Origin, Development, and Evolution of Butterfly Eyespots

Antónia Monteiro

Biological Sciences, National University of Singapore, and Yale-NUS-College, Singapore; email: antonia.monteiro@nus.edu.sg

Annu. Rev. Entomol. 2015. 60:253–71

First published online as a Review in Advance on October 17, 2014

The *Annual Review of Entomology* is online at ento.annualreviews.org

This article's doi: 10.1146/annurev-ento-010814-020942

Keywords

wing patterns, evo-devo, complex traits, constraints, sexual dimorphism

Abstract

This article reviews the latest developments in our understanding of the origin, development, and evolution of nymphalid butterfly eyespots. Recent contributions to this field include insights into the evolutionary and developmental origin of eyespots and their ancestral deployment on the wing, the evolution of eyespot number and eyespot sexual dimorphism, and the identification of genes affecting eyespot development and black pigmentation. I also compare features of old and more recently proposed models of eyespot development and propose a schematic for the genetic regulatory architecture of eyespots. Using this schematic I propose two hypotheses for why we observe limits to morphological diversity across these serially homologous traits.

INTRODUCTION

Homologous trait: a trait in two or more species that derives from the same trait in a common ancestor

Gene regulatory network: functional interactions between a group of genes and their protein products over the course of development to specify a trait or feature in an organism

Eyespots are colorful, conspicuous, and concentric circular markings that butterflies, mostly from the family Nymphalidae, display on the margins of their wings (**Figure 1*a***). Species often differ in the location where these eyespots are displayed; in the total number of eyespots; and in their size, color, and number of rings. In addition, eyespots within a single individual can have different morphologies, and a homologous eyespot can vary between males and females or with environmental rearing conditions.

Understanding eyespots and eyespot diversity requires both ultimate and proximate explanations. The former addresses whether these traits are adaptive in interactions with predators and in finding or securing mates, or are an outcome of neutral evolutionary processes. Proximate explanations involve the origin of the gene regulatory network that differentiates eyespots, the mechanisms that led to the network's repeated deployment and individuation across the wing, its sex-specific modification, and the developmental constraints that limit eyespot color or size diversity.

Progress is being made in unraveling both ultimate and proximate mechanisms for explaining eyespots. After briefly summarizing progress in our understanding of the ultimate mechanisms that select and maintain eyespots on the wing, I focus primarily on summarizing recent work on the mechanistic basis of eyespot origins, development, and evolution and the possible nature of the constraints limiting eyespot color ring diversity.

Figure 1

The origin of eyespots. (*a*) Eyespots, circular bull's-eye markings on the wings (exemplified here by *Orsotriaena medus*), are inferred to have evolved once (*b*), in the lineage sister to the Danainae, concurrently with the origin of expression of a small group of genes at the center of the eyespot pattern and during the larval stages of development. Eyespot presence/absence was mapped for the species depicted on the left of the phylogeny, whereas presence/absence of five proteins resulting from the expression of the five genes in the eyespot centers was mapped for the species depicted in the center and to the right. Expression data (69, 70) are from species marked with asterisks (*). Proteins: Antp, Antennapedia; Dll, Distal-less; En, Engrailed; N, Notch; Sal, Spalt. Photo in panel *a* courtesy of William H. Piel. Panel *b* modified from Ref. 55 with novel insights from Ref. 52.

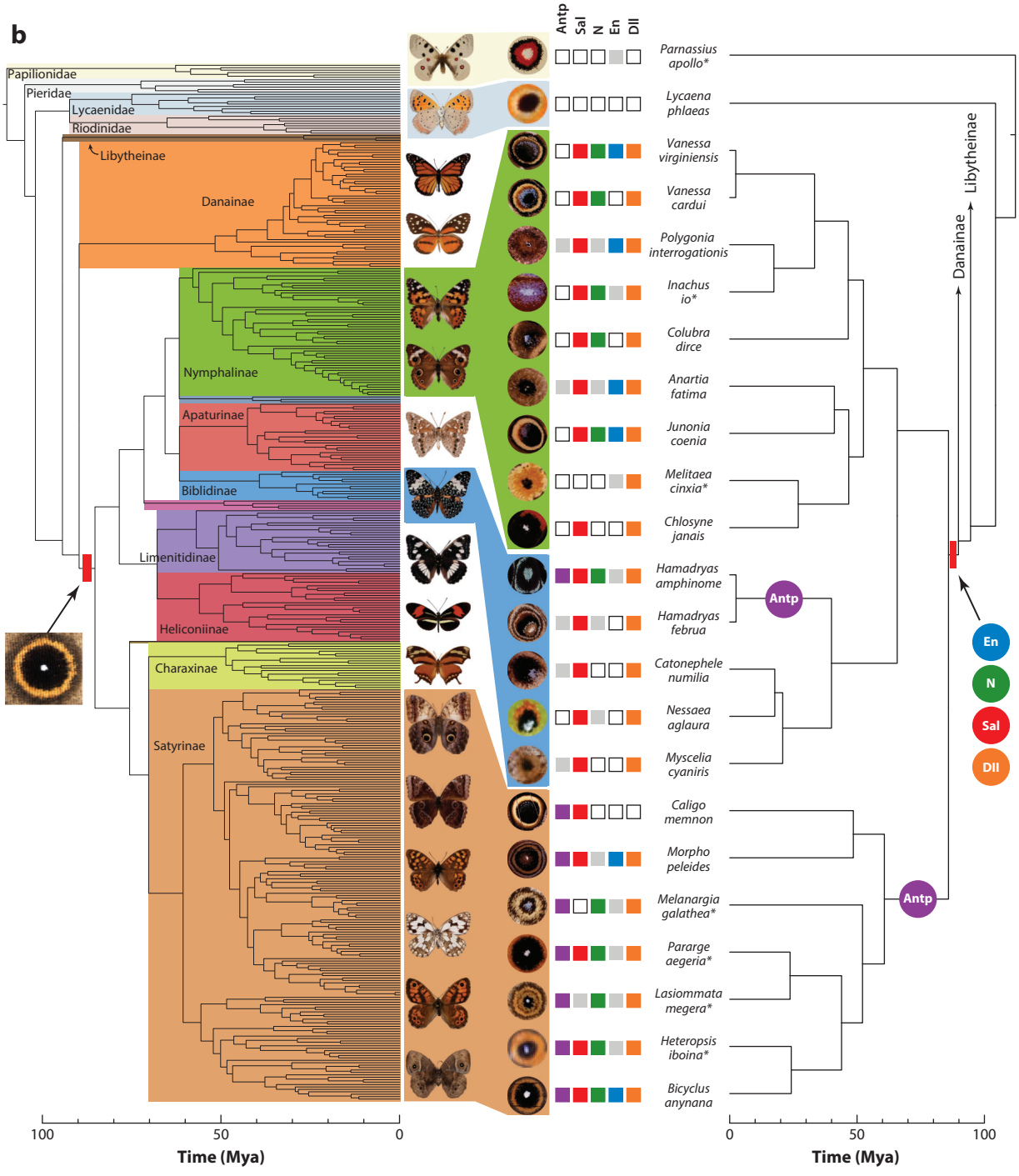


Figure 1

(*Continued*)

ECOLOGICAL FUNCTION OF EYESPOTS

Serial homolog: a repeated trait in a body that develops using the same gene regulatory network

Although the ecological function of butterfly eyespots has been a subject of interest for over a century (19, 64), research in this area has been mostly focused on the role of eyespots in interactions with vertebrate predators (reviewed in 28, 72). Multiple hypotheses have been proposed for the function of eyespots, including predator intimidation (81), predator deterrence (74), and predator deflection to nonvital parts of the body (32). These hypotheses are under active study and debate (36, 73, 75) via experiments with new predators (57, 71, 82), new backgrounds (56), or new light environments (58). In addition, fieldwork involving mark/recapture experiments with either live (63) or paper (76) models is demonstrating that predation on species with eyespots affects males and females differently (63) and that specific characteristics of eyespots, such as conspicuousness, are especially effective at reducing predation (76).

Several years ago, a novel role for eyespots as sexual signals was discovered (14, 18, 66), and associated mechanisms for the maintenance and evolution of eyespot number have been proposed. These mechanisms include the maintenance of eyespots in both sexes via mutual mate choice at different times of the year (65) and male-specific gains in eyespots resulting from a female learning bias in favor of more eyespots in males (86). The novel role in sexual signaling is primarily being observed for eyespots expressed on the hidden, dorsal surfaces of the wings in a single investigated species (66). Eyespots, however, only appeared on this surface many millions of years after they originated on the ventral hindwing (see Origin of Eyespots, below); thus, the sexual signaling function is likely derived, compared with the antipredatory function described above.

ORIGIN OF EYESPOTS

The field of butterfly wing pattern evolution has been, in my view, somewhat constrained by a very influential model, the Nymphalid Ground Plan (50, 51, 61). This model proposes a system of homologies for many of the wing color patterns in nymphalid butterflies, including eyespots, and facilitates the identification of serial homologs across species. However, this model is easily misinterpreted as representing the ancestral nymphalid wing pattern—i.e., with eyespots originating concurrently in all wing sectors. Recent use of the comparative method to map eyespot evolution on a well-established nymphalid phylogeny, however, shows that likely this was not an ancestral pattern.

Eyespots appear to have originated once within the Nymphalidae, at the base of the lineage sister to the Danainae, around 90 Mya (55) (**Figure 1*b***). The presence and absence of eyespots, defined as circular spots with one or more additional concentric rings of a different color (37), were scored in nearly 400 species of nymphalid butterflies belonging to as many different genera and mapped onto a phylogeny constructed with DNA sequence data (83). The lineage where eyespots originated later radiated into a large number of subfamilies, most notably the Satyrinae and Nymphalinae, both containing many members with eyespots. The ancestor to all nymphalids, however, did not have eyespots. Because eyespots are present in other butterfly and moth lineages (37), these data imply that eyespots originated separately and independently in moth lineages and in nymphalid butterflies. Detailed phylogenetic analysis of eyespot distribution outside the nymphalids will be required to estimate the number of times that eyespots have independently originated in the Lepidoptera as a whole.

Follow-up work using the same set of species but with more detailed scoring of eyespot location on the wing confirmed the previous result and also identified the likely ancestral pattern for the first nymphalid butterfly with eyespots. This ancestral pattern appears to have been a set of four or five eyespots on the ventral hindwing. Furthermore, these first eyespots appear to have originated with

the developmental ability to switch on and off independently from each other. This independence at the origin was inferred by the subsequent pattern of gains and losses happening to individual eyespots or small subsets of eyespots within that original cluster, and not to the cluster as a whole (52).

Thus, eyespots appear to have originated on a particular wing (hindwing) and a particular surface (ventral) and only subsequently to have colonized the anterior wing and dorsal surfaces (52). Because there is evidence that eyespots all share roughly the same developmental gene regulatory network, the original restriction of eyespots to only a few wing sectors could be due to the restricted expression of activating transcription factors on the wing or due to the presence of repressing transcription factors in wing sectors originally lacking eyespots. Over time, however, additional sectors and surfaces of the wing became competent to activate the network, and thus total eyespot number and positional diversity increased on the wings of nymphalid butterflies.

The pattern of origin of eyespot serial homology resembles that observed for vertebrate fins: first originating along the dorsal midline, as a dorsal fin (22), then appearing as a pair of anterior pectoral fins, and finally appearing in a more posterior region of the body as pelvic fins (67). Similarly, in flies, the bristle gene regulatory network appears to have been present, at some point in time, in only two positions on the dorsal thorax and to have later evolved novel positional information that allowed bristles to appear in additional positions more anteriorly in the thorax (33).

In addition to having originated in a particular wing surface, eyespots replaced simpler pattern elements that already existed at these locations—simple colored spots centered between veins (52). Currently, a detailed phylogenetic examination of the origin and wing distribution of spots is not available, but cursory examination of specimens suggests that spots are found in most butterfly and many moth lineages and are likely an ancient feature of lepidopteran wings. Eyespots appear, thus, to have originated in the center of wing sectors by using positional information already marking that center and used by simple colored spots.

Transcription factor: a DNA-binding protein with the ability to alter expression of nearby genes by binding to *cis*-regulatory elements

Gene expression: the process of gene activation that leads to transcription of mRNA and then protein synthesis

Co-option: the recruitment of genes, or larger gene networks, to new developmental functions

ORIGIN OF EYESPOTS FROM SPOTS: COMPARATIVE MOLECULAR DATA

What makes an eyespot different from a spot? Aside from having at least an extra ring of color, eyespots look quite similar to spots. Both are usually centered in a wing sector and have a circular appearance. However, a more detailed examination of the gene expression profiles that can be found at the center of spots and eyespots during their development reveals some differences between these traits. Surveys for the expression of a few candidate genes during the larval stage of wing development in nymphalid species with eyespots found that at least two genes (*spalt* and *Distal-less*) were expressed in most eyespot centers (55, 70) (**Figure 1*b***). Out-group species with simple spots or independently evolved eyespots did not express these same genes at the corresponding stage of development. Surprisingly, four of the five genes surveyed appear to have a single origin of expression in the eyespot centers at the same internal branch of the phylogeny that coincides with the origin of eyespots (55) (**Figure 1*b***). This suggests that eyespots may have originated from a network co-option event involving at least these four genes (55). This co-option event may have allowed a simple colored spot to become a multicolored and multiringed eyespot.

Interestingly, after the origin of expression of these four genes, multiple genes' expression was lost from eyespot centers without associated loss of eyespots (**Figure 1*b***). This observation suggests that perhaps following the co-option event, multiple genes did not function in the novel context and their expression was subsequently lost in some lineages. The retention of expression seen for two genes (*Distal-less* and *spalt*) across the phylogeny suggests that they may have functioned from

the very beginning in eyespot development, whereas the retention of expression of other genes across only a subset of lineages suggests that these genes may have gained a novel function in eyespot development in those lineages or that they remain functionless to this day (55).

Morphogen: an unidentified small secreted molecule that affects the differentiation of cells some distance away from the secreting cells, usually in a concentration-dependent manner

If eyespots originated via a network co-option event, as suggested by the phylogenetic study, an important task will be to figure out which pre-existing network was co-opted to function in eyespot development. Several proposals for co-opted networks have been advanced. These include gene networks involved in ventral appendage development (16); in the formation of the anterior-posterior wing compartments (26); in wound healing (42); and, most recently, in wing margin development (25). These circuits/networks all have some genes in common with eyespots, but similarities at other levels, such as at the level of function or regulatory interaction, have yet to be pursued. Empirical tests for deciding which, if any, of these networks was recruited to aid in eyespot origins have been proposed (38, 43) but will need to be executed. At stake is an important challenge for the field of evolutionary biology: Can we demonstrate that the origin of novel complex traits in organisms occurs via abrupt and sudden changes in gene regulatory network architecture? Do phenotypes change gradually, or can some phenotypes change abruptly, via the recruitment of preexisting gene regulatory networks deployed and subsequently modified for novel functions?

Additional aspects of eyespot evolution are also unclear. For instance, how does the co-option of genes to the center of a primitive spot pattern turn spots into eyespots? The main morphological feature distinguishing spots from eyespots, after all, is the external addition of rings to the simpler spot pattern. Intriguingly, several of the genes that are expressed in the center of eyespots are additionally expressed later in development in the rings around the center (15), and it is not impossible that the origins of the two developmental expression domains are interconnected. However, not until additional comparative work has been performed to pinpoint the origin of the novel rings of gene expression around the central cells will we be able to grasp how novel gene expression domains at the center of an eyespot (during the larval stage) correlate with gene expression in the rings at the periphery (during the pupal stage), which distinguishes spots from eyespots.

Recently it was shown that damage applied to cells at the center of simple black spots on the wings of a pierid butterfly (a basal lineage to nymphalids) led to smaller spots (77). This experiment parallels those that have been performed in nymphalids with eyespots, during the pupal stage (12, 23, 48, 62), and suggests that both spots and eyespots have a group of cells at their center that signal to surrounding cells to specify the rest of the pattern. It is quite possible that in the common ancestor of both groups, cells at the center of spots expressed a yet to be identified set of marker or signaling genes that are also expressed in the center of eyespots. These centrally expressed genes may have, in fact, facilitated the co-option of the battery of genes now associated with eyespot centers and with the origin of eyespots.

EYESPOT DIFFERENTIATION: MODELS

As mentioned above, the cells at the center of the eyespot are able to differentiate a complete eyespot around them during the pupal stage, but how this occurs is under debate. What is known, through transplantation experiments in both *Junonia coenia* (48) and *Bicyclus anynana* (24), is that these cells (but not surrounding cells) can differentiate complete eyespots wherever they are placed on the early pupal wing.

The first proposed model to explain how these cells are differentiating the complete eyespot pattern around them is named the gradient model. Here, one or more morphogens, produced in the central cells, diffuse to the surrounding cells (41, 42, 47, 48). The surrounding cells, depending on particular response thresholds to these morphogens, respond to the continuous concentration

gradient in a discrete fashion. High concentrations activate a subset of downstream target genes, whereas lower concentrations activate a smaller subset of (more sensitive) genes. This leads to the differentiation of discrete rings of cells, expressing a different set of transcription factors, that are then fated to produce different colors (15).

Over the years, other competing models have been proposed for the differentiation of an eyespot (20, 60). These models differ in detail, but they all propose that eyespot differentiation depends on multiple diffusible signals, only the first of which is produced by the central cells. Once part of an eyespot is differentiated, either the cells in the immediate first ring (20) or the cells in all the dark rings around the central cells (60) further signal to surrounding cells to differentiate (20) or sharpen (60) the additional color rings. Both of these models, however, fail to explain important experimental data. For instance, damaging the central cells can abolish the first dark ring of colored scales in *B. anynana*, leading to the outer ring of light color differentiating as the first (and only) ring (23, 24). This result cannot be easily explained by serial induction, where the inner (or dark) ring has to differentiate first in order for the outer (light) ring to differentiate. According to the gradient model, however, the differentiation of the outer ring of color in a more central position is expected, because these cells are merely responding to lower concentrations of a morphogen gradient, which are now present closer to the signaling cells because of the untimely arrest of signal production. These lower concentrations of a morphogen can induce the expression of the more sensitive downstream target genes, normally expressed at the periphery of an eyespot, but not the less sensitive genes normally expressed at the center. Also, the expression of multiple transcription factors in consecutive rings (both dark and light rings) around the eyespot center at approximately the same time (15) does not lend support to the alternative model—i.e., the induction model (60), which proposes that dark rings differentiate before light rings. Additional shortcomings of this model relative to the gradient model are presented in **Table 1**.

The simple gradient model, unlike claims to the contrary (59), can potentially also produce variation in eyespot morphology across a wing, such as variation in size, number of rings, and size and color of each ring. This can be achieved if components of this model (the signal and the response to the signal) are differentially modulated by the presence of compartment-specific transcription factors, such as Cubitus interruptus and Engrailed, which are differentially expressed in anterior and posterior butterfly wing compartments, respectively (26), or by the presence of sector-specific transcription factors, such as Spalt (Sal), with more complex expression domains (**Figure 2**). Although other transcription factors, with sector-specific expression domains, have yet to be identified in butterfly wings, multiple such proteins, as well as Spalt, are known to be present in developing *Drosophila* wings and direct the differentiation of veins at the boundary of their expression domains (9, 10). Orthologs of the genes coding for these proteins, once identified in butterfly wings, will become important candidate genes for sector-specific modifiers of eyespot developmental mechanisms across the anterior-posterior axis of a wing (26).

In addition to the models described above, which address signaling from eyespot centers, a different class of models has been proposed for the differentiation of the eyespot centers themselves (49). These models rely on (yet to be identified) mechanisms of reaction-diffusion, with diffusible signals being generated at the wing margin and at the veins. The interaction between these signals as they diffuse eventually leads to the stable expression of one of the signals at the center of each wing sector. Some candidate diffusible molecules are known to be present in butterfly wings, such as Wingless at the margin (16, 25), but other candidate signaling pathways, such as epidermal growth factor receptor–mediated signaling and Decapentaplegic signaling, that are known to be present in developing *Drosophila* veins (11) have yet to be visualized in butterflies.

More recently, these reaction-diffusion models have been updated via the addition of known genes and regulatory interactions from the putatively co-opted networks in *Drosophila* (21, 34).

Table 1 Comparison between the gradient and induction models for eyespot development

Model	Gradient model (15, 42, 48)	Induction model (60)	Data that support either model
Properties of the signal	One or more morphogens diffuse from central cells to surrounding cells.	Multiple signals, produced by the central cells, are set out as waves at different times during development and travel various distances.	Wingless and TGF-β molecules are known morphogens and are present in the center of eyespots early in pupal development (42), offering support to either model.
Properties of the surrounding cells—differentiation	Surrounding cells respond to the central morphogen(s) at different concentration thresholds and at approximately the same time.	The wave signals are interpreted at roughly the same time to differentiate only the dark bands around an eyespot (and parafocal bands).	Multiple transcription factors are expressed in each of the different colored rings in an eyespot at approximately the same time, including light and dark rings (15). This supports the gradient model.
	The light rings of color of an eyespot are assumed to differentiate directly in response to the central morphogen gradient.	The light rings of color in an eyespot are assumed to be determined passively—they are similar to background scales.	Specific transcription factors are expressed in light rings in an eyespot at times equivalent to those in the dark rings. These transcription factors are not expressed in the background scales (15). This supports the gradient model.
Number and type of inducing cells	The eyespot centers are assumed to be the only sources of diffusible signals.	The dark rings are assumed secondary sources of signals, inducing an inhibitory signal in the light rings.	Candidate morphogens have only been localized to the eyespot centers. No molecular evidence currently supports secondary induction signals required for the induction model.
Boundaries between color rings	Sharp boundaries between rings are proposed to be due to cross regulatory interactions among adjacent transcription factors present in some species.	Sharp boundaries between dark and light rings are proposed to be due to a reaction-diffusion interaction between short-range activating and long-range inhibitory signals.	No molecular mechanism is known that lends support to either model.

Abbreviation: TGF-β, transforming growth factor beta.

This latter class of models, however, will need to be tweaked in the future to incorporate the finding that several of the genes that were assumed to be universally expressed in eyespot centers and required for their development seem to be dispensable in multiple lineages (55, 70, 80).

EYESPOT DEVELOPMENT: MOLECULAR DATA

Ligand: a small protein involved in cell-cell communication

Support for any of the models described above ultimately needs to come from developmental perturbation experiments explored at the molecular level. So far, a variety of candidate genes with expression in the eyespot field have been identified (reviewed in 45; **Figure 3**), but only two genes have been tested at the functional level. One of the genes tested codes for the transcription factor Distal-less (Dll), the first molecular maker associated with eyespots (16), and is a gene expressed in most nymphalid eyespots (55, 70). The other gene codes for the ligand Hedgehog (Hh) (26).

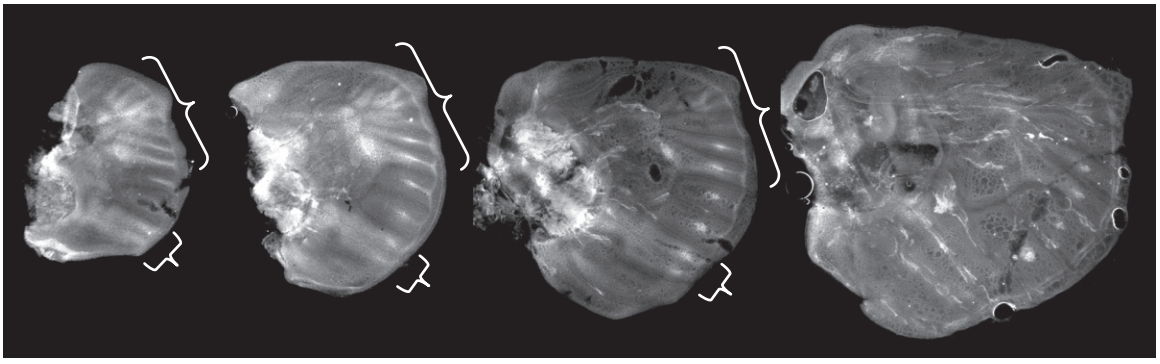


Figure 2

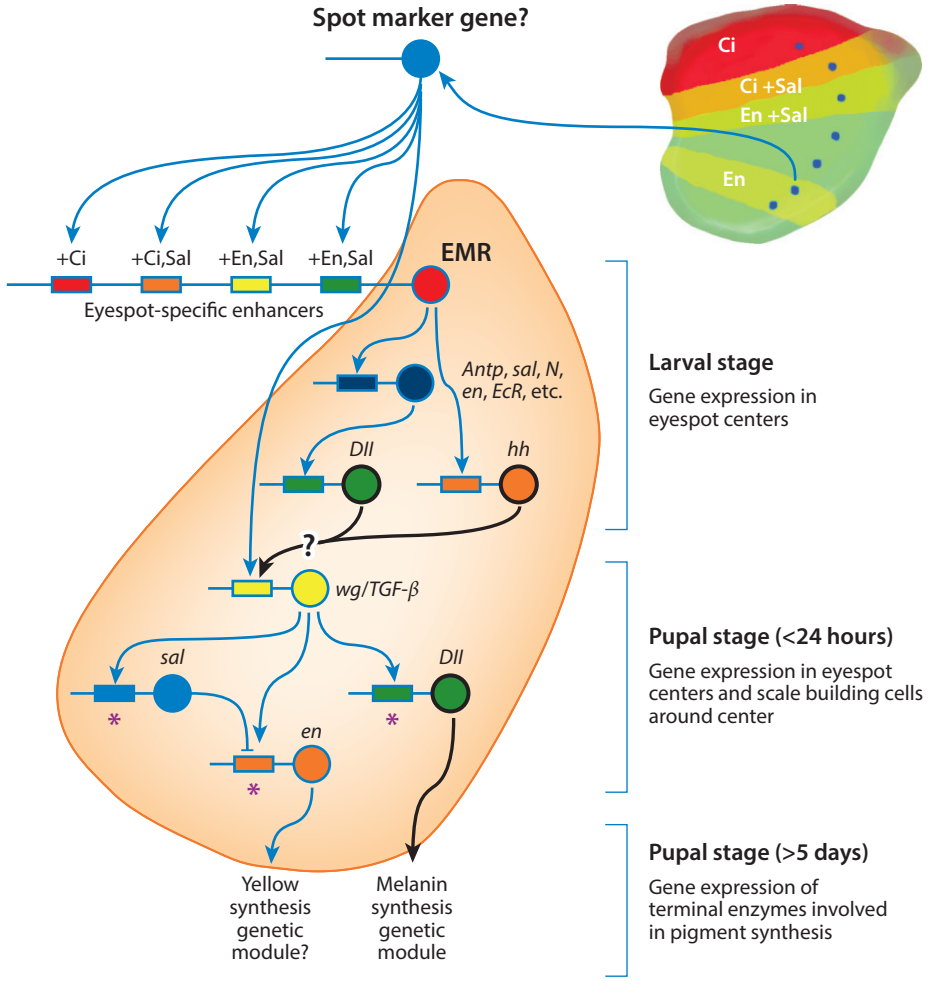
The transcription factor Spalt (Sal) is dynamically expressed in particular wing sectors during the larval stage. Sal is expressed in subsets of both anterior and posterior wing sectors in larval forewings of *Bicyclus anynana* (*braces*). This sector-specific expression, however, weakens as the wings grow larger. Sal's early asymmetric expression may contribute to individuating subsets of serial homologous eyespots across the wing. If functional genes in the eyespot network evolve binding sites for the Sal protein, this could allow the subset of eyespots developing in the sectors expressing Sal to acquire different sizes or positions along the wing margin, relative to eyespots in sectors lacking Sal. Depicted are antibody stains for a laboratory mutant, Spotty, that develops four eyespots on the forewing, instead of the normal two. Sal is additionally expressed in a dynamic pattern associated with the eyespot centers. Anti-Sal antibody was a courtesy of R. Barrio.

Dll has two temporally distinct expression domains in eyespots that appear to serve distinct functions. Expression of *Dll* in the cluster of cells associated with the center of the eyespots begins during the middle of the fifth instar (13). This expression domain persists well into the pupal stage (15, 42). However, around 20 h after pupation, a novel *Dll* expression domain appears around the central group of cells, in the area where a black ring will appear in adult eyespots (15, 42). The function of *Dll* in each of its areas of expression has been addressed with separate experiments.

Overexpressing *Dll* during the fifth instar larvae, using a transgenic *B. anynana* butterfly line with a second coding sequence for *Dll* placed downstream of a heat-shock promoter, led to larger eyespots in the adults (40), whereas downregulating this gene, using the same promoter driving a pin-loop *Dll* RNAi construct (17), led to smaller eyespots (40). Nucleotide variation at *Dll* had been previously associated with the regulation of eyespot size; i.e., individuals selected for larger eyespots disproportionately carried with them a particular nucleotide variant at the *Dll* locus, whereas those selected for small size carried a different nucleotide variant (5). This linkage association study together with the functional study suggests that genetic variation at this locus regulates variation in eyespot size via regulation of *Dll* expression levels at the larval stage of development.

Overexpressing *Dll* during the pupal stage led to eyespots with a wider black center, but similar in overall size to control eyespots, whereas ectopically expressing *Dll* in a patch of wing cells led to ectopic patches of black scales differentiating on the wing (40). These functional experiments show that late *Dll* expression contributes to the differentiation of the black ring of scales but not total eyespot size. In addition, the ectopic experiments indicate that *Dll* is able to activate a complete gene regulatory network leading to black pigmentation—most likely the melanin synthesis pathway. This network involves the coordinated up- and downregulation of multiple genes coding for pigmentation enzymes in individual scale cells (88, 89). These enzymes are responsible for modifying melanin pigment precursors, transported in the hemolymph, into melanin. The discovery that *Dll* can induce black pigment synthesis suggests that *Dll* is a master regulator of this gene regulatory network, at least in the context of the pupal wing (40).

Dll, however, may not be regulating the melanin pigment pathway genes directly. Previous molecular examination of an eyespot mutant in *B. anynana*, almost completely lacking back central scales and displaying golden-yellow scales throughout the eyespot field, suggests that *Dll* may be promoting melanization via the activation of another critical gene, *sal*. In Goldeneye mutants, *Dll* was expressed throughout the eyespot field during the pupal stage; however, *sal*, normally coexpressed with *Dll* in the region of black scales, was missing (15). This suggests that *Dll* can only differentiate black scales as long as *sal* is also expressed in the same cells. Although we currently lack functional data for *sal*, this is an especially interesting gene because it is also associated with black pigmentation in butterfly lineages with more basal branching to the nymphalids, the pierids, whereas *Dll* is not (42, 77). The wing melanization mechanism present in the ancestor of both pierids and *Bicyclus* butterflies is not known, but several scenarios are possible. Perhaps both *sal* and *Dll* were expressed in the black scales of such an ancestor, with *Dll* having a redundant function in differentiating black scales, allowing for the loss of this gene in pierids. Alternatively, a single gene, *sal*, was expressed in black patches in the ancestor, and *Dll* was subsequently co-opted to those patches in the nymphalid lineage. The co-option of *Dll* had to occur upstream of *sal*, enabling it



to activate the melanin pathway via *sal* activation. Only additional comparative functional work will shed light on these alternative hypotheses.

Comparative functional work in other members of the Lepidoptera is currently lacking, but findings from comparative functional research in flies are available. Of particular interest is the recent demonstration that *Dll* overexpression across the wing of *Drosophila biarmipes* led to darker pigmentation, whereas *Dll* downregulation via RNAi removed a dark spot from the wing tip (2). Furthermore, the ectopic expression of another gene, coding for the ligand Wingless, also led to black patches of pigmentation in a different species of *Drosophila* (85). In butterflies, *wingless* is expressed in the eyespot centers during the period of eyespot signaling (<16 h after pupation), slightly before *Dll* and *sal* expression are visualized in the surrounding disc of cells (42). These results are intriguing and suggest that a modular gene regulatory network involved in melanin synthesis—and with the ability to be activated by *Dll*, *sal*, and/or *wingless*—may have evolved prior to the split of the Diptera from the Lepidoptera and been used to specify dark patches of pigmentation in both lineages. Alternatively, the pigmentation network evolved regulatory control by some of the same molecules, independently and in parallel in the fly and butterfly lineages. Only additional comparative functional work will shed light on this issue.

The second eyespot-associated gene that has been functionally tested codes for the ligand Hedgehog (Hh) (80). Hh mRNA was visualized in cells surrounding the eyespot centers of *J. coenia* during late larval development (26). The expression of this gene, however, is lacking in the eyespots of a different species of nymphalid, *B. anynana* (69), indicating that Hh is not associated with eyespot development across all species. In *J. coenia*, however, this gene appears to be functional. The sequestration of Hh ligand via hemolymph injections of a Hh-binding antibody during the larval stages of wing development led to smaller eyespots, indicating that Hh is a positive regulator of eyespot size in this species (80). Comparative expression studies of *hh* are currently lacking, so it is unclear whether this gene was part of the initial set of genes that was co-opted to the eyespot centers concurrently with the origination of eyespots and whose expression in eyespots was subsequently

Figure 3

Proposed model for the genetic and regulatory architecture of the eyespot gene regulatory network. This model is based on an earlier model (44) but incorporates new knowledge about the function of two genes (*Distal-less*, *Dll*; and *hedgehog*, *hh*), highlighted with bold black outlines, as well as additional insights from new gene expression studies. Other depicted genes are expressed in eyespots, but their function has yet to be investigated. A spot marker gene and an eyespot master regulatory (EMR) gene have not yet been identified and are hypothesized to be involved in spot and eyespot development, respectively. The co-option of a network of prewired genes to the location of primitive spots may have been facilitated by the expression of a spot marker gene (*blue*), primitively marking the center of each wing sector. The spot gene can alternatively represent a suite of veins, activators, and repressors that may be involved in differentiating cells at the center of a wing sector. The co-option of the network to particular wing sectors may be due to the evolution of novel positional information (i.e., novel enhancers) in the EMR gene that allowed it to respond to the spot gene as well as to the particular combination of transcription factors present in that wing sector, such as Engrailed (En), Spalt (Sal), and Cubitus interruptus (Ci) (*right*). The presence of sector-specific enhancers in the EMR gene, receiving input from the sector-specific transcription factors, may help eyespots evolve unique identities with regard to size. The co-option of the EMR gene to the spot location brought with it a network of other genes (highlighted with an orange background) that helped create an eyespot. Regulatory interactions depicted in blue are inferred but have not been validated. The presence of single enhancers for genes in the middle of the network (highlighted with asterisks), coupled with the potential absence of sector-specific transcription factors during later stages of pupal development, may prevent eyespots from acquiring distinct identities with regard to color composition in later stages of eyespot development. Other abbreviations: *Antp*, *Antennapedia*; *EcR*, *Ecdysone receptor*; *N*, *Notch*; *TGF-β*, *Transforming growth factor beta*; *wg*, *wingless*.

lost in *B. anynana*, or whether *hh* was co-opted to eyespots only in some derived lineages, as appears to be the case with the hox gene *Antennapedia* (55, 70). Either of these evolutionary scenarios suggests that the gene was not initially required for eyespot origination but became functional only in derived lineages, such as the one leading to *J. coenia* (80).

Aside from being implicated by functional experiments, a few other loci and signaling pathways are implicated in eyespot development owing to examination of mutations that lead to alterations both in eyespot morphology and in other traits where the developmental players are well known. For instance, three separate mutations that map to the same locus in *B. anynana* cause large aberrations in eyespot size and/or color composition in heterozygotes but also disrupt early embryonic segmentation when in homozygote condition (68). Because the observed embryonic defects are similar to those in individuals with mutations for any of a series of regulators of the Wingless signaling pathway in *Drosophila*, this pathway was indirectly implicated in eyespot development in butterflies (68).

Hox genes: genes that regulate the identity of body regions along the anterior-posterior axis across all bilaterians

Enhancer: a *cis*-regulatory element; i.e., a discrete region of DNA that, when bound by transcription factors, directs transcription of nearby genes in specific patterns at specific times in development

Selector gene: a gene that controls cell fate

EVOLUTION OF EYESPOT NUMBER, MORPHOLOGY, AND SEXUAL DIMORPHISM

Whereas the number of wing sectors that became competent to differentiate eyespots has increased over evolutionary time, detailed analyses of eyespot number evolution within single genera show that species are both losing and gaining eyespots from multiple wing surfaces at a fast pace (27, 54). In the subtribe Junoniini, for instance, eyespots on the dorsal hindwing have been lost and regained in the same wing sectors multiple times. The same is happening in the genus *Bicyclus* (54). Furthermore, in *Bicyclus*, eyespot number is evolving at sex-specific rates and at a faster rate (more losses and gains) on the dorsal and forewing surfaces compared with the ventral or hindwing surfaces of the wing, where eyespots are more stably expressed (54). Presumably these surface- and sex-specific rates of eyespot losses and gains relate to the eyespot's dual role in sexual signaling and predator interactions in this genus; eyespots on private surfaces (the dorsal surfaces and the forewing, which are often hidden at rest) appear to be evolving under labile patterns of sexual selection, whereas those on exposed surfaces (i.e., the ventral surfaces and the hindwing) are under strong stabilizing selection in both sexes (54).

The molecular basis for the rapid appearance and disappearance of eyespots in particular sectors of the wing is currently not understood. Presumably, some set of mutations enable or disable the eyespot gene regulatory network from functioning in specific wing sectors and act as on-off switches (44). The disabling mutations are likely reversible because eyespots are able to reappear in those sectors after having been absent (27, 54). One possibility for such lability could be transposable elements jumping in and out of important regulatory loci, alternately rendering them nonfunctional and then functional. Genetic data supporting this or any other explanation for eyespot number evolution are currently lacking. The affected loci could be of two types. Either they are sector-specific enhancers of a master regulatory gene (MRG) responsible for deploying the complete network in particular sectors of the wing (**Figure 3**), or the loci are protein-coding sequences of selector genes that are differentially expressed in particular sectors of the wing and that interact with the eyespot gene regulatory network to up- or downregulate it just in those sectors (**Figures 2** and **3**). Disruptions to the first type of loci (enhancers of a MRG) will presumably lead to fewer pleiotropic effects compared with disruptions in genes expressed in wing sectors, as these latter genes are likely also involved in positioning wing veins (9, 10).

A recent survey of presence and absence of eyespots across nearly 450 nymphalids found a large degree of sexual dimorphism in these traits: Nearly 80% of the species with eyespots (278) were

sexually dimorphic for eyespot number (78). The sexual dimorphism happens in two directions: In the majority of cases females display more eyespots; in the minority males do. In addition, dorsal surfaces of both forewings and hindwings and ventral surfaces of forewings display relatively few eyespots but are disproportionately more sexually dimorphic than ventral hindwing surfaces (78).

Sexual dimorphism in eyespots can result either from natural selection acting differentially in each sex, as shown recently in *Junonia evarete* (63), or from the action of sexual selection, as observed in *B. anynana* (65, 66, 86). Additional experiments testing both modes of selection in a broader set of species are needed. Only a much larger comparative study will allow us to understand how eyespot function has been evolving and has been partitioned across wing surfaces over time.

At the proximate level, sexual dimorphism in eyespots indicates that the eyespot gene regulatory network has gained the ability to be modified in a sex-specific fashion, presumably via input from genes from the sex determination pathway (29, 87). One such gene, *doublesex*, was recently shown to be a key regulator for the development of male- and female-specific patterns in a species of *Papilio* butterfly (30), and preliminary data show that *doublesex* is expressed in eyespots. Future work will head further in this direction. In addition, given that in genera such as *Bicyclus* and *Junonia* eyespots can originate in both sexes and then be lost in a single sex, or, alternatively, can originate from the very beginning in a single sex (53), studying the molecular basis of eyespot presence/absence across species should be coupled with a close understanding of the molecular mechanisms leading to sex-specific expression of eyespots.

DEVELOPMENTAL SYSTEMS BIASING AND CONSTRAINING THE EVOLUTION OF EYESPOTS

There is great morphological diversity of eyespots across butterfly species; however, recent studies have highlighted that certain types of diversity are rare and may stem from developmental constraints. The studies in question probed for particular types of genetic diversity within a species and related their findings to the morphological diversity observed in closely related species. The studies started with the observation that when directional artificial selection targets features of a single eyespot, most other eyespots change in a coordinated way. For instance, selection directed at size led to all eyespots becoming either larger or smaller (46), whereas selection directed at color ring composition led to all eyespots becoming either more black or more gold—i.e., they have a narrow exterior gold ring and a broad central disc of black scales, or the reverse, respectively (39). Given the correlated changes observed across all eyespots, the key studies that followed asked whether populations harbor genetic variation that is eyespot-specific; i.e., whether certain eyespots can vary independently from other eyespots in the same animal. Interestingly, these studies found that eyespots have dedicated genetic variation for size but not for color composition (1, 4, 6–8): Different sizes of eyespots can evolve, but not different color ring compositions. In addition, presence/absence of eyespot-specific genetic variation for these two traits within a species perfectly correlated with presence/absence of eyespot morphological diversity across closely related species (1).

Three recent key discoveries may inform the nature of these constraints. One is that variation in gene expression (*Dll* and *hh*) during the larval stage, rather than the pupal stage, alters eyespot size (40, 80); another is that eyespots originated as a small series of individualized modules that later colonized new wings and wing surfaces, presumably via *cis*-regulatory evolution of an eyespot master regulatory (EMR) gene (52). The third insight is that selector genes that may be involved in positioning wing veins in butterflies (such as *Spalt* expressed early in larval wings) appear to lose their sector-specific expression in older larval wings, before the pupal stage (**Figure 2**), when the eyespot rings differentiate (15).

Below I propose two (nonexclusive) testable hypotheses about why there should be limited eyespot-specific genetic variation affecting eyespot color composition but not size:

1. **The lack of selector gene expression differences along the anterior-posterior axis in pupal versus larval wings.** Multiple studies that have looked at the differentiation of serial homologs along a body axis have documented the requirement for differential expression of selector genes, such as hox genes, along that axis. These genes interact with serial homologs present in that region of the body and modify their development independently of the flanking serial homologs (3, 31, 79, 84). Perhaps important transcription factors, expressed only in certain wing sectors in *Drosophila* and involved in vein differentiation, are also present in the butterfly wing and are functioning in a similar fashion as hox genes—giving each sector of the wing blade a discrete identity. One example is *engrailed*, and a second example is *spalt*; both are essential genes for vein patterning in *Drosophila* (9), and both are expressed in only a subset of wing sectors in both fly and butterfly larval wings (**Figure 2**). These genes, owing to their asymmetric expression across the wing blade, could potentially be involved in modifying the expression of eyespot genes in a sector-specific fashion—for instance, leading to a sector-dependent regulation of genes involved in the control of eyespot size. The sector-specific expression of *spalt*, however, seems to disappear in late larval wings (**Figure 2**), perhaps because it is no longer required for vein positioning at this stage of development. If several sector-specific genes, such as *spalt*, are absent from the pupal wing, this may create a developmental constraint. This constraint would prevent the eyespot gene regulatory network from being further modulated in different sectors of the wing, especially at the critical point in development when eyespot color composition is being determined. Mutations entering the system, and altering color ring genes at this stage of development, would have the same effect across all eyespot modules, leading to more gold or more black eyespots across the entire wing blade.
2. **Sector-specific enhancers in a putative EMR gene may be appropriate mutational targets for size regulation but not color composition.** An alternative, but not exclusive, hypothesis to the one above could be related to the mechanism through which eyespots have increased in number on the wing since their origin. Perhaps the sequential appearance of eyespots on hindwings, forewings, and dorsal surface sectors involved the evolution of novel enhancer sequences in only a few key regulators (e.g., in an EMR gene) that allowed the network to be expressed in the *trans*-regulatory environment of the novel wing sectors (**Figure 3**). If these enhancers are discrete sequences, as is often the case with the enhancers of master regulators of serially repeated traits (33, 35), molecular evolution within each enhancer could allow for modulations of EMR gene expression independently in each wing sector and lead to sector-specific variation in eyespot size. In contrast, genes that are downstream in the network do not necessarily evolve sector-specific enhancers when the network is recruited to novel wing locations; they reuse the same old enhancers in the novel context (38, 43). This mechanism of eyespot network co-option translates to the absence of sector-specific enhancers in genes such as *Dll*, *sal*, and *en*, which differentiate the eyespot rings during later pupal stages of development (**Figure 1**). The absence of sector-specific dedicated enhancers for these genes would explain why there is no genetic variation available to change the color composition of an eyespot in opposite directions in different sectors of the wing.

CONCLUSIONS AND FUTURE DIRECTIONS

The natural history of eyespots is complex, only partly understood, and rich with potential for future discoveries. The recent use of well-established phylogenies and the comparative method

has pinpointed the origin of these complex traits in nymphalids to approximately 90 Mya. These analyses have also shown that eyespots started as a few units on the ventral hindwing that progressively got co-opted to new wing sectors over evolutionary time. The retention of eyespots in many lineages after their origin is explained by the important ecological role of eyespots in interactions with predators, primarily based on their conspicuousness and bull's-eye morphology of highly contrasting colors. Subsequent to their origin, eyespots moved to dorsal wing surfaces, where they currently function in sexual signaling. The high degree of sexual dimorphism (across multiple surfaces) indicates that sex-specific patterns of natural selection and/or sexual selection have been playing important roles in the diversification and maintenance of these traits. However, more research in needed to dissect which of these two forces plays a dominant role and whether these roles shift across lineages. In addition, there is endless research ahead for those mostly interested in the ecological significance of variation in eyespot number, position, and size across nymphalids. Associated with eyespot origins is the concurrent origin of expression of multiple genes in the center of these traits. Functional studies show that two of the genes that have been tested so far function in eyespot development, and one is sufficient to modify scale color when expressed ectopically on the pupal wing. Future comparative transcriptomic studies performed across out-group species without spots and in-group species with eyespots should provide a more comprehensive picture of eyespot network evolution and identify the subset of genes that must be contributing to eyespot origins. Another priority for the field is to map genetic variants involved in eyespot number variation and to identify the loci that may have had key roles in eyespot origins and that are potentially still involved in altering eyespot number across closely related species. A separate endeavor is to understand how eyespots in other lineages of moths and butterflies originated. Finally, this review highlights multiple untested models and hypotheses for eyespot development that beg for more empirical support. In summary, there is plenty of work ahead for those wishing to contribute to unraveling the fascinating evolutionary and developmental puzzle surrounding the origin and diversification of eyespots in the wings of Lepidoptera.

SUMMARY POINTS

1. Eyespots serve both to deter or deflect attacks by predators and to attract mates.
2. There was a single origin of eyespots within the nymphalid lineage of butterflies, roughly 90 Mya.
3. Eyespots originated as a cluster of four or five units on the ventral hindwing, and they only later appeared on the forewing and dorsal wing surfaces.
4. Multiple genes became expressed in eyespot centers concurrently with eyespot origins, suggesting a network co-option event.
5. One of the genes whose expression was retained in eyespots, *Distal-less*, is a positive regulator of eyespot size. *Distal-less* is also sufficient to differentiate black scales on the pupal wing.
6. Rates of gains and losses of eyespots vary between males and females and also between wing surfaces.
7. More work is necessary to explore the molecular basis of eyespot number evolution, sexual dimorphism, and the constraint in eyespot color composition observed for eyespots on the same individual.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Many of the insights described in this review stem from the work of outstanding postdoctoral fellows and graduate and undergraduate students with whom I have had the pleasure of collaborating over the years. I have learned much from them. In particular, much of the work surrounding eyespot origins is due to the contributions of Jeffrey Oliver; functional work was driven by Jeff Marcus, Bin Chen, Diane Ramos, Xiaoling Tong, Anna Lindemann, Andrew Stoehr, Joseph Walker, and Gary Glaser; and ecological work by Kendra Robertson, Katie Costanzo, Katy Prudic, Erica Westerman, and Andrea Hodgins-Davis. Thanks also to Ondrej Podlaha for helping with conceptual thinking around co-option and to William Piel for providing comments on the manuscript. Research in the lab is currently funded by the National University of Singapore and Yale-NUS-College, Singapore, and Singapore Ministry of Education Academic Research Fund Tier 1 grant R-154-000-602-112.

LITERATURE CITED

1. Allen CE, Beldade P, Zwaan BJ, Brakefield PM. 2008. Differences in the selection response of serially repeated color pattern characters: standing variation, development, and evolution. *BMC Evol. Biol.* 8:94
2. Arnoult L, Su KFY, Manoel D, Minervino C, Magrina J, et al. 2013. Emergence and diversification of fly pigmentation through evolution of a gene regulatory module. *Science* 339:1423–26
3. Averof M, Patel NH. 1997. Crustacean appendage evolution associated with changes in *Hox* gene expression. *Nature* 388:682–86
4. Beldade P, Brakefield PM. 2003. Concerted evolution and developmental integration in modular butterfly wing patterns. *Evol. Dev.* 5:169–79
5. Beldade P, Brakefield PM, Long AD. 2002. Contribution of *Distal-less* to quantitative variation in butterfly eyespots. *Nature* 415:315–17
6. Beldade P, French V, Brakefield PM. 2008. Developmental and genetic mechanisms for evolutionary diversification of serial repeats: eyespot size in *Bicyclus anynana* butterflies. *J. Exp. Zool. B* 310:191–201
7. Beldade P, Koops K, Brakefield PM. 2002. Developmental constraints versus flexibility in morphological evolution. *Nature* 416:844–47
8. Beldade P, Koops K, Brakefield PM. 2002. Modularity, individuality, and evo-devo in butterfly wings. *Proc. Natl. Acad. Sci. USA* 99:14262–67
9. Biehs B, Sturtevant MA, Bier E. 1998. Boundaries in the *Drosophila* wing imaginal disc organize vein-specific genetic programs. *Development* 125:4245–57
10. Bier E. 2000. Drawing lines in the *Drosophila* wing: initiation of wing vein development. *Curr. Opin. Genet. Dev.* 10:393–98
11. Blair SS. 2007. Wing vein patterning in *Drosophila* and the analysis of intercellular signaling. *Annu. Rev. Cell Dev. Biol.* 23:293–319
12. Brakefield PM, French V. 1995. Eyespot development on butterfly wings: the epidermal response to damage. *Dev. Biol.* 168:98–111
13. Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden PJ, et al. 1996. Development, plasticity, and evolution of butterfly eyespot patterns. *Nature* 384:236–42
14. Breuker CJ, Brakefield PM. 2002. Female choice depends on size but not symmetry of dorsal eyespots in the butterfly *Bicyclus anynana*. *Proc. R. Soc. B* 269:1233–39
15. Brunetti CR, Selegue JE, Monteiro A, French V, Brakefield PM, Carroll SB. 2001. The generation and diversification of butterfly eyespot color patterns. *Curr. Biol.* 11:1578–85

16. Carroll SB, Gates J, Keys DN, Paddock SW, Panganiban GEF, et al. 1994. Pattern formation and eyespot determination in butterfly wings. *Science* 265:109–14
17. Chen B, Hrycaj S, Schinko JB, Podlaha O, Wimmer EA, et al. 2011. Pogostick: a new versatile *piggyBac* vector for inducible gene over-expression and down-regulation in emerging model systems. *PLOS ONE* 6:e18659
18. Costanzo K, Monteiro A. 2007. The use of chemical and visual cues in female choice in the butterfly *Bicyclus anynana*. *Proc. R. Soc. B* 274:845–51
19. Darwin C. 1871. *The Descent of Man and Selection in Relation to Sex*. London: John Murray
20. Dilao R, Sainhas J. 2004. Modelling butterfly wing eyespot patterns. *Proc. R. Soc. B* 271:1565–69
21. Evans TM, Marcus JM. 2006. A simulation study of the genetic regulatory hierarchy for butterfly eyespot focus determination. *Evol. Dev.* 8:273–83
22. Freitas R, Zhang GJ, Cohn MJ. 2006. Evidence that mechanisms of fin development evolved in the midline of early vertebrates. *Nature* 442:1033–37
23. French V, Brakefield PM. 1992. The development of eyespot patterns on butterfly wings: morphogen sources or sinks? *Development* 116:103–9
24. French V, Brakefield PM. 1995. Eyespot development on butterfly wings: the focal signal. *Dev. Biol.* 168:112–23
25. Held LI. 2013. Rethinking butterfly eyespots. *Evol. Biol.* 40:158–68
26. Keys DN, Lewis DL, Selegue JE, Pearson BJ, Goodrich LV, et al. 1999. Recruitment of a *hedgehog* regulatory circuit in butterfly eyespot evolution. *Science* 283:532–34
27. Kodandaramaiah U. 2009. Eyespot evolution: phylogenetic insights from *Junonia* and related butterfly genera (Nymphalidae: Junoniini). *Evol. Dev.* 11:489–97
28. Kodandaramaiah U. 2011. The evolutionary significance of butterfly eyespots. *Behav. Ecol.* 22:1264–71
29. Kopp A. 2012. *Dmrt* genes in the development and evolution of sexual dimorphism. *Trends Genet.* 28:175–84
30. Kunte K, Zhang W, Tenger-Trolander A, Palmer DH, Martin A, et al. 2014. *doublesex* is a mimicry supergene. *Nature* 507:229–32
31. Liubicich DM, Serano JM, Pavlopoulos A, Kontarakis Z, Protas ME, et al. 2009. Knockdown of *Parhyale Ultrabithorax* recapitulates evolutionary changes in crustacean appendage morphology. *Proc. Natl. Acad. Sci. USA* 106:13892–96
32. Lyytinen A, Brakefield PM, Mappes J. 2003. Significance of butterfly eyespots as an anti-predator device in ground-based and aerial attacks. *Oikos* 100:373–79
33. Marcellini S, Simpson P. 2006. Two or four bristles: functional evolution of an enhancer of *scute* in Drosophilidae. *PLOS Biol.* 4:2252–61
34. Marcus JM, Evans TM. 2008. A simulation study of mutations in the genetic regulatory hierarchy for butterfly eyespot focus determination. *Biosystems* 93:250–55
35. McGregor AP, Orgogozo V, Delon I, Zanet J, Srinivasan DG, et al. 2007. Morphological evolution through multiple *cis*-regulatory mutations at a single gene. *Nature* 448:587–90
36. Merilaita S, Vallin A, Kodandaramaiah U, Dimitrova M, Ruuskanen S, Laaksonen T. 2011. Number of eyespots and their intimidating effect on naive predators in the peacock butterfly. *Behav. Ecol.* 22:1326–31
37. Monteiro A. 2008. Alternative models for the evolution of eyespots and of serial homology on lepidopteran wings. *BioEssays* 30:358–66
38. Monteiro A. 2012. Gene regulatory networks reused to build novel traits. *BioEssays* 34:181–86
39. Monteiro A, Brakefield P, French V. 1997. Butterfly eyespots: the genetics and development of the color rings. *Evol. Int. J. Org. Evol.* 51:1207–16
40. Monteiro A, Chen B, Ramos DM, Oliver JC, Tong X, et al. 2013. *Distal-less* regulates eyespot patterns and melanization in *Bicyclus* butterflies. *J. Exp. Zool. B* 320:321–31
41. Monteiro A, French V, Smit G, Brakefield P, Metz J. 2001. Butterfly eyespot patterns: evidence for specification by a morphogen diffusion gradient. *Acta Biotheor.* 49:77–88
42. Monteiro A, Glaser G, Stockslager S, Glansdorp N, Ramos D. 2006. Comparative insights into questions of lepidopteran wing pattern homology. *BMC Dev. Biol.* 6:52
43. Monteiro A, Podlaha O. 2009. Wings, horns, and butterfly eyespots: How do complex traits evolve? *PLOS Biol.* 7(2):e1000037

44. Monteiro A, Prijs J, Bax M, Hakkaart T, Brakefield P. 2003. Mutants highlight the modular control of butterfly eyespot patterns. *Evol. Dev.* 5:180–87
45. Monteiro A, Prudic KL. 2010. Multiple approaches to study color pattern evolution in butterflies. *Trends Evol. Biol.* 2:e2
46. Monteiro AF, Brakefield PM, French V. 1994. The evolutionary genetics and developmental basis of wing pattern variation in the butterfly *Bicyclus anynana*. *Evol. Int. J. Org. Evol.* 48:1147–57
47. Nijhout HF. 1978. Wing pattern formation in Lepidoptera—a model. *J. Exp. Zool.* 206:119–36
48. Nijhout HF. 1980. Pattern formation on lepidopteran wings: determination of an eyespot. *Dev. Biol.* 80:267–74
49. Nijhout HF. 1990. A comprehensive model for colour pattern formation in butterflies. *Proc. R. Soc. B* 239:81–113
50. Nijhout HF. 1991. *The Development and Evolution of Butterfly Wing Patterns*. Washington, DC: Smithson. Inst. Press
51. Nijhout HF. 2001. Elements of butterfly wing patterns. *J. Exp. Zool.* 291:213–25
52. Oliver JC, Beaulieu JM, Gall LF, Piel WH, Monteiro A. 2014. Nymphalid eyespot serial homologues originate as a few individualized modules. *Proc. R. Soc. B* 281:20133262
53. Oliver JC, Monteiro A. 2011. On the origins of sexual dimorphism in butterflies. *Proc. R. Soc. B* 278:1981–88
54. Oliver JC, Robertson KA, Monteiro A. 2009. Accomodating natural and sexual selection in butterfly wing pattern evolution. *Proc. R. Soc. B* 276:2369–75
55. Oliver JC, Tong X-L, Gall LF, Piel WH, Monteiro A. 2012. A single origin for nymphalid butterfly eyespots followed by widespread loss of associated gene expression. *PLOS Genet.* 8:e1002893
56. Olofsson M, Jakobsson S, Wiklund C. 2013. Bird attacks on a butterfly with marginal eyespots and the role of prey concealment against the background. *Biol. J. Linn. Soc.* 109:290–97
57. Olofsson M, Lovlie H, Tibblin J, Jakobsson S, Wiklund C. 2013. Eyespot display in the peacock butterfly triggers antipredator behaviors in naive adult fowl. *Behav. Ecol.* 24:305–10
58. Olofsson M, Vallin A, Jakobsson S, Wiklund C. 2010. Marginal eyespots on butterfly wings deflect bird attacks under low light intensities with UV wavelengths. *PLOS ONE* 5(5):e10798
59. Otaki JM. 2011. Color-pattern analysis of eyespots in butterfly wings: a critical examination of morphogen gradient models. *Zool. Sci.* 28:403–13
60. Otaki JM. 2011. Generation of butterfly wing eyespot patterns: a model for morphological determination of eyespot and parafocal element. *Zool. Sci.* 28:817–27
61. Otaki JM. 2012. Color pattern analysis of nymphalid butterfly wings: revision of the nymphalid ground-plan. *Zool. Sci.* 29:568–76
62. Otaki JM, Ogasawara T, Yamamoto H. 2005. Morphological comparison of pupal wing cuticle patterns in butterflies. *Zool. Sci.* 22:21–34
63. Pinheiro CEG, Antezana MA, Machado LP. 2014. Evidence for the deflective function of eyespots in wild *Junonia evarete* Cramer (Lepidoptera, Nymphalidae). *Neotrop. Entomol.* 43:39–47
64. Poulton EB. 1890. *The Colours of Animals: Their Meaning and Use, Especially Considered in the Case of Insects*. London: Kegan Paul, Trench, Trübner
65. Prudic KL, Jeon C, Cao H, Monteiro A. 2011. Developmental plasticity in sexual roles of butterfly species drives mutual sexual ornamentation. *Science* 331:73–75
66. Robertson KA, Monteiro A. 2005. Female *Bicyclus anynana* butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. *Proc. R. Soc. B* 272:1541–46
67. Ruvinsky I, Gibson-Brown JJ. 2000. Genetic and developmental bases of serial homology in vertebrate limb evolution. *Development* 127:5233–44
68. Saenko SV, Brakefield PM, Beldade P. 2010. Single locus affects embryonic segment polarity and multiple aspects of an adult evolutionary novelty. *BMC Biol.* 8:111d
69. Saenko SV, Marialva MSP, Beldade P. 2011. Involvement of the conserved *Hox* gene *Antennapedia* in the development and evolution of a novel trait. *EvoDevo* 2:9
70. Shirai LT, Saenko SV, Keller RA, Jeronimo MA, Brakefield PM, et al. 2012. Evolutionary history of the recruitment of conserved developmental genes in association to the formation and diversification of a novel trait. *BMC Evol. Biol.* 12:21

71. Sourakov A. 2013. Two heads are better than one: False head allows *Calycopis cecrops* (Lycaenidae) to escape predation by a jumping spider, *Phidippus pulcherrimus* (Salticidae). *J. Nat. Hist.* 47:1047–54
72. Stevens M. 2005. The role of eyespots as anti-predator mechanisms, principally demonstrated in the Lepidoptera. *Biol. Rev.* 80:573–88
73. Stevens M, Hardman CJ, Stubbins CL. 2008. Conspicuousness, not eye mimicry, makes "eyespots" effective antipredator signals. *Behav. Ecol.* 19:525–31
74. Stevens M, Hopkins E, Hinde W, Adcock A, Connolly Y, et al. 2007. Field experiments on the effectiveness of 'eyespots' as predator deterrents. *Anim. Behav.* 74:1215–27
75. Stevens M, Ruxton GD. 2014. Do animal eyespots really mimic eyes? *Curr. Zool.* 60:26–36
76. Stevens M, Stubbins CL, Hardman CJ. 2008. The anti-predator function of 'eyespots' on camouflaged and conspicuous prey. *Behav. Ecol. Sociobiol.* 62:1787–93
77. Stoehr AM, Walker JF, Monteiro A. 2013. Spalt expression and the development of melanic color patterns in pierid butterflies. *EvoDevo* 4:6
78. Tokita CK, Oliver JC, Monteiro A. 2013. A survey of eyespot sexual dimorphism across nymphalid butterflies. *Int. J. Evol. Biol.* 2013:926702
79. Tomoyasu Y, Wheeler SR, Denell RE. 2005. *Ultrabithorax* is required for membranous wing identity in the beetle *Tribolium castaneum*. *Nature* 433:643–47
80. Tong X, Lindemann A, Monteiro A. 2012. Differential involvement of Hedgehog signaling in butterfly wing and eyespot development. *PLOS ONE* 7:e51087
81. Vallin A, Jakobsson S, Lind J, Wiklund C. 2005. Prey survival by predator intimidation: an experimental study of peacock butterfly defence against blue tits. *Proc. R. Soc. B* 272:1203–7
82. Vlieger L, Brakefield PM. 2007. The deflection hypothesis: Eyespots on the margins of butterfly wings do not influence predation by lizards. *Biol. J. Linn. Soc.* 92:661–67
83. Wahlberg N, Leneveu J, Kodandaramaiah U, Pena C, Nylin S, et al. 2009. Nymphalid butterflies diversify following near demise at the cretaceous/tertiary boundary. *Proc. R. Soc. B* 276:4295–302
84. Weatherbee SD, Halder G, Kim J, Hudson A, Carroll S. 1998. Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the *Drosophila* haltere. *Genes Dev.* 12:1474–82
85. Werner T, Koshikawa S, Williams TM, Carroll SB. 2010. Generation of a novel wing colour pattern by the Wingless morphogen. *Nature* 464:1143–48
86. Westerman EL, Hodgins-Davis A, Dinwiddie A, Monteiro A. 2012. Biased learning affects mate choice in a butterfly. *Proc. Natl. Acad. Sci. USA* 109:10948–53
87. Williams TM, Carroll SB. 2009. Genetic and molecular insights into the development and evolution of sexual dimorphism. *Nat. Rev. Genet.* 10:797–804
88. Wittkopp PJ, Beldade P. 2009. Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. *Semin. Cell Dev. Biol.* 20:65–71
89. Wittkopp PJ, True JR, Carroll SB. 2002. Reciprocal functions of the *Drosophila* yellow and ebony proteins in the development and evolution of pigment patterns. *Development* 129:1849–58

Annual Review of Entomology

Volume 60, 2015

Contents

Indexes

Errata

An online log of corrections to *Annual Review of Entomology* articles may be found at http://www.annualreviews.org/errata/ento