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# **Eco-evo-devo advances with butterfly eyespots** Patrícia Beldade<sup>1,2</sup> and Antónia Monteiro<sup>3,4</sup>



Eyespots on the wings of different nymphalid butterflies have become valued models in eco-evo-devo. They are ecologically significant, evolutionarily diverse, and developmentally tractable. Their study has provided valuable insight about the genetic and developmental basis of inter-specific diversity and intra-specific variation, as well as into other key themes in evoevo-devo: evolutionary novelty, developmental constraints, and phenotypic plasticity. Here we provide an overview of ecoevo-devo studies of butterfly eyespots, highlighting previous reviews, and focusing on both the most recent advances and the open questions expected to be solved in the future.

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The colour patterns on butterfly wings have fascinated biologists and lay people, and inspired authors, artists, and advertisers. These patterns arise from the spatial distribution of projections of epidermal cells, called scales, which are monochromatic and are arranged like tiles on a roof on each side of a wing. Most studies of butterfly wing patterns have focused on species from the family Nymphalidae, where colour patterns are composed of different types of pattern elements repeated along the antero-posterior axis of the wing [1,2]. Among the types of pattern elements, eyespots have received considerable research attention, likely due to both their appearance and developmental tractability using surgical manipulations of developing wings. Eyespots are composed of concentric rings of different colours and get their name because their appearance can be reminiscent of vertebrate eyes. Eyespots display enormous diversity (Figure 1), and have served as excellent models for a range of eco-evo-devo studies.

# Ecological significance Predator avoidance

A variety of field and laboratory experiments have provided evidence that eyespots offer protection against predators. Eyespots often appear on wings in two main configurations: few and large versus many and small [4], believed to correspond to different functions in predator avoidance. Few and large eyespots work as intimidation elements that scare off predators [5,6], whereas many and small eyespots divert predator's attacks towards the wing margin and away from the body [7–11]. The actual colour of eyespot rings [12<sup>•</sup>] and the UV signal at eyespot centers [13] are believed to affect their conspicuousness to predators. Predators tested so far in the lab include birds [6-8], mantids [9], lizards [14], and geckos and skinks [11]. However, we are far from knowing what the actual natural predator species are for different eyespot-bearing butterflies, and how evespots appear to them.

### Mate-signaling

Eyespots are also used in sexual signaling. Experiments with the seasonal forms of *Bicyclus anynana* have shown that wet-season females prefer males with intact dorsal UV-reflective eyespot centers, and dry season males prefer females with intact ventral and dorsal eyespot centers [15,16]. To date, the role of eyespots in mate choice experiments has only been tested in this species, leaving scope for broader experimental analysis, targeting other species and variation in properties other than eyespot centers. In particular, it would be valuable to study species with sexually dimorphic eyespot patterns (e.g. in Ref. [17]). Advances in the study of butterfly vision [18] are expected to aid in understanding the involvement of eyespots in intra-specific communication.

### Development Stages

Eyespot development can be divided into a sequence of four sequential steps (Figure 2a), including two separate patterning processes (steps 1 and 2 in Figure 2a). First, in late larval wings, after veins have differentiated, eyespot centers differentiate in the center of each eyespot-bearing wing sector (Figure 3a). This presumably involves positional information conferred by the wing margin and/or wing veins, which can function as sources or sinks of diffusible signals. Second, in early pupal wings, cells at the presumptive eyespot centers work as organizers





Diversity of eyespot patterns across nymphalid butterflies, where eyespots have originated and diversified. Sections of the ventral surface of the hindwing of various nymphalid species illustrate variation in different aspects of eyespot patterns, including eyespot number (e.g. 2 in panel m and 8 in panel k), position (e.g. panel e versus j; and different eyespots within panel I), size (e.g. panel c versus n), as well as number and colour of eyespot rings (e.g. panel g versus h). (a) *Megisto cymela*; (b) *Cithaerias pireta*; (c) *Taenaris catops*; (d) *Hamadryas arinome*; (e) *Caligo eurilochus*; (f) *Morpho portis*; (g) *Orsotriaena medus*; (h) *Mycalesis patnia*; (i) *Melitaea cinxia*; (j) *Moduza procris*; (k) *Agrias hewitsonius*; (l) *Asterocampa leilia*; (m) *Dynamine serina*; (n) *Lethe minerva*; (o) *Diaethria clymena*; (p) *Mycalesis terminus*. Images from Ref. [3].

providing surrounding cells with information about their distance to the center, which will later translate to different colour rings. This process presumably involves cells at the eyespot center working as sources (or sinks) of signals (Figure 3b) that lead to the activation of different transcription factors (step 3 in Figure 2a) that activate the production of pigments of different colours (step 4 in Figure 2a) in rings around the center. Facilitated by the fact that eyespot patterning is a two-dimensional process, different studies have modelled the two patterning processes (Figure 3). While experimental data are still patchy, studies that combine modelling with analysis of gene expression and gene function (see Ref. [21<sup>•</sup>]) hold great promise to help narrow the gap between models and data.

# Genes

Much of what is known about the genes involved in eyespot development came from studies of candidate genes, with analysis of expression patterns (using *in situ* 





#### Eyespot development and eyespot variants.

(a) Steps in eyespot formation, including genes implicated in each of them and whose function has been confirmed by gene manipulations [19°,20,21°,22–27]. Analysis of gene expression and function in developing wings have identified a number of genes involved in eyespot development, including transcription factors Antennapedia (Antp), Distalless (DII), Ultrabithorax (Ubx), and Spalt (SaI), the signaling molecule Wingless (Wg), the Ecdysone Receptor (EcR), and melanogenesis enzymes Ebony (E), Yellow (Y), Dopadercaboxylase (Ddc) and Aralkylamine *N*-Acetyltransferase (aaNAT). Wg, marked with '?' might have a role in either steps 1 or 2. For each of the steps, the box to the left represents a wing section bordered by wing veins and the wing margin at the center of which an eyespot will form (see also Figure 3). The presumptive eyespot center (grey circle) is established in larval wings (step 1), and it signals to the cells around it in early pupal wings (gradient of grey in step 2). In response to these signals, epidermal cells express specific transcription factors in rings around the center (grey rings in step 3) and become committed to synthesizing different colour pigments in late pupal wings (colour rings in step 4). (b) Sections of the ventral surface of hindwings of 'wildtype' and various spontaneous mutants isolated in *B. anynana* laboratory populations illustrate intra-specific variation in multiple aspects of eyespot patterns (see extended mutant collection in Ref. [28]), including number (*Cyclops* and *3* + 4), shape (*Cyclops* and *comet*), size of some or all eyespots (*067*, *Pineye*, *Bigeye*), colour rings (*Frodo*), as well as wing pigmentation (*No Pigment*). Future work will continue to link phenotypic variants (b) to changes in development and its underlying genes (a).

hybridization and/or immunohistochemistry) and of gene function (using transgenesis for inducing ectopic expression or repression, and, more recently, using CRISPR-Cas9 for inducing loss-of-function mutations). The latter method has already functionally implicated several candidate genes in each of the four steps of evespot development (examples in Figure 2a), including the Hox genes Antennapedia (Antp) and Ultrabithorax (Ubx) in evespot center establishment [19<sup>•</sup>]. Testing the involvement of other candidate genes is undoubtedly forthcoming and will include the putative role of Decapentaplegic (Dpp) as a long-range signal. It will also include the roles of Spalt (Sal) and Engrailed paralogs (En) as patterning genes that respond to that signal and activate synthesis of different pigments in rings around the center. Importantly, as the eyespotpatterning function of more and more genes is revealed, it will be crucial to be able to understand the interactions between them. Achieving this will require

establishing how they are organized in networks, as well as identifying the regulatory elements that underlie such organization.

# Variation and diversification Phenotypes

Eyespot patterns differ greatly across species (Figure 1) and also within species, with differences between sexes, geographical and seasonal populations, as well as between individuals of the same sex and the same population (Figure 2b). Variation has been documented in eyespot number, shape, position, and size, as well as in eyespot ring number, colour, relative width, and symmetry. Comparative studies across species have identified patterns of eyespot variation and co-variation, while experimental studies, in a few laboratory models, have linked phenotypic variation to variation in the mechanisms underlying eyespot development (Figure 2). Much of what we know about the genetic and developmental basis of eyespot





Models of positional information for placing eyespot centers in larval wings and eyespot rings around those centers in pupal wings. The diagram to the right of each type of model represents one wing sector at the center of which an eyespot will be found in adult wings (see also Figure 2a). (a) Models for how two types of signaling molecules (activator and inhibitor, or activator and substrate) define concentration gradients and determine where eyespot foci will differentiate in larval wings, at the center of each wing-sector bordered by veins and the wing margin. The models differ in boundary conditions, with each of the boundaries working as sources or sinks of the activator signal. Models also differ in outcome, including whether maximum concentration of the two signals are co-localized or anti-localized, and if a steady-state is generated. a1: [29,30], a2: [31], a3: [21\*]. The reaction-diffusion model of a1 uses two chemical morphogens, an 'activator' that activates itself and a 'repressor' that represses the activator. Both substances can diffuse freely but the inhibitor diffuses at a higher rate. The grass-fire model of a2 uses a substrate (or fuel) that is transformed into a product as the two substances diffuse over the field, more like model a3, which also uses a substrate and a product. In a1, starting from a system at steady state, extra activator is produced along wing veins. In a2, from a system at steady state, the fuel starts to be burned along the wing veins, and at higher rates at more distal positions. In a3, the substrate, initially uniformly distributed, starts to be converted into a product along the wing margin. The a3 model is the only one supported by gene expression and gene-functional perturbation data. (b) Models for how evespot centers generate information to place concentric rings of different colours around them. The models differ in what way presumptive eyespot centers work as eyespot organizers: acting places where diffusible signals are produced (b1: [29]) or degraded (b2: [32]) to generate gradients of signal concentration or acting as mechanical sources of physical distortions that lead to calcium waves (b3: [33]). The gradient model supports results from disruptions of central signaling cells that lead to the differentiation of only the outer rings of colour (e.g. inner ring cells responding to lower morphogen concentrations). The sink model (b2) is supported by damage experiments to the epidermis leading to ectopic eyespots. There is still no experimental support for the b3 model, only measurements of physical properties of central cells and of spontaneous calcium waves.

variation relied on studies of captive *B. anynana* populations, including phenotypic variants generated by artificial selection on quantitative variation and spontaneous mutations of large effect (Figure 2b). Through a combination of gene expression, gene functional analyses, and tissue micro-dissections, we have been able to associate variation in eyespot phenotype to the different steps of eyespot development. For example, changes in eyespot number and shape are reflected in changes in eyespot size and colour ring size can reflect changes in signal strength and/or epidermal response thresholds in pupal wing development [34]. Through a combination of experimental crosses, genetic mapping, and analysis of gene expression and gene function, researchers have also been able to map loci harbouring allelic variation responsible for variation in eyespot phenotype, including the contribution of Distal-less (Dll) to quantitative variation in eyespot size [35], multiple loci contributing to variation in eyespot number [36\*], and mapping eyespot mutants [37].

### Origin

Eyespots vary in number and location on butterfly wings and the patterns of phenotypic variation have a strong phylogenetic signal. Ancestral-state reconstructions have proposed that eyespots originated first on ventral surfaces of hindwings and later appeared in forewings and on dorsal surfaces [38–40]. The current genetic model of eyespot evolution across wings and wing surfaces proposes an initial co-option of a gene network that required Ultrabithorax (Ubx) input to function, a gene that is expressed on insect hindwings but not forewings, followed by the input from Antennapedia (Antp) to be moved to the forewing [19<sup>•</sup>], and finally the repression of Apterous A (ApA) in eyespot centers on dorsal wing surfaces to allow eyespots to emerge there too [41].

# Eyespots as models for key eco-evo-devo themes Plasticity

Evespot size plasticity in response to temperature in satyrid butterflies is a classic example of developmental plasticity and seasonal polyphenism. In Bicyclus butterflies, for instance, high temperatures promote the development of large conspicuous eyespots, whereas low temperatures lead to small eyespots with rings of duller colours [12°,27,42–44]. Previous studies established an association between seasonal plasticity in eyespot patterns and alternative seasonal strategies for predator avoidance (see Box 1 in Ref. [44]). Evespot size plasticity is mediated by temperature effects on the levels and dynamics of the molting hormone, 20E, which regulates evespot size via signaling through the EcR receptor present in cells at the eyespot center [26,27,45]. A recent comparative study shed new light onto the evolutionary origin of thermal plasticity in eyespot development [46<sup>•</sup>]. This study with 13 eyespot-bearing and outgroup species showed that while all species showed increases in 20E levels with increasing temperature, and many expressed EcR in their presumptive evespot centers, increases in eyespot size in response to increasing temperature only occurred in satyrids. This finding led to the suggestion that an essential functional connection between EcR signaling and eyespot development genes evolved only along the satyrid lineage. We also do not know what genes involved in eyespot development are downstream of an active 20E-EcR complex that allows 20E to affect the size of eyespots. Techniques applied recently to butterflies, such as Chip-Seq [47], should aid in those explorations.

### Novelty

Nymphalid eyespots provide an example of the genetic and developmental origin of novelty. Many genetic commonalities have been identified between eyespot development and more ancestral developmental processes, including those implicated in appendage, embryonic, and wing patterning, as well as in wound healing [48– 51]. It has been argued that these commonalities reflect the co-option of 'old genes' for the evolution of new traits, but it seems more likely it is 'old gene networks', rather than individual genes, that were co-opted for eyespot formation. Most of the conserved genes proposed to have been recruited for eyespot development play some role in insect wing development (Figure 2), with the exception of Antp, which is not expressed in the epidermis of the developing wing blade of other insects [52]. This case illustrates how co-option can work through the acquisition of novel expression patterns into the same or novel tissues. Open questions about the origin of eyespots include which and by what mechanism the co-option of 'old genes' and/or 'old gene networks' occurred, and how these gene networks evolved in association with the diversification of eyespot patterns.

### Modularity

Evespots appear as serially repeated elements and provide a great system to ask about patterns and mechanisms underlying the diversification of serial repeats [4,40,53]. The nymphalid ground plan, with different types of pattern elements each running along the anterior-posterior axis of the wings, entail the idea of modularity, with strong associations between repeated elements of the same type (e.g. different eyespots on same wing surface) and independence between elements of different types (e.g. eyespots and band elements). Studies of B. anynana eyespots have tested the extent to which the shared developmental and genetic mechanisms between serially repeated evespots might constrain evespot evolution. Artificial selection in captive *B. anynana* populations showed that some properties of eyespots, such as size, can more easily diverge across eyespots on the same wing surface than other properties, such as colour ring composition [54]. This developmental bias against independent evolution of colour-composition of eyespots on the same wing was confirmed in a recent comparative study across Mycalesina butterflies [55<sup>•</sup>]. However, this study also identified a lineage where this constraint has been lifted, presumably through the evolution of wing sector-specific responses to eyespot-inducing signals. Differences between eyespot size versus colour composition in how easily independent changes can be generated are believed to depend on whether the signalresponse steps of eyespot formation (steps 2 and 3 in Figure 2a) are properties of each eyespot-bearing wing sector versus properties of the whole wing epidermis [54,56]. Eyespot-specific modifications, in genetic terms, may depend on the existence or persistence of expression (from larval to the pupal stage) of wing sector-specific genes, which interact with genes of the eyespot-regulatory network [49]. A series of candidate wing sector-specific genes have been identified recently to function in setting up veins in early larval wing development [57,58<sup>•</sup>]. Future work will continue to attempt to identify and characterize both wing sector-specific genes and how they interact with evespot development genes to clarify the genetic mechanisms underlying the diversification between serial repeats.

### Perspectives for future studies Genes

We have identified some of the open questions pertaining to both the proximate and ultimate mechanisms behind the formation and diversification of butterfly eyespots.

Here we lay down perspectives for future studies. As for many other model traits, new technological advances have catapulted our understanding of the genetic basis of the development and evolution of evespots. CRISPR-Cas9-based approaches have been used to test the function of specific candidate genes [19,20,21,24,42,59,60] and can also be helpful in testing the function of candidate regulatory elements and in elucidating the organization of those genes in networks. NGS-based approaches can be used to address questions at different levels, including: 1) using RNA-seq to ask about how temperature (or other environmental factors) affects gene expression in association with eyespot plasticity, 2) ATAC-seq to identify regulatory regions of genes involved in eyespot development, 3) GWAS and genome scans to map the genetic basis of natural eyespot variation, and 4) techniques not yet used to study wing patterns such as single cell RNA-seq [61], and DBiT-seq [62] for high spatial resolution of gene expression detection. In addition, visualization techniques such as single cell multi-probe FISH [63] and live imaging of pattern development [64] and gene expression patterns [65<sup>•</sup>] will be important to establish the dynamics of eyespot pattern formation.

### Evo-Devo

Evespot studies can widen the phylogenetic and phenotypic breadth of case-studies that are needed to resolve outstanding key questions in evo-devo about the genetic basis of phenotypic diversification (database in Ref. [66<sup>•</sup>]). Are there 'hotspot genes' repeatedly associated to phenotypic variation in different species? Several proposed hotspot genes have been implicated in variation of lepidopteran wing patterns (e.g. optix, cortex, and WntA; [67]), but they have yet to be tested in relation to evespot development. Do the genes responsible for evolutionarily relevant variation have 'special positions' within gene networks (cf. [68]) or within genomes? Are the DNA sequence polymorphisms responsible for evolutionarily relevant variation more often in coding versus regulatory sequence? Duplication of regulatory elements and their subfunctionalization into eyespot-specific roles [69] can allow for mutations in pleiotropic genes to have eyespotspecific effects. To what extent is there overlap between: 1) genes bearing alleles of large effect responsible for mutants studied in the laboratory and those bearing alleles of subtle effect responsible for segregating variation in natural populations; 2) genes responsible for intraspecific variation and genes responsible for inter-species differences? Studies of butterfly eyespots can add valuable data towards answering all these questions and towards identifying general patterns in evo-devo.

## Fitness

Beyond acquiring a deeper understanding of the genetic and developmental basis of variation and diversity, eyespots can aid in efforts to elucidate genotype-phenotype-fitness maps. It will be crucial to have better insight into the selective agents that shape eyespot evolution in natural populations. Achieving this will require progress on different fronts. It will be valuable to clarify how evespots look like both by butterflies and their predators. Identification of the latter will be crucial and barcoding of stomach contents may aid in identifying which species are predators of butterflies with eyespots. On the other hand, an examination of how location of predator-induced damage in wild-flying butterflies (e.g. [11,70<sup>•</sup>]) relates to the position of eyespots may help us understand the ecological functions of evespots in predator avoidance. It is precisely the possibility of integrating concepts and approaches at different levels that render eyespots an interesting study system to link variation in genotype to variation in phenotype to variation in fitness.

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### **Conflict of interest statement**

The authors contributed equality to all aspects of manuscript, conceptualization, writing, and figures. Authors declare no conflict of interests.

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