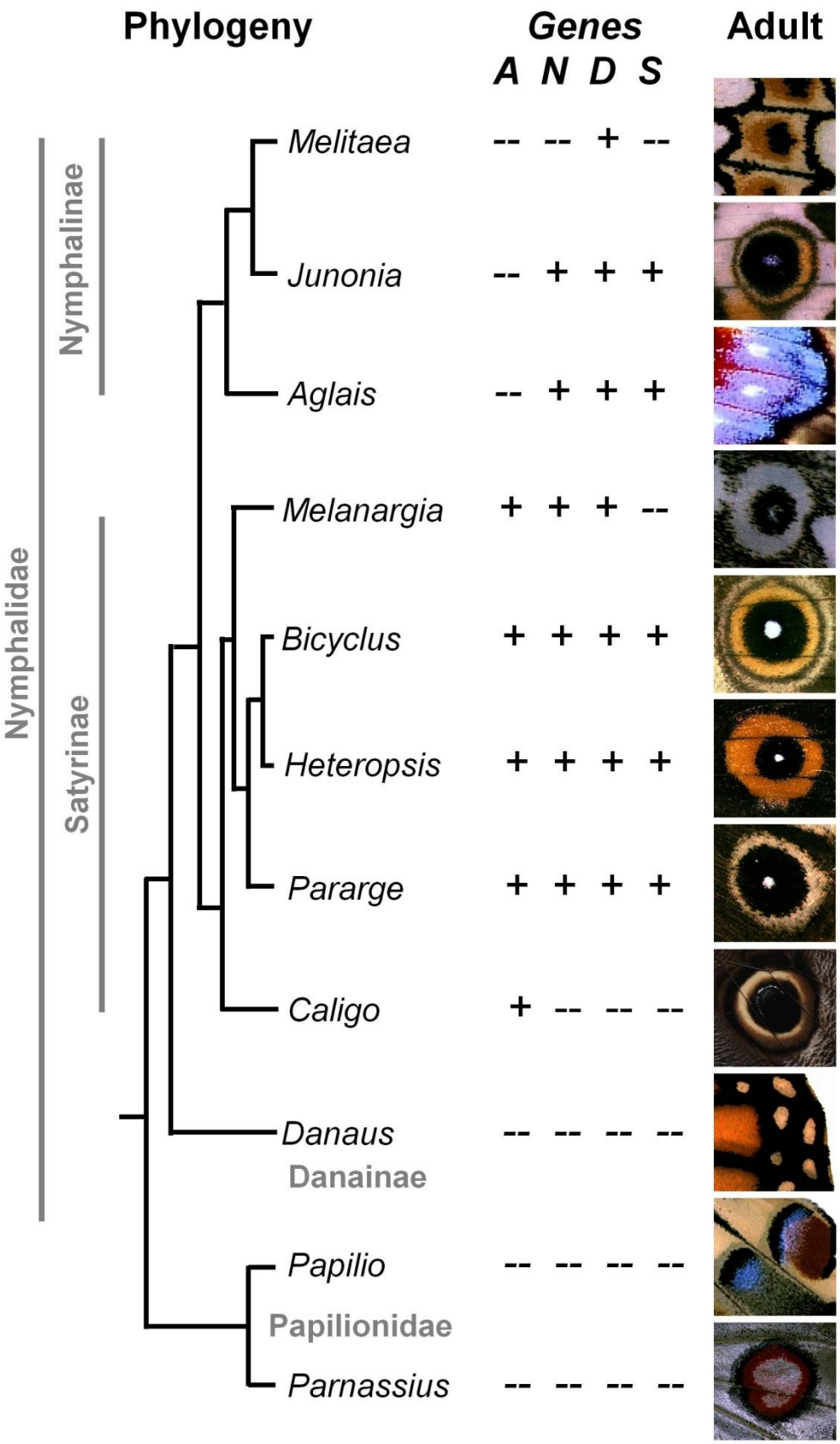

Figure 1. Genes associated with (eye)spot patterns in larval wing discs.
The phylogram is derived from nymphalid phylogeny by Wahlberg *et al*. (2009). Expression patterns of four genes associated with eyespot focus determination in *B. anynana* (A, *Antp*; N, *Notch*; D, *Distal-less*; S, *spalt*) were examined in ten other species. In total, nine members of the family Nymphalidae (three nymphalines, five satyrines and one danaine), as well as two papilionids, were used to establish a relationship between gene expression and (eye)spots.

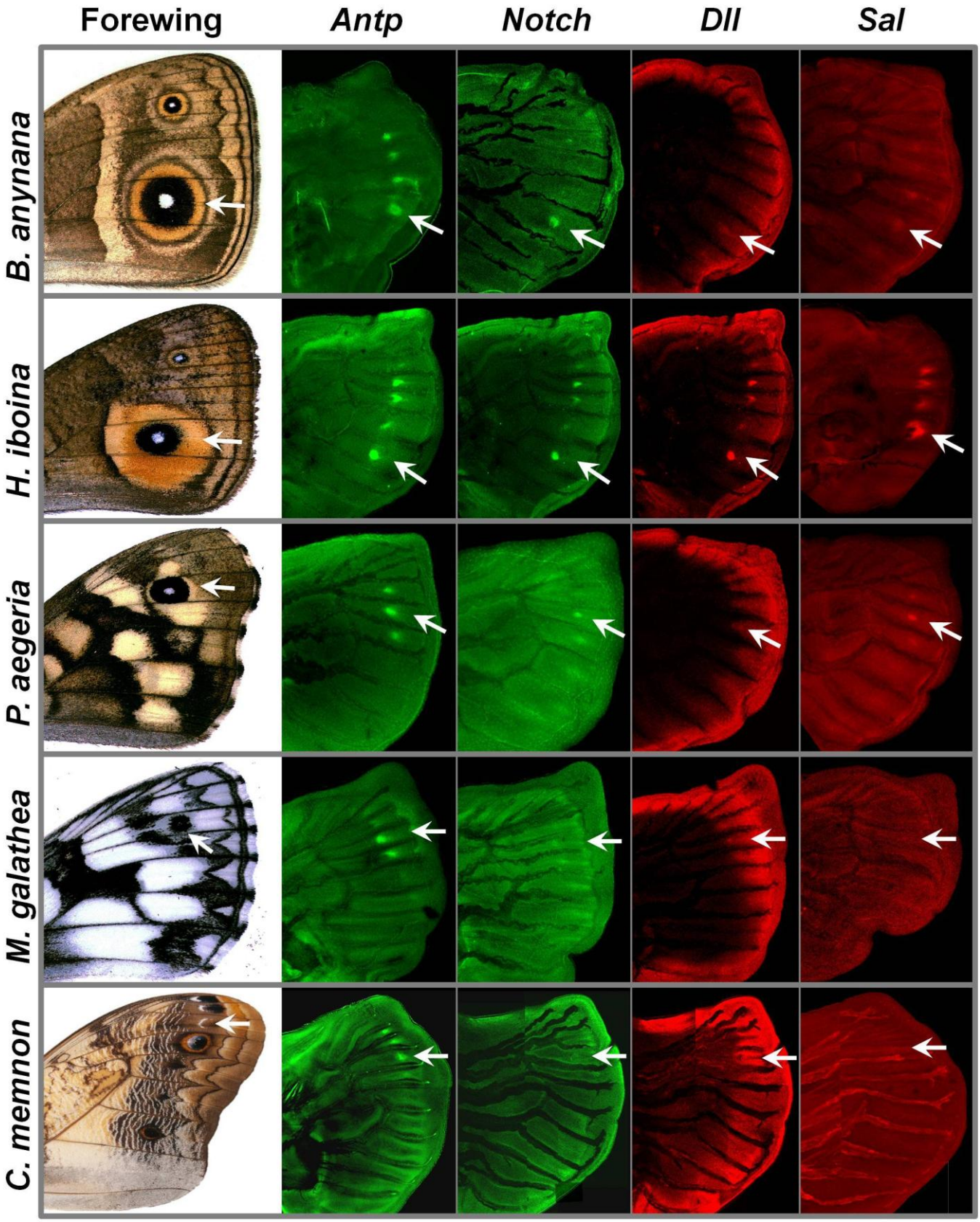

Figure 2a. Expression patterns of *Antp*, *N*, *Dll* and *sal* in the forewings of Satyrinae.
Only *Antp* is expressed in all five species in presumptive eyespot centres; its upregulation precedes activation of *N*, *Dll* or *sal* transcription in these wing areas. No *sal* upregulation is observed in eyespot foci in *M. galathea*, and only Antp is associated with eyespot areas in *C. memnon*. White arrows provide reference between panels for a specific eyespot.

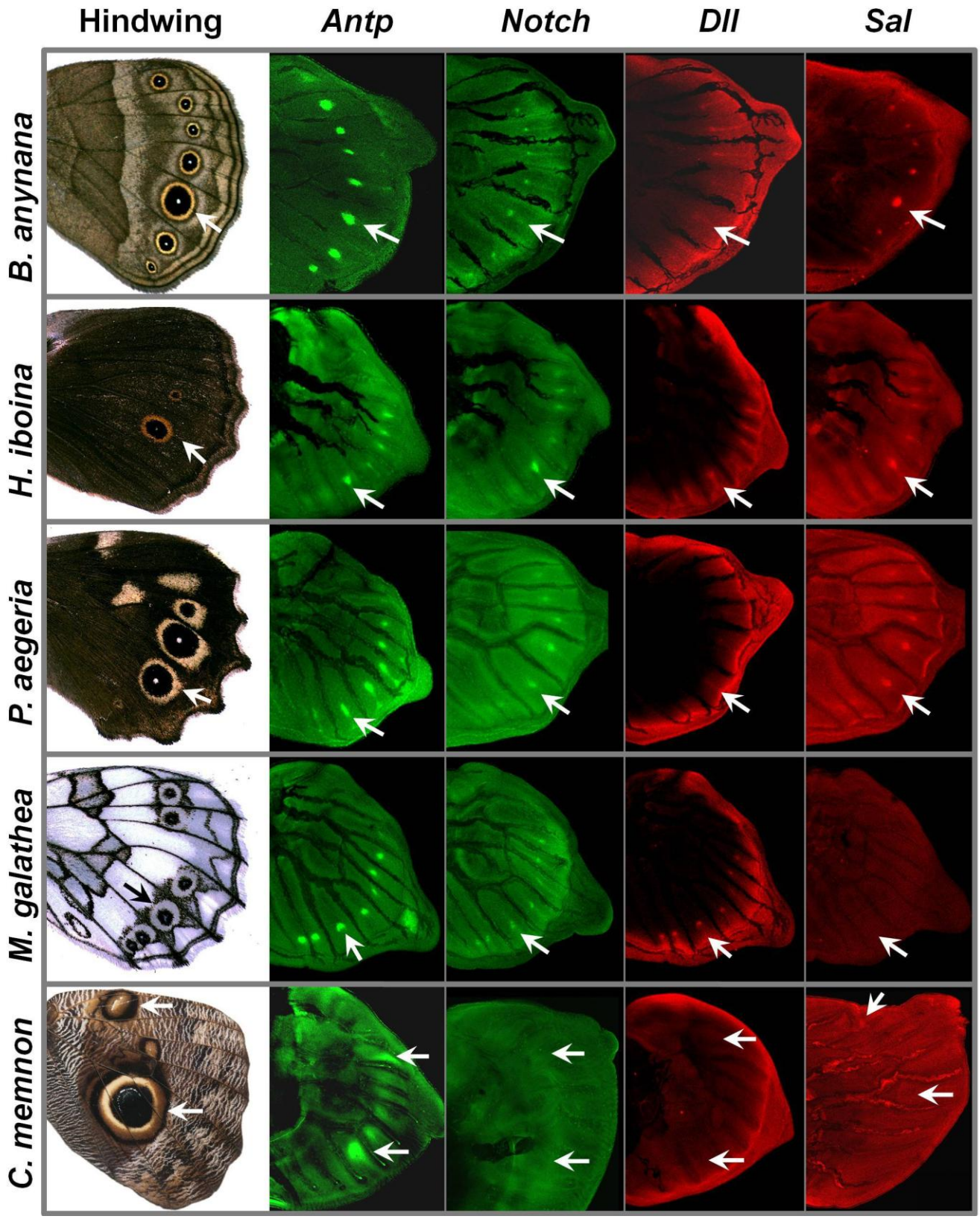

Figure 2b. Expression patterns of *Antp*, *N*, *Dll* and *sal* in the forewings of Satyrinae (see legend to Figure 2a).

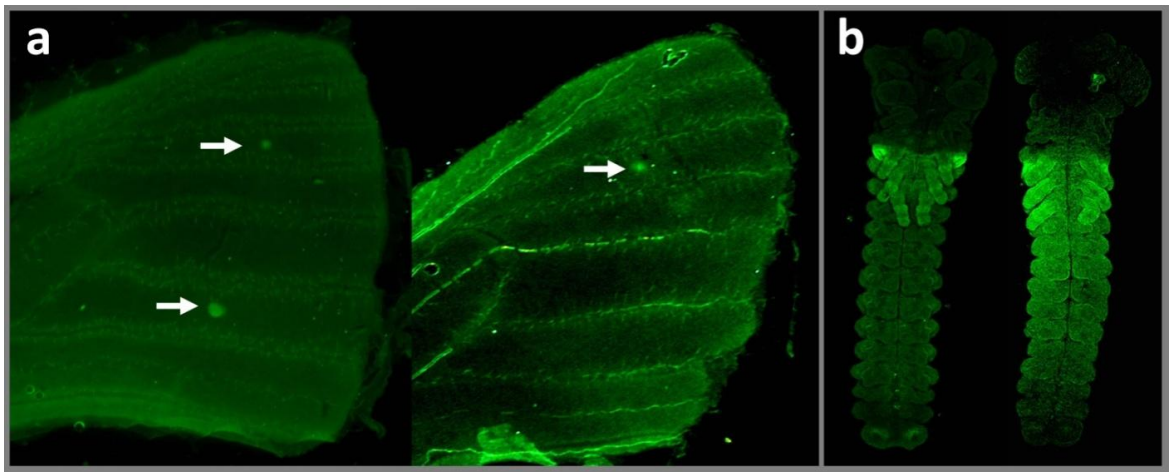

Figure 3. Expression of *Antp* in pupal forewings and embryos.
a. Strong upregulation of *Antp* in eyespot foci (arrows) in the forewings of *H. iboina* (left) and *P. aegeria* (right) 36 – 48 hour old pupae; the number of eyespot foci marked by *Antp* expression corresponds to the number of eyespots on the adult forewings. **b.** Embryos of *B. anynana* and *J. coenia* at 25-30% developmental time show identical patterns of *Antp* expression; the highest levels of protein are detected in the thorax (ventral view, anterior is up).

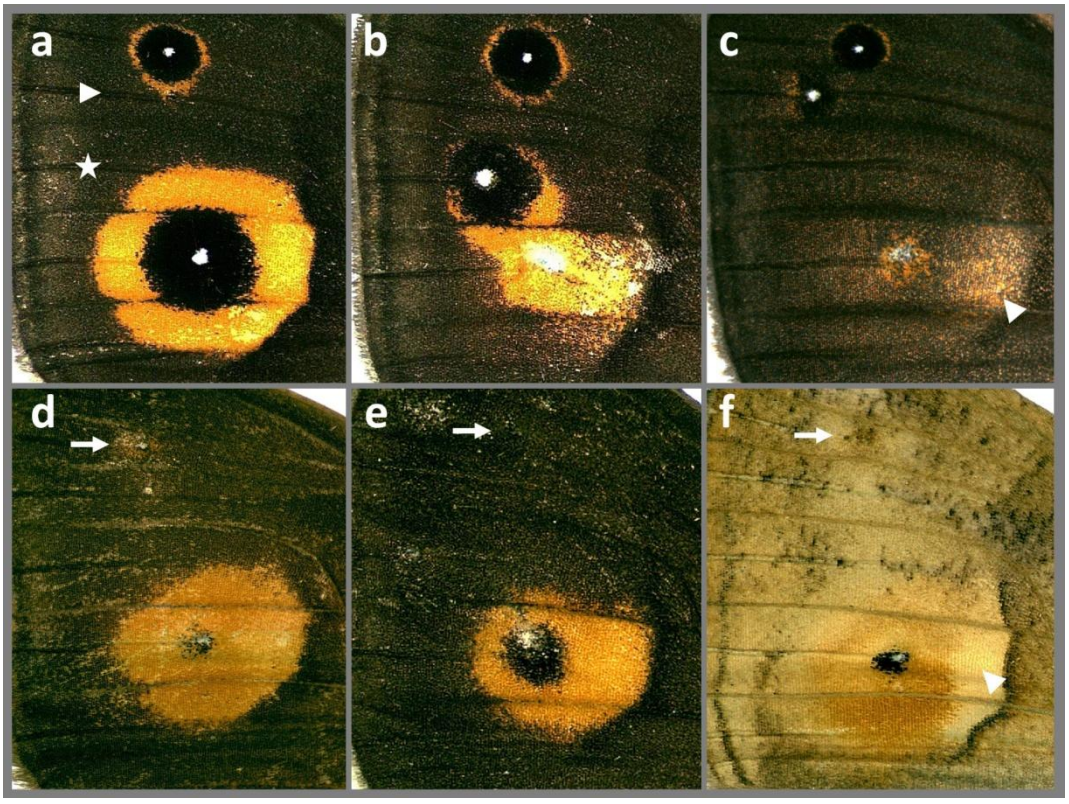

Figure 5. Surgical manipulations on *H. iboina* forewings.
a. Wild-type dorsal forewing with small anterior and large posterior eyespots. **b.** Grafting of *B. anynana* focal tissue into a position indicated with (*) in **a** was able to induce eyespot formation in *H. iboina*; removing posterior eyespot focus did not completely eliminate orange scales. **c.** Interspecific graft in a more anterior position on the wing (arrowhead in **a**) resulted in formation of ectopic eyespot; posterior eyespot disappeared but its proximal part still contains orange scales (arrowhead). **d** and **e.** Damage of eyespot foci at 3 – 6 hours after pupation inhibited anterior eyespot formation (arrows), but posterior eyespots never disappeared completely. **f.** Similar effect was observed on the ventral side of the damaged wings (compare to undamaged ventral forewing in Fig. 2a). In all images anterior is up and distal is to the left.

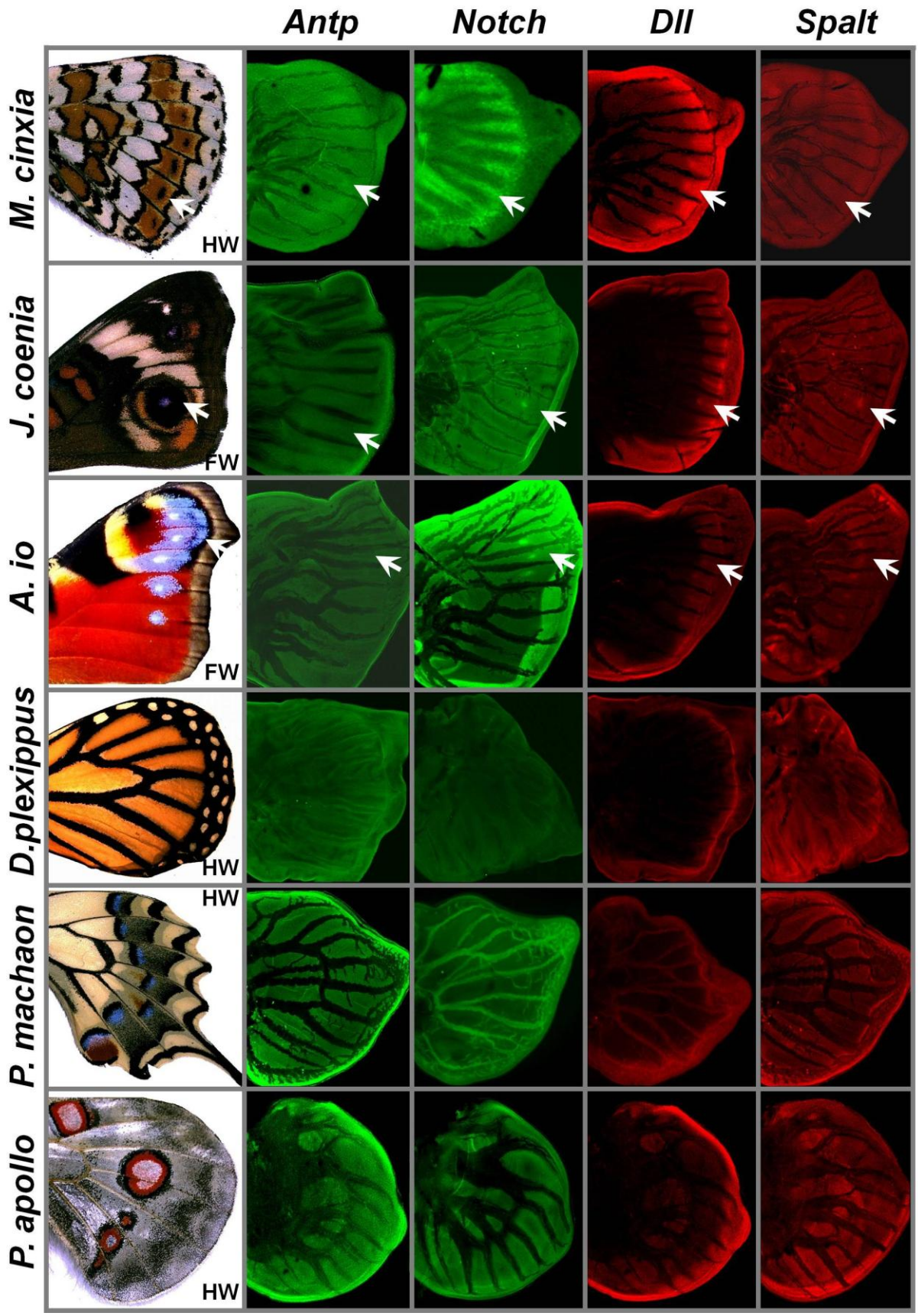

Figure 4. Expression patterns of *Antp*, *N*, *Dll* and *sal* in different species of Nymphalinae, Danainae and Papilionidae.

Only *Dll* is expressed in eyespot foci in all three Nymphalinae examined, and none of the studied genes is associated with (eye)spot patterns in the representatives of the more basal Danainae and Papilionidae. White arrows provide reference between panels for a specific eyespot.

Only *Antp* was expressed in all five satyrines in the areas of larval wings that correspond to future eyespot centres. In *B. anynana* (N = 75), *H. iboina* (N = 7), *P. aegeria* (N = 10) and *M. galathea* (N = 11), its upregulation in eyespot foci preceded activation of *N*, *Dll* or *sal* transcription (Fig. 2). Interestingly, in these species *Antp* was expressed also in some wings cells (*i.e.* regions of wing separated by veins) that do not typically bear eyespots. For example, four forewing cells in *B. anynana* and *H. iboina*, and three forewing cells in *M. galathea* and *P. aegeria* were marked by *Antp*, whereas the adults typically have one or two forewing eyespots, respectively (Fig. 2a). Its expression levels were, however, gradually reduced over time in those wing cells that do not produce eyespots (Fig. 3a). Expression patterns of *N* and *Dll* in *H. iboina*, *P. aegeria* and *M. galathea* were similar to those previously described for *B. anynana* (Reed & Serfas 2004; see also Chapter 5). These two genes were initially upregulated in intervein midlines, and later in eyespot foci, although focal expression of *Dll* in *P. aegeria* was never as strong as in the other three species. Expression of *sal* was observed in eyespot foci in *B. anynana*, *H. iboina* and *P. aegeria* (consistent with previous work in *J. coenia*, see Reed, Chen & Nijhout 2007), but not in *M. galathea*. It is possible that upregulation of *sal* in focal cells occurs at a later stage, or that this gene is not involved in determination of eyespot foci in *Melanargia*. Remarkably, neither *N*, *Dll* or *sal* were upregulated in eyespot areas in *C. memnon* (N = 7), even in the wings discs of late last instar larvae. *Antp*, on the contrary, was detected in all examined wings in those eyespots that contain white crescent-shaped pupils, which presumably corresponds to white foci of other satyrines.

N, Dll and Sal, but not of Antp, are associated with (eye)spots in Nymphalinae

The second large nymphalid subfamily of the Nymphalinae contains several species used as models in studies of dispersal and metapopulation dynamics (*e.g.* Haag *et al*. 2005), interactions with host plants (*e.g.* Weingartner, Wahlberg & Nylin 2006) and predators (*e.g.* Vallin *et al*. 2005), and the developmental basis of wing pattern formation (*e.g.* Nijhout 1980; Carroll *et al*. 1994). The latter has been studied mainly in *J. coenia*, the forewing eyespots of which not only resemble marginal eyespots of satyrines in appearance (*i.e.* are made up of a white focus, a black inner disc and a gold outer ring, Fig. 4), but also express some of the same signaling molecules and transcription factors in the eyespot field during critical steps of pattern formation (Brakefield *et al*. 1996; Brunetti *et al*. 2001; Reed & Serfas 2004). There is considerable variation in the number, position and colour composition of (eye)spots in the tribe Junoniini (Kodandaramaiah 2009), as well as in the whole subfamily Nymphalinae. Some representatives of this taxon have peripheral bands running alongside the wing

margin in an antero-posterior direction and marked with intervening black, undifferentiated spots in each wing cell (*e.g. Melitea cinxia*, Fig. 4), while others have large conspicuous spots formed by scales of bright contrasting colours (*e.g. Aglais io*, Fig. 4).

Analysis of the expression patterns of *Antp*, *N*, *Dll* and *sal* in larval wings of these nymphalines (summarized in Fig. 4) revealed that the development of their (eye)spot patterns share at least one transcription factor, Dll. Upregulated levels of this protein were detected in the areas corresponding to small black spots of *M. cinxia* (N = 12), eyespot foci of *J. coenia* (N = 85), and five white spots on the forewings of *A. io* (N = 56). Moreover, focal cells of *J. coenia* and cells of the future white spots of *A. io* express *N* and *sal*, but none of the examined genes are upregulated in the epidermal cells in those wing areas that bear large conspicuous eyespot-like patterns in *A. io*. Remarkably, eyespots of *J. coenia* and white spots of *A. io* do not appear to be associated with the expression of *Antp*, the Hox gene that is upregulated in the future eyespot centres in all satyrines examined in this study (Fig. 2). This conclusion was reached only after extensive analysis of a large number of *J. coenia* and *A. io* larval wings, covering all stages of the last instar wing development. Immunostainings in the embryos of *B. anynana* and *J. coenia* with the anti-Antp antibody produced similar patterns (Fig. 3b), consistent with those previously described in the Lepidopteran *Bombyx mori* (Nagata *et al*. 1996). This shows that the antibody used here does work in Nymphalinae, and that the absence of Antp in eyespot focal cells is not because the antibody fails to recognize its target.

(Eye)spots of Papilionidae and Danainae are not associated with any of the studied genes

Many representatives of the basal nymphalid family Danainae, which includes such well-known species as the monarch butterfly (*Danaus plexippus*, Fig. 4), bear patches of single colour along the wing margin that could represent early morphological stages of eyespot evolution (Nijhout 1991, Brakefield *et al*. 1996). For instance, nymphalid eyespots may have evolved from such primitive spots, which might have been expressing some of the 'eyespot' genes and later acquired signaling activity through co-option of Wg and Dpp signaling pathways (Monteiro 2008). Alternatively, eyespots might have evolved as early as in the common ancestor of all 'true' butterflies (superfamily Papilionoidea), since series of marginal spots or eyespots are found also in the representatives of the basal butterfly family Papilionidae. Members of this family often have red, blue or black spots on their wings, and in some species, for example *Parnassius apollo* (Fig. 4), they include a white center surrounded by red and black rings, and thus resemble nymphalid eyespots. Immunostainings of larval wings in *D. plexippus*

(N = 5), *P. apollo* (N = 15) and *Papilio machaon* (N = 15) revealed, however, that neither *Antp*, *N*, *Dll* nor *sal* were expressed in the wing epidermis in distinct patterns that could be associated with adult (eye)spot patterns (Fig. 4). These patterns are likely to be produced by some other, as yet unknown, genetic mechanisms.

Surgical manipulations suggest conservation of signal/response components of eyespot formation

Comparative studies of gene expression patterns in nymphalid butterflies have demonstrated that (partially) similar genetic machinery is involved in establishing eyespot organizing centres in larval wings (*e.g.* Reed & Serfas 2004, and this thesis) and in defining the cellular domains that will later produce different colour pigments in pupal wings (*e.g.* Brunetti *et al*. 2001). We examined whether the cellular interactions underlying the signal/response components of eyespot formation in this group of butterflies were similar as well. Reciprocal grafts (transplantations) of the signaling focus of the large dorsal forewing eyespot were performed between young pupae of *B. anynana* and either *H. iboina*, *P. aegeria* or *J. coenia*. Grafts into *P. aegeria* and *J. coenia* did not heal properly, perhaps due to immune system incompatibility, but the focal transplantations between the more closely related *B. anynana* and *H. iboina* (Peña *et al*. 2006) were successful and resulted in the production of well-defined ectopic eyespots in 7 out of 49 individuals (Fig. 5b,c). This indicates that either the focal cells of these two satyrines produce the same signaling molecule(s), or that their wing epithelia are competent to recognize both types of signal and respond to them in a threshold-dependent manner.

Unexpectedly, removing of the large posterior forewing eyespot focus which typically leads to a complete elimination of this eyespot in *B. anynana* (*e.g.* French & Brakefield 1995) did not have such an effect in *H. iboina*. The effects observed in all operated individuals varied from decrease of only orange, or orange and black rings, to loss of the black ring and the preservation of the outer orange ring (Fig. 5b) or a patch of orange scales proximally from the eyespot centre (arrowhead in Fig. 5c). In a similar way, focal ablations in 3 – 6 hour old *H. iboina* pupae resulted in patterns different from those reported previously for other species. Typically, damage of the forewing eyespot centres in young pupae also strongly reduces, or even completely eliminates eyespots in *B. anynana* (French & Brakefield 1992) and *J. coenia* (Nijhout 1980), indicating that these foci are crucial for the development of colour rings. In *H. iboina*, damage to the foci of the small anterior eyespots produced similar effects (white arrows in Fig. 5), but cauteries of the large posterior eyespot foci resulted in smaller eyespots (Fig. 5e), sometimes lacking black scales (Fig. 5d), but never in complete loss of eyespots. Similar effects were observed on the ventral side of

the wings, where patches of yellow/orange scales remained at the proximal side of large posterior eyespots after all focal ablation (arrowhead in Fig. 5f). These observations suggest that additional mechanisms might exist in *H. iboina* pupal wings that instruct cells to produce yellow/orange pigment in this part of the forewing. For instance, the cells in the midline might produce a signal that induces formation of the yellow patch just proximal to the posterior eyespot focus (arrowheads in Fig. 5c,f). This patch underlies the 'normal' eyespot and is probably not, or only to a lesser extent, affected by the damage applied to eyespot focus. In fact, many satyrine butterflies that do not have posterior forewing eyespots bear patches of light colour in these positions instead, *e.g. P. aegeria* and *M. galathea* (Fig. 2a). These patches might be produced by the same type of mechanism as indicated above for *H. iboina*.

The origin, conservation and modification of eyespot patterning mechanisms

Previous research showed that two transcription factors, Dll and En, are present in eyespot centres of nymphalid butterflies and saturniid moths, and raised the possibility that concentric rings of colour that utilize En/Dll circuit may have evolved as early as in the common ancestor of these two lineages (Monteiro *et al*. 2006; Monteiro 2008). Our study of gene expression patterns in the basal butterfly family Papilionidae and in the basal nymphalid *D. plexippus* shows, however, that their (eye)spots do not share similar genes with nymphalid eyespots. It is likely that eyespots of higher Nymphalidae evolved by co-option of multiple signaling pathways and transcription factors, and that some of these were used independently to form eyespots in saturniid moths. This illustrates that conserved developmental genes can be co-opted in the evolution of various novel traits, in this case the lepidopteran wing patterns.

Here we showed that different genes underlie eyespot patterning even in more closely related butterflies, such as the representatives of the subfamilies Nymphalinae and Satyrinae (both belonging to the family Nymphalidae). While eyespot focus determination is associated with the expression of *N* and *Dll* in both lineages, the Hh signaling pathway has been implicated in eyespot formation only in Nymphalinae (see Chapter 5), and the Hox gene *Antp* appears to be expressed in eyespot foci only in Satyrinae. Eyespots of nymphalins and satyrines either arose independently but by using at least some of the same developmental genes, or could have originated in a common ancestor but diversified substantially in the underlying genetic machinery. Differences in the expression patterns of 'eyespot' genes were found even within Satyrinae (*e.g.* Sal protein was not detected in eyespot foci of *M. galathea* in this study), suggesting that the mechanisms of eyespot development are flexible, and open to tinkering at the level of the loss and gain of regulating genes. Experimental analysis of gene function is necessary to confirm that the genes expressed in eyespot foci are

actually involved in eyespot formation, but is unfortunately not yet easily accessible in butterflies (see Monteiro & Prudic 2010).

Butterfly eyespot patterns show spectacular variation in number and position on the wing. Here we showed that expression of *Antp* correlates with eyespot number in Satyrinae. We hypothesize that high levels of *Antp* can mark those positions on larval wings that have the potential to produce eyespots. For example, additional eyespots on the forewing are typical for *B. anynana* individuals bearing the *Spotty* mutation (Brakefield & French 1993), and are occasionally detected not only in *B. anynana*, but also in a closely related *B. safitza* (Brakefield *et al*. 1996) and in *H. iboina* butterflies (*pers. obs.*). Mutation such as *Spotty* might have occurred in a locus that directly or indirectly regulates expression levels of *Antp* in forewing eyespot foci, acting similarly to the *Missing* locus, which presumably regulates accumulation of N and Dll in eyespot foci on the hindwing (Reed & Serfas 2004). It will be important to unravel the identity of such 'key regulators' of the eyespot developmental program, as they can tell us a lot about the modular regulation and the evolution of serially homologous eyespot patterns (Monteiro *et al*. 2007; Monteiro 2008).

Eyespot size is another aspect of butterfly wing patterning that shows substantial variation within and between species (Monteiro, Brakefield & French 1994; Beldade, Koops & Brakefield 2002; Brakefield & Roskam 2006), and depends on focal signal strength and epidermal cell sensitivity (Beldade, French & Brakefield 2008). The results of surgical manipulations in *H. iboina*, a satyrine butterfly with very large and conspicuous posterior forewing eyespots, suggested that additional mechanisms might regulate formation of the orange eyespot ring in this species. It will be an exciting finding if future work can confirm that this novel pattern has evolved in a lineage of Madagascan species of *Heteropsis* by combining two different patterning mechanisms. Moreover, large posterior eyespots of this species probably represent an ecological novelty since these conspicuous marking are combined with a ritualized 'flashing' display of opening and closing the wings on disturbance, characteristic of eyespots that function in startling predators away rather than in deflecting, or misdirecting predator attacks (see Stevens 2005). The flexibility in the mechanisms of the formation of eyespot-like markings is also indicated by our finding that fore- and hindwing eyespots of *C. memnon* share at least one focal marker protein with all other examined Satyrinae. While forewing eyespots are located along the wing margin in this species, those on the hindwing are shifted towards the wing basis (Fig. 2). The fact that Antp is associated with all these patterns suggests that butterfly eyespots are highly flexible in terms of size, colour scheme and position on the wing.

CONCLUDING REMARKS

We investigated which components of eyespot developmental network as identified in the lab model *B. anynana* were associated with (eye)spot patterns in ten other species that belong to butterfly families Nymphalidae and Papilionidae. Wings of all these butterflies bear spots or eyespot-like patterns that differ in colour, size, number, or position. We searched for correlations between these patterns and the expression of four 'eyespot' genes in larval wings, during the early stages of pattern formation. First, we found that (eye)spots in the more basal butterfly lineages (*i.e.* Danainae and Papilionidae) do not share similar genetic mechanisms with nymphalid eyespots. It is therefore likely that these pattern elements and the eyespots of higher Nymphalidae (*i.e.* subfamilies Satyrinae and Nymphalinae) evolved independently, by co-option of different genes. It remains to be investigated which mechanisms are responsible for colour pattern formation in danaids and papilionids. We also found that the genetic mechanisms underlying eyespot patterning, even in relatively closely related butterflies, seem to differ considerably. Upregulation of the Hox gene *Antp*, for instance, was associated with eyespot centres in all Satyrinae, but in none of the Nymphalinae examined here. Eyespots could have arisen independently in these subfamilies by using at least some of the same genes, or could have originated in a common ancestor and diversified substantially in the underlying genetic machinery. Variation in expression patterns was detected even within subfamilies, *e.g.* not all genes upregulated in eyespot foci in *B. anynana* were found in other satyrines. These observations suggest that the mechanisms of eyespot development are highly flexible, and that different components of eyespot patterning network can operate in different lineages, resulting in the spectacular diversity of these patterns ultimately shaped by by the action of natural and/or sexual selection.

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