The generation and diversification of butterfly eyespot color patterns

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Background: A fundamental challenge of evolutionary and developmental biology is understanding how new characters arise and change. The recently derived eyespots on butterfly wings vary extensively in number and pattern between species and play important roles in predator avoidance. Eyespots form through the activity of inductive organizers (foci) at the center of developing eyespot fields. Foci are the proposed source of a morphogen, the levels of which determine the color of surrounding wing scale cells. However, it is unknown how reception of the focal signal translates into rings of different-colored scales, nor how different color schemes arise in different species.

Results: We have identified several transcription factors, including butterfly homologs of the *Drosophila* Engrailed/Invected and Spalt proteins, that are deployed in concentric territories corresponding to the future rings of pigmented scales that compose the adult eyespot. We have isolated a new *Bicyclus anynana* wing pattern mutant, *Goldeneye*, in which the scales of one inner color ring become the color of a different ring. These changes correlate with shifts in transcription factor expression, suggesting that *Goldeneye* affects an early regulatory step in eyespot color patterning. In different butterfly species, the same transcription factors are expressed in eyespot fields, but in different relative spatial domains that correlate with divergent eyespot color schemes.

Conclusions: Our results suggest that signaling from the focus induces nested rings of regulatory gene expression that subsequently control the final color pattern. Furthermore, the remarkably plastic regulatory interactions downstream of focal signaling have facilitated the evolution of eyespot diversity.

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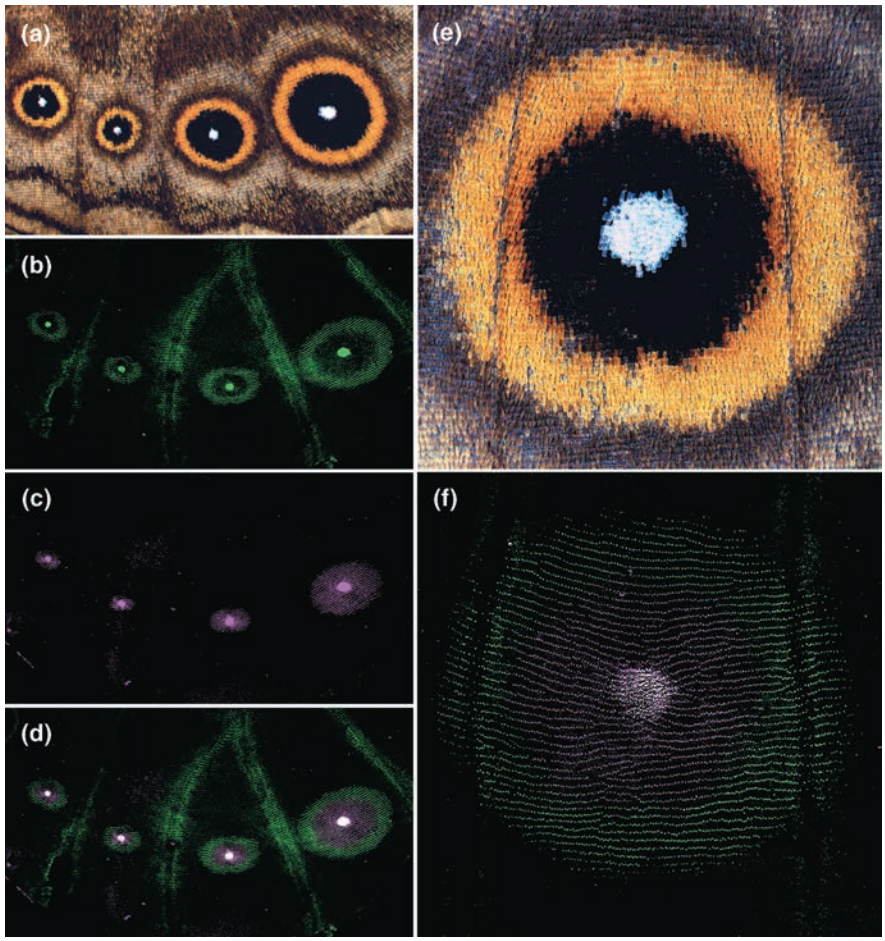
Background

The evolution of new morphological characters has played a key role in the diversification of particular animal groups. Among the insects, for example, the evolution of wings and powered flight catalyzed their radiation as the most speciose animal taxon. Further innovations in wing architecture and patterning have played important roles in the evolution of various insect groups, such as the beetles, flies, and moths and butterflies. Among the latter, the evolution of scale-covered wings, pigmentation, and spatial patterning systems has led to a spectacular variety of wing color patterns composed of several independently evolving sets of elements [1, 2].

One of the more recently derived and better-studied pattern elements on butterfly wings are the eyespots that play crucial roles in interactions with predators [3]. The formation of eyespot patterns is controlled by a developmental organizer (the focus), which induces surrounding cells to synthesize specific pigments [4–7]. Transplantation and ablation experiments, studies of gene expression in different butterfly species, and analyses of wing pattern mutants have suggested that eyespot development is progressively specified in four stages [8]. In the first stage, during the last larval instar, such prepatterns of gene activity as the expression of the transcription factor *Distal-less* (*Dll*) reflect the equivalent potential for pattern formation in each subdivision of the wing [8, 9]. In the second stage, foci are established within specific subdivisions of the larval wing. Focus formation correlates with *Dll* and *engrailed* (*en*)/*invected* (*inv*) expression within the center of each eyespot field and appears to be established through the action of members of the *hedgehog* (*hh*) signal transduction pathway [10]. In the third stage, in the early pupa, signaling from the focus induces surrounding cells to adopt different color fates and in the final, late pupal stage, the adult color pattern is realized as the scales become pigmented. However, neither gene expression studies nor mutants

Figure 1



Engrailed/Invected and Spalt protein expression defines territories on the *B. anynana* pupal wing that correlate with the concentric rings of adult eyespots. **(a)** A portion of the ventral hindwing surface of an adult *B. anynana* encompassing four of the seven eyespots. **(b–d)** Expression of En/Inv (green) and Sal (purple) protein in the *B. anynana* hindwing corresponds to the region shown in (a) at 16 hr after pupation. (d) En/Inv and Sal proteins are coexpressed in a densely staining central spot. Immediately surrounding this spot is (c,d) a ring of Sal protein, and (b,d) En/Inv is expressed in a ring outside of the Sal expression domain. The rings of expression of En/Inv and Sal correlate with rings of colored scales on the adult *B. anynana* wing. **(e)** A high-magnification view of the ventral surface of a *B. anynana* forewing disc. (e) The white spot, black ring, and gold ring on the adult wing correlate with **(f)** the expression domains of En/Inv/Sal, Sal, and En/Inv, respectively.

have revealed information about the specification of eyespot color patterns during these latter two stages.

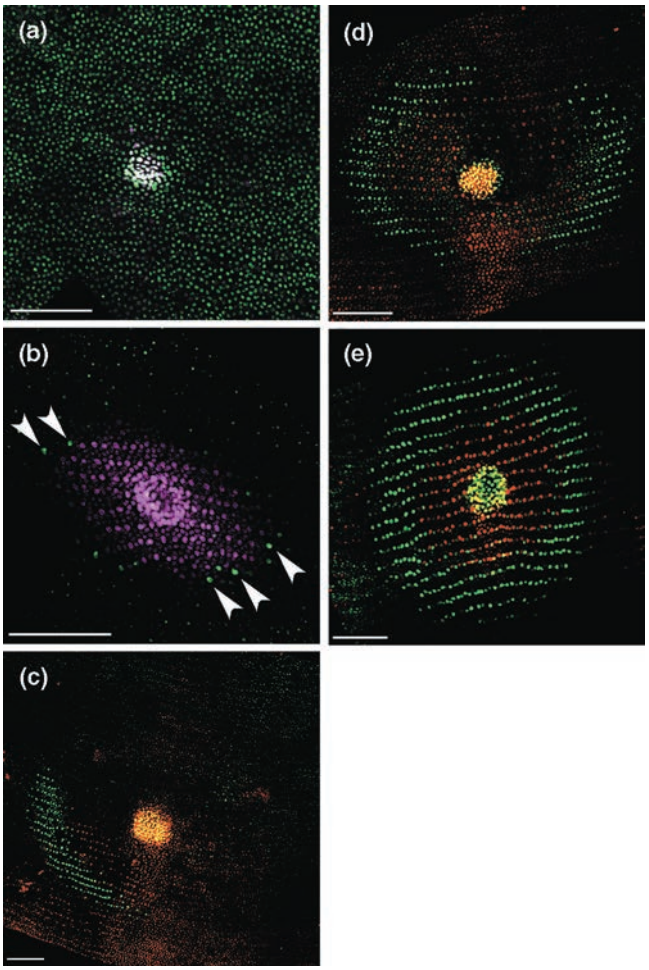
A number of theoretical models have been proposed to explain the production of concentric rings of pigmented scales in cells surrounding the focus [7, 11, 12]. In some models, scale cells directly interpret the levels of the focal morphogen [12], but it is also possible that some regulatory genes are interposed between the reception of the focal signal and the expression of structural genes involved in pigmentation. Furthermore, it is not understood how the great diversity of butterfly eyespot color schemes is generated. Among the ways in which eyespot diversity could arise are through differences in focal signaling or through differences in the species-specific responses to focal signaling of either pigmentation genes or regulatory genes. In this study, we provide genetic and molecular evidence that at least one tier of regulatory proteins is interposed between focal signaling and scale color differentiation. Differences in eyespot patterns appear to arise both at the level of these regulatory proteins and in the downstream response of pigmentation genes.

Results

Engrailed/Invected and Spalt protein expression in the pupal wing marks the future eyespot color pattern

To distinguish between different potential mechanisms of eyespot development and evolution, we sought to identify candidate genes involved in eyespot color pattern formation. We screened for gene products that were expressed during the period of scale cell differentiation (12 to 36 hours after pupation [11]) and that had patterns that correlated with the concentric rings of *Bicyclus anynana* eyespots. Among the various proteins and transcripts we surveyed (these included Cubitus interruptus, Schnurri, SMAD, Brinker, *aristaless*, *dachshund*, and *teashirt*; data not shown), only the Engrailed/Invected (Engrailed and/or Invected, hereafter denoted by En/Inv) [10], Dll [8], and Spalt (Sal) transcription factors were expressed in patterns of scale-forming cells that correlated with eyespot formation. All three proteins were expressed in cells in the region of the focus at the center of each eyespot field (Figures 1b,c and 2e). Remarkably, a second domain of En/Inv expression arose in the 16 hour pupal wing in a

Figure 2



The expression of En/Inv, Sal, and Dll on the wings of *B. anynana* follows a temporal progression. **(a)** The first gene expression observed in the developing pupal eyespot field is in cells at the center of the eyespot field that express both En/Inv (green) and Sal (purple; coexpression is white). At this stage, scale-building cells are not yet present. **(b)** After the formation of the central spot, scale-building cells differentiate and express Sal (purple) in a second domain, while En/Inv expression appears in a few cells at the periphery of this Sal domain (arrowheads). **(c)** En/Inv expression then expands around the ring of Sal. **(d,e)** At the stage when the En/Inv domain (green) is almost complete, Dll (red) begins to be expressed and fills the same territory as Sal (not shown). Scale bars in (a)–(e) are 150 μm.

distinct ring of cells outside of the focal region and at the periphery of each eyespot field (Figure 1b). In addition, Sal was expressed in rings of cells between the focal region and the ring of En/Inv-expressing cells (Figure 1c,d). Based upon physical landmarks of the developing wing and by comparison of the relative size and position of the concentric rings of gene expression patterns with the colored rings of the adult eyespot, we found correlations between protein expression patterns and the three colored rings of *B. anynana* eyespots. The En/Inv, Sal, and Dll [8] expression in the focus corresponds to the white center in the adult eyespot (Figures 1e,1f and 2e). The territory marked by Sal and Dll expression, but not En/Inv expression, appears to correspond to the domain of the black ring of scales in the adult eyespot (Figures 1e,1f and 2e). Additionally, the outer ring of En/Inv expression correlates with the position of the gold ring of scales in the adult wing (Figure 1e,f). We have not yet discovered a gene product for which the pattern of expression correlates with the outermost dark-brown ring of scales.

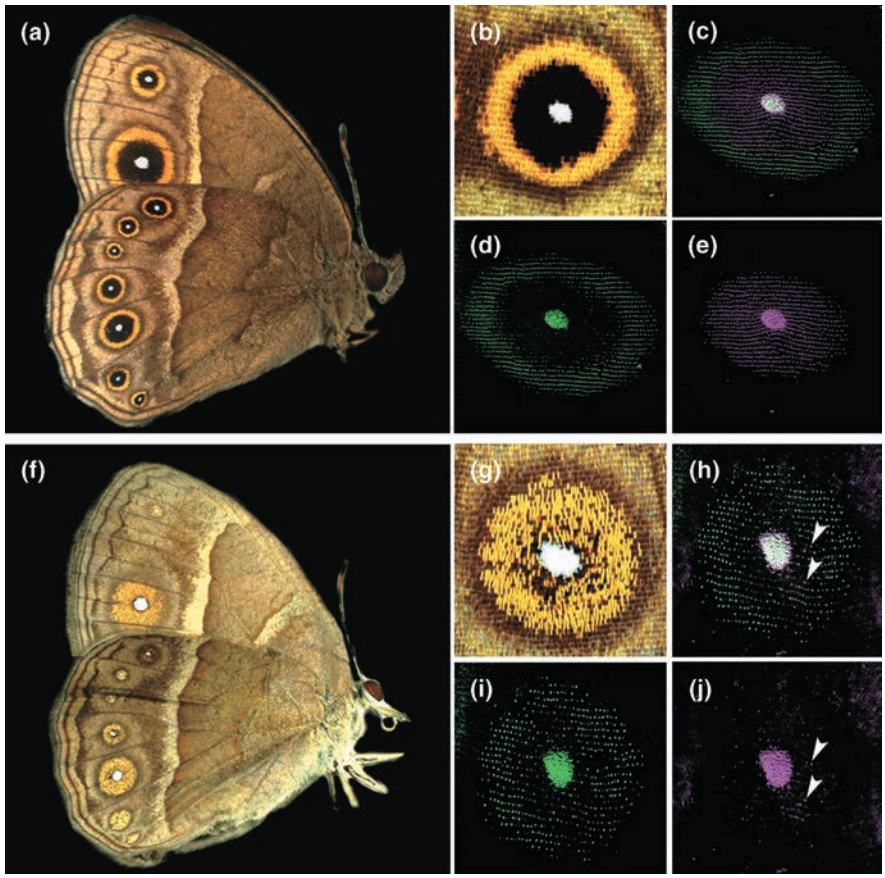
In order to gain insight into how these concentric rings of gene expression are established, we examined the formation of the En/Inv, Sal, and Dll expression patterns over time. Initially, En/Inv, Sal, and Dll were coexpressed in epithelial cells at the center of the eyespot field (Figure 2a,c). During this stage, scale-building cells had not yet begun to differentiate from the disc epithelium (Figure 2a). As the scale-building cells differentiated, Sal was the first of the transcription factors to be expressed in the scale-building cells and formed a ring surrounding the central spot (Figure 2b). Once Sal expression filled this region, En/Inv expression began in cells just at the boundary of the Sal domain (Figure 2b, arrows). Unlike Sal expression, the initial activation of En/Inv expression was not uniform around its circumference. En/Inv expression appeared at one or two sites and then expanded over time to form a ring around the Sal domain (Figure 2c,d). Dll expression expanded after Sal and En/Inv expression and eventually coincided with the Sal domain (Figure 2e).

From these observations of the temporal and spatial relationships between En/Inv, Sal, and Dll expression, we can make two important inferences. First, the switch from synchronous coincident expression of these three proteins in the center of the eyespot field to their asynchronous, nonoverlapping expression in the outer rings of the field suggests that they are under different regulatory controls when the foci are first established than when the eyespot field is elaborated. Second, the sequential appearance of the rings, in particular the expression of En/Inv in cells just outside of the Sal domain, suggests that one mechanism for generating concentric patterns of gene expression may be to exclude the expression of one gene from another's domain.

Goldeneye, a new mutant that specifically alters the eyespot color scheme

To further test the correlation between these protein expression domains and the adult color pattern scheme, and to further examine whether there may be regulatory interactions that govern their establishment, we examined the expression of En/Inv, Sal, and Dll in developing wings of *Goldeneye* butterflies, a newly discovered spontaneous autosomal dominant mutant of *B. anynana*. In *Goldeneye* mutants, the region of black scales of each adult eyespot is reduced to just a few scales around the central white

Figure 3



The expression of En/Inv and Sal is altered in the *B. anynana* eyespot pattern mutant *Goldeneye*. **(a)** Ventral view of adult wild-type *B. anynana* wings and **(b)** a high-magnification view of one of the ventral hindwing eyespots. **(c–e)** Double labeling reveals the rings of expression of En/Inv (green) and Sal (purple) at 16 hr after pupation in wild-type wings. **(f)** Adult *Goldeneye* mutant (ventral view) and **(g)** a high-magnification view of a ventral hindwing eyespot. In the *Goldeneye* mutant, the black ring of scales is almost completely gone and is replaced by gold-colored scales. **(h–j)** Corresponding to the changes in scale coloration, the expression patterns of En/Inv (green) and Sal (purple) are also altered. (j) Sal is almost completely absent outside of the central spot. (h,j) Arrowheads point to small clusters of Sal-positive scale-building cells. (i) En/Inv expression has expanded to fill the domain that would be occupied by Sal in a wild-type eyespot, and (h) the few Sal-positive scale-building cells remain En/Inv negative. There also appears to be a few cells that do not express either Sal or En/Inv; these may correspond to the few dark-brown scales that are interspersed among the gold scales in the *Goldeneye* mutant.

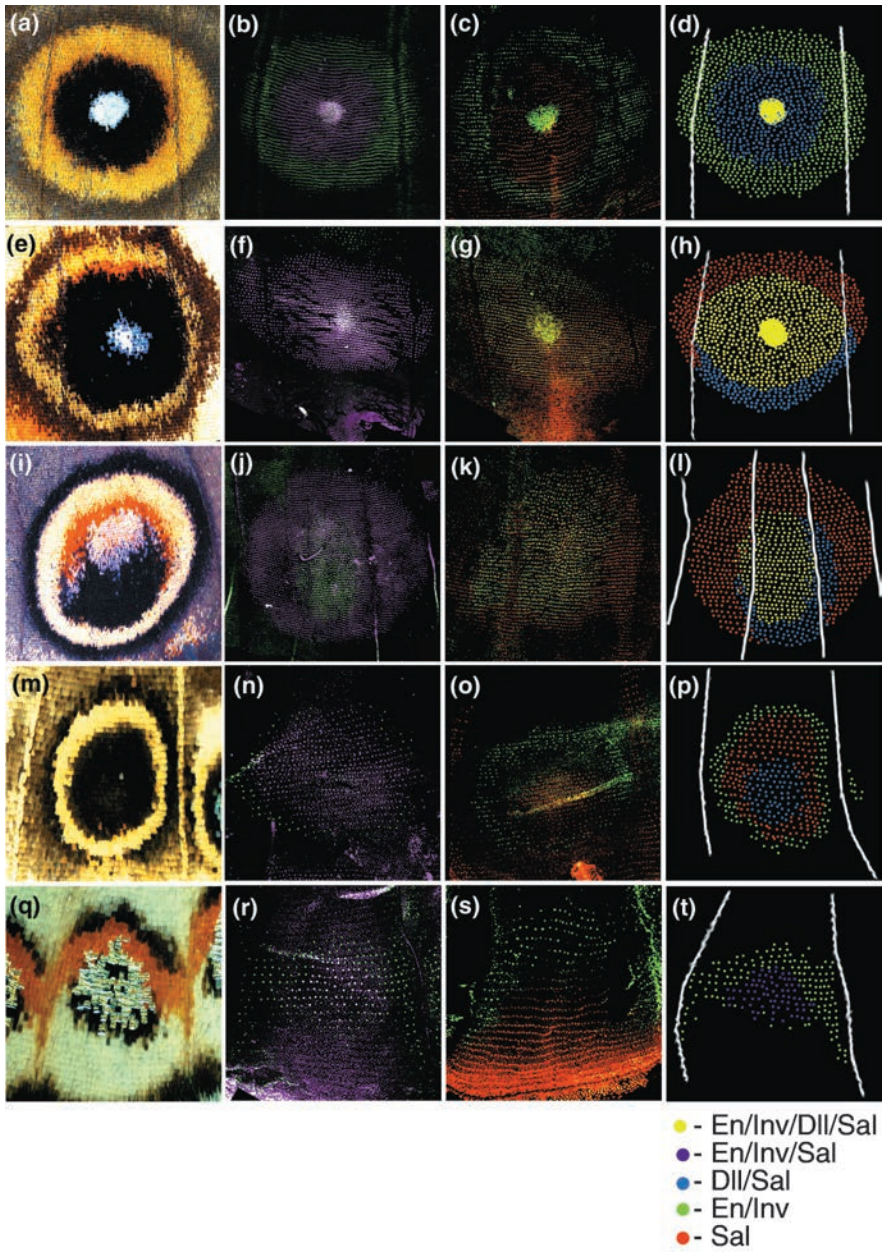
spot, and the outer gold ring is expanded into the region previously occupied by the black scales (Figure 3f,g). If En/Inv, Sal, and Dll are involved in defining territories on the adult wing, then the loss of the black ring and the concomitant expansion of the gold ring in *Goldeneye* mutants could be reflected by changes in the expression patterns of these genes. Alternatively, it is possible that *Goldeneye* affects a process downstream or independent of these transcription factors.

We found that the expression of En/Inv was altered in *Goldeneye* pupal wings and correlated with the pattern of gold scales on the adult wing. En/Inv expression was patchy and encompassed the entire eyespot field outside of the center domain (Figure 3i). These results apparently reflect the patchy appearance of gold scales on the adult (Figure 3g). Furthermore, Sal expression was almost completely lost outside of the central spot, with only occasional Sal-expressing scale cells (Figure 3h,j, arrowheads), consistent with the paucity of black scales on the adult mutant wing. Interestingly, Dll expression in *Goldeneye* mutants is expanded and encompasses the entire En/Inv expression domain (data not shown). These results indicate that the Goldeneye mutation acts upstream of all three transcription factors. The reciprocal changes in En/Inv and Sal expression in *Goldeneye* mutants are consistent with the correlation that En/Inv (but not Sal) is expressed in scale-building cells destined to give rise to gold scales, whereas scale-building cells that express Sal (but not En/Inv) give rise to black scales. Furthermore, the mutually exclusive expression of Sal and En/Inv in these rings in wild-type and *Goldeneye* wings suggests that the boundaries between these territories may be established by regulatory mechanisms that exclude expression of each gene from the other's expression domain.

Eyespot color pattern diversity is correlated with the diversification of transcription factor expression patterns

The association between the switch in the color scheme of *Goldeneye* eyespots and changes in regulatory protein expression prompted us to investigate how these proteins are expressed in other butterfly species with different eyespot color schemes. There is spectacular variation in the coloration and shape of butterfly eyespots [7]. A number of observations have suggested that eyespot color pattern diversity arises during the latter stages of eyespot development. The transplantation of eyespot foci be-

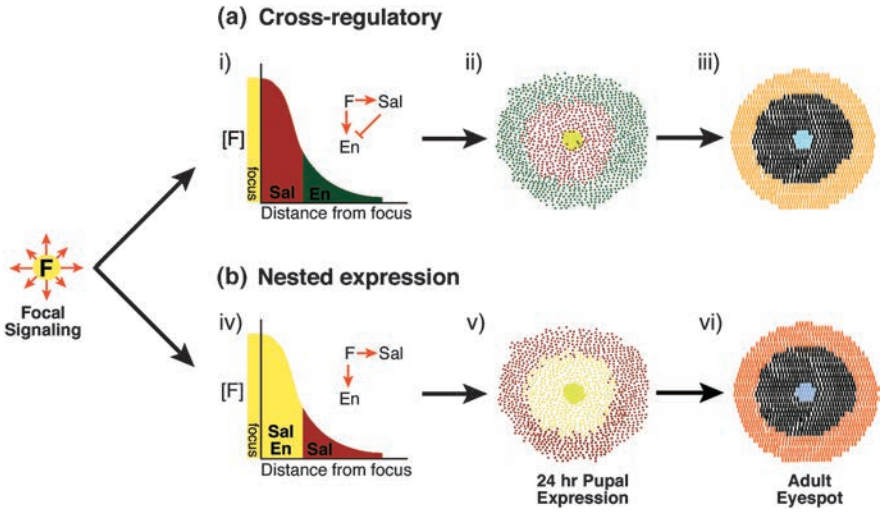
Figure 4



The diversity of eyespot patterns is the result of altering the expression of both regulatory and pigmentation genes. **(a,e,i,m,q)** Adult eyespots are shown on the left-hand panels. **(b,f,j,n,r)** Double labeling for En/Inv (green) and Sal (purple) expression at 16–20 hr after pupation is shown in the second column from the left, and **(c,g,k,o,s)** double labeling for En/Inv (green) and Dll (red) expression is shown in the third column from the left; **(d,h,l,p,t)** schematics of the expression patterns of En/Inv, Sal, and Dll are summarized in the right-hand panels. The species and eyespots shown are from (a–d) *B. anynana*, (e–h) *P. coenia* forewing and (i–l) hindwing, (m–p) *V. cardui*, and (q–t) *L. melissa*. In the schematics, yellow indicates the coexpression of En/Inv, Sal, and Dll; purple represents the coexpression of En/Inv and Sal; blue represents the coexpression of Dll and Sal; green represents En/Inv expression alone; and red indicates Sal expression alone. Note that the spatial relationships among the expression of all three proteins differ between species but still correlate with the diverse color schemes. In addition, the expression of one protein (e.g., En/Inv) correlates with different colors in different species (compare [a], [m], and [q] with [d], [p], and [t]).

tween species (cited in [1]) or of selected lines of *B. anynana* differing in eyespot color composition induces eyespot patterns characteristic of the host animal (not the donor), suggesting that the response to the focal signal (not the signal itself) is different between species [13]. It is possible that the differences in cells' responses to focal signaling could arise as a result of changes in the expression patterns of regulators. Alternatively, direct responses to focal signaling may be similar between species, but the regulators may interact with different downstream genes involved in scale pigmentation and structure. To determine when during development differences arise between the eyespot color schemes of various species, we compared the expression patterns of En/Inv, Sal, and Dll in *B. anynana* (Nymphalidae, Satyrinae), *Precis coenia* (Nymphalidae, Nymphalinae), *Vanessa cardui* (Nymphalidae, Nymphalinae), and *Lycaeides melissa* (Lycaenidae, Lycaeninae). In each of the examined species, which represent two different families of butterflies and three different genera within the Nymphalidae, the expression patterns of En/Inv, Sal, and Dll are different, yet they mark territories in the pupal wing that often correlate with color pattern schemes on the adult wing (summarized in Figure 4).

Figure 5



Generation and diversification of butterfly eyespot color patterns. Two models for the generation of concentric rings of gene expression in response to focal signaling are depicted. Both models involve threshold responses to focal signaling but differ in the role of crossregulatory interactions. **(a)** Crossregulatory interactions may occur in *B. anynana*. Focal cells expressing En/Inv/Sal (yellow) signal (concentration of focal signal [F]) to surrounding cells, which (i) activate En/Inv (green) and Sal (red), which then interact to (ii) produce the mutually exclusive domains of Sal and En/Inv. **(b)** In *P. coenia*, En/Inv and Sal have different threshold responses to the focal signal, (iv) producing nested patterns of En/Inv/Sal (yellow) and Sal (red) expression. (v) In *P. coenia*, no repression between Sal and En/Inv occurs. (iii and vi) Within defined territories of the eyespot fields, pigmentation genes are activated in a species-specific manner.

For example, in *P. coenia*, the Sal territory in the pupal wing marks the entire area encompassed by the adult eyespot (Figure 4c–f). In addition, the coexpression of En/Inv, Sal, and Dll in *P. coenia* forewings in an asymmetric patch of scales at the center of the pupal eyespot corresponds to the white/blue scales at the center of the adult eyespot (Figure 4c,d). The coexpression of the same genes in scale-building cells outside of this central spot correlates with the black ring of scales on the adult (Figure 4c,d). In *V. cardui*, a species closely related to *P. coenia*, En/Inv is expressed in an outer ring of scale-building cells that correlates with the black ring of scales in the adult eyespot. However, in *L. melissa*, a crescent of En/Inv expression correlates with the future position of orange scales on the adult (Figure 4i,j), and En/Inv and Sal coexpression correlates with the metallic-looking patch of scales at the center of the eyespot field (Figure 4i,j).

We deduce that, in addition to these differences in the expression domains of transcription factors, the regulation of structural genes involved in scale pigmentation has also diverged. For example, the expression of En/Inv alone correlates with specific eyespot rings in *B. anynana*, *V. cardui*, and *L. melissa*, but these scales are gold, black, and orange, respectively. We used a Sindbis viral expression system [14] to test whether Dll or En expression alone is sufficient to alter scale pigmentation in *P. coenia* and found that neither protein appears to be able to switch pigmentation type on its own. One possible explanation for this observation may be that a combinatorial mechanism involving multiple transcription factors controls individual pigmentation types.

We also note that the color scheme can differ between wing surfaces within a species. For example, the relative territories of expression of En/Inv, Sal, and Dll are similar between *P. coenia* forewings and hindwings (Figure 4d,f), yet the adult color schemes are different (Figure 4c,e). This latter observation suggests that in eyespot fields in which regulatory genes respond in a similar way to the focal signal, differences can exist in the responses of genes involved in pigmentation or scale morphology between different wing surfaces. One modifier of these responses in the *P. coenia* hindwing is the Ultrabithorax protein [14, 15]. From this comparative data, we conclude that eyespot color pattern diversity is generated by regulatory differences at two distinct stages of eyespot development that evolve independently of each other: (1) during the focal signaling stage, through the generation of different combinations and patterns of expression of regulatory genes such as *en/inv*, *sal*, and *Dll*; and (2) during the scale differentiation stage, through differences in the response of pigmentation genes to the upstream regulators.

Discussion

The generation and diversification of butterfly eyespot patterns

Dll, En/Inv, and Sal play critical roles in the regulation of embryonic and adult patterning in *Drosophila*. Most of these roles are highly conserved in other insects, including butterflies, in which their expression has been compared. However, the deployment of these proteins in butterfly eyespot developmental fields is an evolutionary novelty. It is notable that transcription factors that are deployed in such a conserved manner in most insects differ so greatly in their expression in the developing eyespots of various butterfly species. The developmental and comparative data presented here indicate that eyespot color pat-

tern formation and diversity is governed by a novel and remarkably plastic genetic regulatory system in which both regulatory and pigmentation gene expression can evolve independently.

Transplantation experiments have demonstrated that one central feature of this regulatory system is a long-range signaling activity that emanates from the focus; the levels of this activity determine the eventual color of developing scales [5, 7, 13, 16]. Our results indicate that at least one tier of spatially regulated transcription factors is interposed between focal signaling and scale color differentiation. How the graded distribution of a focal signal is translated into the concentric territories of En/Inv, Sal, and Dll expression is therefore of special interest. In *B. anynana*, we suggest that this occurs through response thresholds of, and negative cross-regulation among, genes regulated by the signal (Figure 5a). For example, one of the simplest explanations for the exclusion of En/Inv and Sal expression from each other's territories outside of the focus could be the repression of one gene by the product of the other (Figure 5a). The reciprocal effects of the *Goldeneye* mutation on En/Inv and Sal expression are strongly suggestive of negative crossregulation. The establishment, through negative crossregulation, of distinct spatial domains of downstream genes in response to a single activator is a common theme illustrated by the subdivision of the *Drosophila* embryonic mesoderm and neuroectoderm and of the proximodistal axis of *Drosophila* limb fields [17–19]. In *P. coenia*, however, the nested nonexclusive expression of Sal and En/Inv suggests that here these genes do not crossregulate (Figure 5b). Rather, the nested expression pattern outside of the focus is most simply explained by different threshold responses of these two genes to the focal signal (Figure 5b); these responses are analogous to the threshold responses of genes to long-range signals in the Xenopus mesoderm [20, 21] and the *Drosophila* imaginal wing field [22, 23].

The origin of eyespots

The deployment of En/Inv, Sal, and Dll in all of the species we examined also raises some interesting possible scenarios regarding the origin and diversification of eyespots and the evolution of the underlying genetic regulatory system that controls eyespot pattern formation. It has been proposed that eyespots have a single origin and are derived from simpler spot patterns of uniform color that evolved into organizing centers [2]. Because all three proteins are deployed in color-correlated patterns in this well-diverged group of butterflies, it is likely that these genes were recruited into the developmental program early during the evolution of eyespots. Furthermore, it is intriguing that while the three proteins have distinct expression patterns during scale differentiation, they are coexpressed during focus formation. It is tempting to speculate, on the basis of the data presented here, that the evolution of eyespots in response to diverse selective environments involved the modification of the deployment of genes that were originally expressed in simpler spot patterns into additional concentric patterns organized around and by cells in the center of the eyespot field.

Materials and methods

Antibodies

Rabbit anti-*P. coenia* Dll antibody and the crossreactive 4F11 monoclonal antibody that recognize the En and Inv proteins have been described previously [8, 24]. *P. coenia* possesses definitive Engrailed and Invected orthologs; both proteins are likely to be recognized by the antibody [9, 10]. Rat and rabbit antibodies against the *Drosophila* Sal protein [25] detect the same patterns in butterfly wings, and we therefore assume that they recognize the *bona fide* butterfly Sal protein.

Immunohistochemistry

Butterfly 12–24 hr pupal wings were fixed for 30 min in 0.1 M PIPES (pH 6.9), 1 mM EGTA, 1% Triton X-100, 2 mM $MgSO_4$, and 1.8% formaldehyde. To prevent nonspecific binding, we blocked the wings for 2 hr in 50 mM Tris (pH 6.8), 150 mM NaCl, 0.5% NP40, and 5 mg/ml bovine serum albumin (BSA). The wings were incubated overnight in 50 mM Tris (pH 6.8), 150 mM NaCl, 0.5% NP40, and 1 mg/ml BSA (wash buffer) containing monoclonal antibody 4F11 against En/Inv (1:5) [24] and either rabbit anti-Dll (1:100) [8] or rabbit anti-Sal (1:200) [25]. The wings were washed four times with wash buffer and incubated for 2 hr with wash buffer containing goat anti-mouse FITC (1:200, Jackson Laboratories) and goat anti-rabbit Cy5 (1:200, Jackson Laboratories). The wings were washed four times in wash buffer and mounted in Vectashield (Vector Laboratories), and images were collected on an MRC600 laser-scanning confocal microscope.

Isolation of the Goldeneye *mutant*

The *Goldeneye* mutant was isolated as a spontaneous autosomal dominant mutation in a large population that is maintained at Leiden and displays no other pattern defects in heterozygotes. It is lethal when homozygous.

Butterfly husbandry

P. coenia and *V. cardui* (obtained from the Carolina Biological Supply Company) butterflies were reared at 28°C under a 16L:8D photoperiod. *P. coenia* larvae were fed an artificial diet containing *Plantago lanceolata* [11], and *V. cardui* were fed an artificial diet supplied by the Carolina Biological Supply Company . *B. anynana* were raised under a 12L:12D photoperiod at 28°C, and the larvae fed on maize plants. *L. melissa* eggs were collected in Mt. Rose, Nevada, and the larvae were fed a diet of clover leaves..

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