Evo-Devo of Butterfly Wing Patterns

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Abstract

The evolution and development of lepidopteran wing patterns are a valuable system for understanding cellular development and differentiation of phenotypes with clear ecological functions. Butterfly wings are only two cells thick, with single epithelia making up the dorsal and ventral wing surfaces. The color patterns found on lepidopteran wings are formed from a mosaic of scale cells containing pigments or with structural coloration caused by the reflection of particular light wavelengths by ridges found on scale surfaces. Butterfly color patterns are laid down on top of a preexisting developmental genetic architecture that directs wing development and which determines the shape of each wing, allows for the functional specialization of forewings and hindwings and of dorsal and ventral wing surfaces, and which specifies the positions of the longitudinal veins on the wing. This architecture also provides a mechanism by which different regions on the wing can be regulated independently of one another. The Nymphalid ground plan is an archetype that can be used to compare and homologize color patterns from different Lepidoptera species. Many genes related to the determination of many color pattern elements within

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the Nymphalid ground plan have been identified, and for border ocelli (or eyespot) patterns in particular, a large genetic regulatory network for pattern formation and differentiation has been assembled. Many Lepidoptera model species have contributed to our understanding of butterfly color pattern development and evolution, but recent comparative genomic work examining of intraspecific and interspecific variation in *Heliconius* has identified new genes with important roles in pattern development.

Introduction

The color patterns on the wings of butterflies and moths (order Lepidoptera) are among the most striking patterns in nature, with demonstrated roles in mate choice, thermoregulation, and predator avoidance (including the substrategies of camouflage, disruptive coloration, attack deflection, aposematism, and mimicry). Lepidopteran wing color patterns are among the most complex of any insect and are made up of overlapping wing scales that project above the surface of the wing membrane like tiles or shingles on a roof as seen in Fig. 1. Other insect wings lack scales and primarily feature melanin pigments, while butterfly and moth wings are colored by combinations of pigments produced by the ommochrome (yellow, orange, red, and brown), melanin (grey, black, brown, tan, and yellow), pteridine (white), and urate granule (white) biosynthetic pathways in the scales, as well as by structural colors (including iridescent blue, green, and violet). Structural colors are produced by microscopic ridges composed of layered lamellae on the surfaces of the scales that appear to be produced by the regulation of the polymerization of actin filaments during scale development (Dinwiddie et al. 2014; Parnell et al. 2018). On the whole, Lepidopteran color patterns are particularly suitable for study because they are complex, yet consist of clearly defined subunits, exist primarily in two dimensions, and are structurally simple; features that have helped

Fig. 1 Tiled scales on the forewing of the nymphalid butterfly *Precis octavia* containing ommochrome (orange) and melanin (black) pigments or showing iridescent blue structural coloration
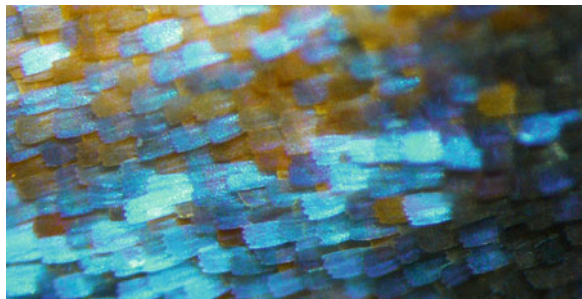
make them an important model system in the field of evolution and development (Marcus 2005).

Lepidopteran wing scales differentiate during metamorphosis in the latter part of pupal stage of development and measurable quantities of pigments are produced in scale cells during this period. However, the groups of adjacent cells in the developing wing tissues (also known as wing imaginal discs) that are regulated in a coordinated fashion and express the same pigments to form color pattern elements are developmentally determined in the last (often the 5th) larval instar of the caterpillar and in the early pupal stage (Fig. 2). The wing imaginal discs are established much earlier in development and are usually discernable under the
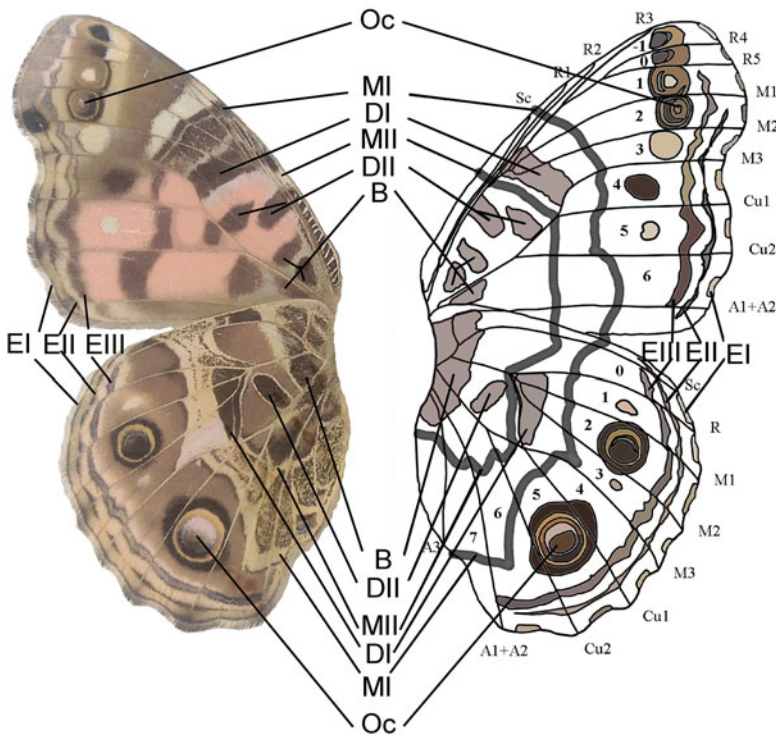

Fig. 2 The Nymphalid ground plan for Lepidopteran wing color pattern organization as expressed on the ventral wing surfaces of the butterfly, *Vanessa braziliensis* (family Nymphalidae), after Abbasi and Marcus (2015, 2017) and labeled using the nomenclature of Schwanwitsch (1924). The ground plan color pattern elements are sometimes divided into three major divisions: the basal symmetry system [consisting of the Basalis (B) pattern element], the central symmetry system [centered on Discalis I (DI) and including Discalis II (DII) and two Medial bands (MII and MI)], and the border symmetry system at the wing margin [containing the Border ocelli (OC, often referred to as eyespots) and the Externa patterns (E): including parafocal (EIII), submarginal (EII), and marginal (EI) elements]. The wing veins in *Vanessa* are also labeled including the Subcosta (Sc); Radius (R); Media (M); Cubitus (Cu); and Anal (A). The wing sectors are numbered in the forewing and hindwing after Abbasi and Marcus (2015, 2017)

microscope by the third larval instar. They rest just inside of the larval body wall in a dorso-lateral position in the second and third thoracic segments. This position makes them surgically accessible and they can easily be manipulated in vivo or removed for in vitro experiments during the larval and pupal stages of development. Adult insect wings, including those of Lepidoptera, consist primarily of dead tissue, and so all developmental processes cease almost immediately after adult emergence from the pupal case as the wings harden and the cells that make up the dorsal and ventral wing epithelia die.

Nymphalid Ground Plan and Wing Patterning in Three Dimensions

The typical color pattern elements on the wings of most butterfly species and also many moths can be understood as derivatives of the Nymphalid ground plan (Fig. 2). The Nymphalid ground plan was originally proposed by Schwanwitsch (1924) and reintroduced into the recent scientific discourse by Nijhout (1991) as a paradigm for understanding the development and determining homologies among the patterns found in different species of Lepidoptera. Individual color pattern elements are sometimes grouped into three "symmetry systems," within which color pattern elements often show correlated phenotypes and may have shared developmental characteristics (Otaki 2012). Yet, discrete color pattern elements within each symmetry system can vary in size, color, and position between species and also between sexes or morphs in polymorphic species (Nijhout 1991). The complete set of the color pattern elements that constitute the Nymphalid ground plan may never have all co-occurred in any individual lepidopteran species, so it should be thought of as an archetype (a conceptual framework) rather than as an ancestral state for color patterns.

The location of each color pattern element within the Nymphalid ground plan is determined by the preexisting developmental architecture of the wings: the cellular signals and patterns of gene expression that establish wing identity (forewing vs. hindwing) and the dorsal-ventral, anterior-posterior, and proximal-distal axes of the wing (Fig. 3). Hindwings become differentiated from the forewings through the expression of the hox gene *Ultrabithorax,* which is otherwise involved in patterning the third thoracic segment of the insect anterior-posterior body axis (Weatherbee et al. 1999). Insect wings are also divided into dorsal and ventral developmental compartments by expression of Apterous, a transcription factor that is expressed in the dorsal wing epithelium (Prakash and Monteiro 2018). The actions and interactions of the two transcription factors Ultrabithorax and Apterous permit the independent upregulation or downregulation of downstream targets on any combination of the four wing surfaces, establishing the inherent capacity for each wing surface to take on different color pattern phenotypes and visual signaling functions.

Within each wing surface, the placement of color pattern elements is specified by signals associated with patterning with the proximal-distal and anterior-posterior
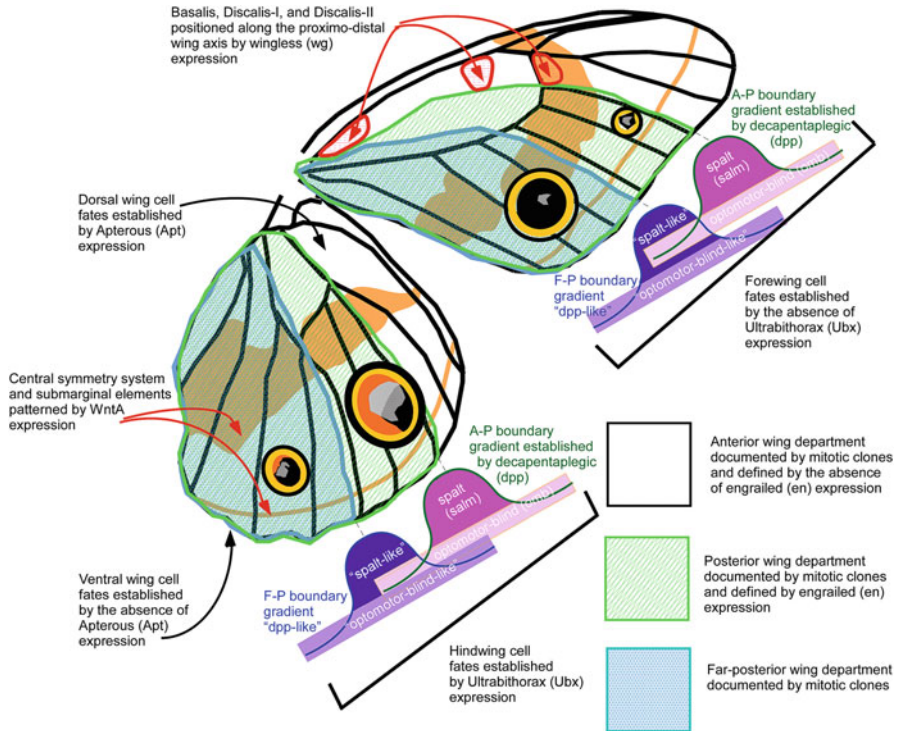
Fig. 3 Establishing the Nymphalid ground plan in three dimensions. Color patterns in species corresponding to the typical Nymphalid ground plan are regulated by transcription factors Apterous (which is expressed in dorsal wing surfaces and specifies dorsal versus ventral cell fates, establishing the dorsal-ventral wing axis (Prakash and Monteiro 2018)), Ultrabithorax (which is expressed in the hindwing and specifies hindwing versus forewing cell fates (Weatherbee et al. 1999)), and Engrailed (which is expressed in the posterior wing compartment and specified anterior versus posterior wing cell fates, establishing the anterior-posterior wing axis (Keys et al. 1999)), as well as the signaling proteins decapentaplegic and the not-yet characterized "decapentaplegic-like" (which also contribute to anterior-posterior axis specification), and wingless and Wnt-A (which specify the color pattern elements along the proximal-distal wing axis) (Carroll et al. 1994; Martin and Reed 2014). Though the expression of these proteins, as well as downstream genes upregulated in response to their expression (including *Spalt, optomotor blind,* and genes with analogous functions (Zhang and Reed 2016)), it is possible for the genetic regulatory apparatus of the wing to place color patterns at precise locations on particular wing surfaces (Abbasi and Marcus 2017)

axes (Fig. 3). The placement of color patterns along the proximal-distal axis is patterned primarily by Wnt ligands, especially wingless (wg) and WntA (Carroll et al. 1994; Martin et al. 2012; Martin and Reed 2014). The size and location of Basalis, Discalis-I, and Discalis-II pattern elements appear to be determined by wingless expression, while the Medial-I and Medial-II of the central symmetry system and the submarginal band (EII) of the border symmetry system are associated with WntA signaling (Fig. 3). Often the final colors of the patterns in the adult butterfly wing produced by wg and WntA signaling are different, suggesting that

these very similar ligands differentially interact with their receptors or have differential effects on downstream components of the signal transduction pathways to produce alternative outcomes for adult wing pigmentation (Martin and Reed 2014). Wnt ligands not only activate signal transduction through the canonical pathway (involving β-catenin), but sometimes also through the c-Jun N-terminal kinase (JNK), and Ca2+ alternative signaling pathways, suggesting one possible mechanism by which similar ligands could produce different color pattern outcomes. However, it is not yet known which signaling pathways are triggered by the Wnt signals on the wings of butterflies (Özsu and Monteiro 2017).

Anterior-posterior axis patterning in insect wings is initiated by the expression of Engrailed transcription factor in the posterior portion of each body segment early in embryogenesis (Abbasi and Marcus 2017). Engrailed expression is maintained throughout development, defining the far posterior compartment of the insect wing (Fig. 3). Engrailed upregulates the expression of hedgehog (hh), a short-range signaling molecule that binds to receptors in a band of cells immediately anterior to the posterior compartment, which respond by producing a longer-range signaling molecule decapentaplegic (dpp) (Keys et al. 1999) at the A-P boundary. High concentrations of dpp result in expression of the transcription factor spalt (sal), while lower concentrations of dpp are sufficient to drive expression of a second transcription factor optomotor-blind (omb).

It has recently been proposed (Abbasi and Marcus 2017) that there is an additional developmental compartment in the far posterior of the wing in Lepidoptera, which further subdivides the wing. This additional compartment boundary may establish a second gradient of a dpp-like signaling molecule and a second set of nested domains of gene expression (sal-like at high concentrations of ligand, omb-like at lower concentrations) similar to those that have been described more anteriorly (Fig. 3). Collectively, these domains of expression in the developing wing are used to define the locations of the incipient wing veins (which serve as channels for blood flow to provide nutrients to wing cells and through which trachea grow permitting cellular gas exchange), and subdividing the wing into a series of sectors. It has also been pointed out that the combination of transcription factors expressed within each wing sector according to this scheme is unique, and might serve as a combinatorial code or "address" by which each wing sector can be targeted individually by the machinery of wing development and consequently express unique color pattern phenotypes. This is most easily observed in the variation among border ocelli (eyespot) phenotypes between wings, wing surfaces, and wing sectors shown by individual butterflies (Figs. 2 and 3).

The Development of Eyespots/Border Ocelli

Of all lepidopteran color patterns that make up the Nymphalid ground plan, our mechanistic understanding of the details of development is greatest for the border ocelli or eyespots. There are more candidate genes with known expression patterns in border ocelli and more gene products with demonstrated effects on border ocelli

phenotypes than are known for any other lepidopteran color pattern (Özsu et al. 2017; Özsu and Monteiro 2017), which has allowed for the construction of computational models for the determination of these phenotypes (Evans and Marcus 2006; Marcus and Evans 2008).

Among the first gene products to be studied extensively with respect to border ocelli was the transcription factor Distal-less, which functions to define the wing margin in many insects, but is also involved in specifying the focus (center) of border ocelli in butterflies and moths (Fig. 4) (Brakefield et al. 1996; Monteiro et al. 2006). The cells that make up the eyespot focus are located in an indentation relative to the rest of the wing epithelium and appear to have an accelerated cell cycle relative to other wing cells. This makes the locations of foci visually identifiable long before pattern differentiation even without staining for genetic markers (Iwasaki et al.
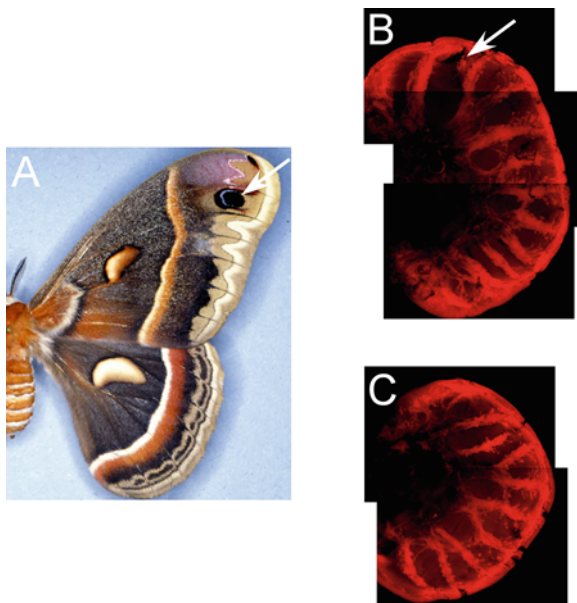

Fig. 4 (**a**) The cecropia moth, *Hyalophora cecropia* (family Saturniidae), has a small border ocellus (white arrow) in the anterior forewing but lacks border ocelli in the hindwing. (**b**) *Hyalophora* late fifth larval instar forewing disc showing expression of Distal-less transcription factor protein in the incipient eyespot focus (white arrow) as visualized with an anti-Distal-less polyclonal primary antibody and a fluorescent TRITC-conjugated mouse anti-rabbit monoclonal secondary antibody, as in butterfly eyespots (Brakefield et al. 1996). (**c**) *Hyalophora* moths lack border ocelli in the adult hindwing and lack foci of Distal-less expression in late fifth larval instar hindwing discs treated in the same way as described above. The tracheae that define the position of the wing veins in the imaginal discs and which are present along the wing imaginal discs margins are autofluorescent. Thus, the fluorescence of the tracheae in panels **b** and **c** is independent of the presence of the primary antibody and is not an indication of Distal-less expression. As in many other Saturniid moths (Monteiro et al. 2006), late fifth larval wing imaginal discs in *H. cecropia* are large (over 1 cm^2 each), so each figure panel is a mosaic of two or three fluorescent photo micrographs taken at 5× magnification

2017). The coincidence of eyespot foci with physical indentations in the wing epithelium facilitated classic experiments involving focus ablation or transplantation that established the focus as the developmental organizer for border ocelli (Nijhout 1991).

In its role as a developmental organizer, the cells of the eyespot focus release a signal that contributes to pattering the surrounding wing tissue into concentric rings (Marcus 2005). The molecular identity of this signal is not yet known, but a large number of gene products involved in specifying the size and location of the focus have been identified (Fig. 5) (Evans and Marcus 2006; Marcus and Evans 2008). Early models for the creation of the concentric rings of the eyespot were based on different focal signal concentration threshold responses associated with each color ring (Brakefield et al. 1996). More recent models suggest that the incipient rings may also be producing ligands in response to the signals from the eyespot focus (Otaki 2011), generating feedback loops that further define and consolidate the ring-like patterns. In the African bush brown butterfly, *Bicyclus anynana* (Nymphalidae: Satyrinae), there is evidence during the early pupal stage (but not during late larval development when the primary focal signal is produced) that wingless ligand is secreted transiently by the focal cells to interact with the surrounding tissue, and in response, a transient signal from the TGF-β family (which includes dpp) is being received by the cells of the eyespot focus from the surrounding tissue (Fig. 5) (Monteiro et al. 2006). This may correspond to the phenomenon of developmental pattern reinforcement in border ocelli identified by Otaki (2011). Once established, each ring of the border ocellus expresses a different combination (or level of expression) of transcription factors (Brunetti et al. 2001), which are suspected to directly or indirectly upregulate components of the biosynthetic pathways required for the production of pigments.

In *Bicyclus anynana,* the border ocellus in the adult butterfly is composed of a white focus, surrounded by an inner black ring and an outer yellow ring. It is thought that the focus is colored by white, ultra-violet reflective pteridine pigments, while both the black and yellow rings are made up of different forms of melanin (Fig. 5) (Matsuoka and Monteiro 2018). The color of butterfly scale cells containing different forms of melanin may be determined by which components of the pathway have been upregulated and/or by the availability of different melanin precursors in the hemolymph relative to the timing of scale cell differentiation (a process regulated by Notch (N) signaling) during the pupal stage (Marcus 2005). Finally, it should be noted that in some other butterfly species, red ommochrome pigments are made in eyespot rings (Brunetti et al. 2001; Marcus 2005) suggesting that other biosynthetic pathways can also be recruited and expressed as part of this genetic regulatory network.

Heliconius and the Importance of Extreme Phenotypes

While the wing color patterns found in most groups of butterflies (and many moths) can be mapped on to the Nymphalid ground plan with relative ease, there are some notable exceptions (Nijhout 1991; Schachat and Brown 2016).
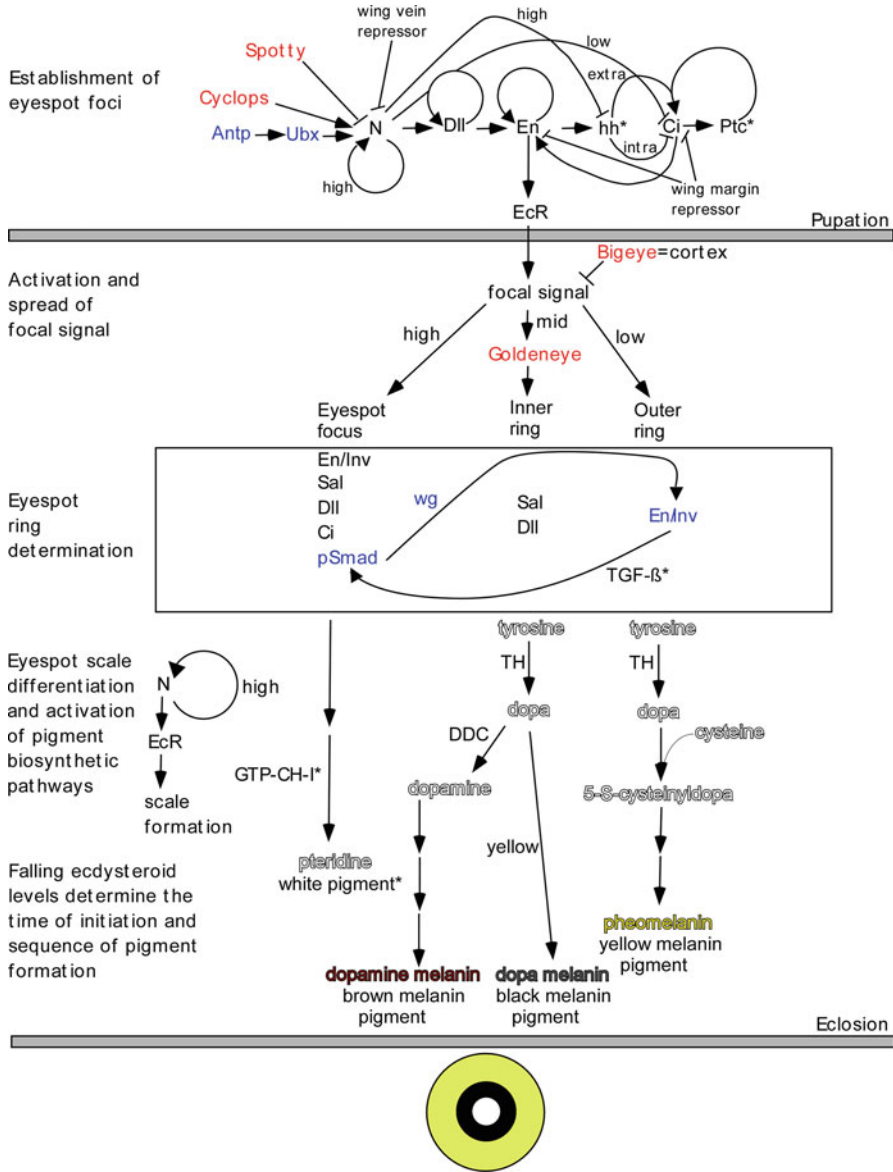
Fig. 5 Developmental model for the formation of border oscelli (also known as eyespots) in *Bicyclus anynana* (Nymphalidae: Satyrinae). Genes labeled in red are spontaneous mutants that appear to function in color pattern development, but with one exception (*Bigeye* appears to be a mutation in the *cortex* locus) have not been characterized at the molecular level. The inferred role of these mutations is based on the assumption that these are loss of function mutations. Genes labeled in blue are components of the hierarchy that are known from *B. anynana*, but for which similar patterns of expression do not appear to be present in two other commonly used model species for eyespot development: *Junonia coenia* and *Vanessa cardui* (both Nymphalidae: Nymphalinae). Pigments and pigment precursors are indicated by hollow letters. Gene products and pigments known from these other models and suspected to be present in *B. anynana* are indicated by asterisks.

Among these exceptions, perhaps best studied are the *Heliconius* butterflies, which for many decades were among the most difficult constellations of color patterns (used for aposematic coloration and involved in Müllerian mimicry rings) to homologize with patterns found elsewhere in the Lepidoptera (Nijhout and Wray 1988). Yet, in spite of these early difficulties (now mostly resolved), the intraspecific and interspecific color pattern variation found in *Heliconius* in combination with advances in genome sequencing have proven to be a powerful tool for uncovering the developmental genetic architecture underlying many color patterns across the Lepidoptera (Heliconius Genome Consortium 2012).

Using a combination of controlled crosses and genome sequencing, it has been determined that most of the major color pattern variation found in *Heliconius* can be attributed to genetic variation (mostly in the regulatory regions) of four genes (Fig. 6). Two of these genes regulate the width of the black melanized bands on the wing. As mentioned above, the gene encoding the secreted ligand *WntA* regulates the proximal-distal width of Discalis I. This gene has shown to correspond to *Heliconius* locus *Sd* (Martin et al. 2012). A second *Heliconius* locus *Yb* regulates the proximal-distal width of several wing bands and has been mapped to the gene that encodes the cell cycle regulator *cortex* (Nadeau et al. 2016). *Cortex* has also been implicated in altering the timing of scale cell development relative to the availability of melanin precursors with effects on the number of highly melanized cells on the wings of *Biston betularia* moths (van't Hof et al. 2016). More generally, *cortex* may be modulating the timing of the maturation of blocks of scale cells relative to other patterning events taking place on the wing as a means of establishing color pattern elements with similar phenotypic characteristics.

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Fig. 5 (continued) The presence of TGF-β signaling being received by the cells of pupal eyespot foci is inferred from the localization of the activated downstream protein pSMAD in those cells (Monteiro et al. 2006). Attempts to detect upregulated expression of Ptc and hh in the vicinity of the eyespot foci of *B. anynana* have not been successful (Tong et al. 2012), but both of these gene products are found in association with *Junonia* eyespot foci (Marcus 2005; Evans and Marcus 2006) and mRNAs from both of these genes have been detected in appropriately staged wing tissue transcriptomes from *Bicyclus* (Özsu and Monteiro 2017). Eyespot development can be broken down into four stages (Brakefield et al. 1996; Nijhout 1996): (1) the number and position of eyespot foci are established, (2) a focal signal is secreted from the eyespot focus and that patterns the surrounding tissue (note: the molecular identity of the focal signal is undetermined), (3) the rings of the eyespot are then determined in response to different concentrations of the focal signal. Finally, (4) the wing scales that contain the pigment differentiate and the pigment molecules are synthesized in response to falling ecdysteroid levels. White pteridine pigments are produced first, followed by yellow melanin pigments, with black and brown melanin pigments being produced last. (Abbreviations: *Antp* Antennapedia, *Ubx* Ultrabiothorax, *N* Notch, *Dll* Distalless, *En* Engrailed, *Inv* Invected, *hh* hedgehog, *Ptc* Patched, *Ci* Cubitus interruptus, *EcR* Ecdysone receptor, *Sal* Spalt, *wg* wingless, *TGF-β* transforming growth factor beta, *pSMAD* phospho-SMAD, *GTP-CH I* GTP cyclohydrolase I, *TH* tyrosine hydroxylase, *DDC* DOPA decarboxylase.) Genetic interactions shown here have been previously reported in (Marcus 2005; Evans and Marcus 2006; Marcus and Evans 2008), supplemented by data from more recent literature (Monteiro et al. 2006; Tong et al. 2014; Nadeau et al. 2016; Özsu et al. 2017; Özsu and Monteiro 2017; Matsuoka and Monteiro 2018)

Two other *Heliconius* genes are responsible for determining the color of specific color pattern elements (Fig. 6). Locus *D* in *H. erato* and *H. melpomene* and locus *G* in *H. cydno* have all been shown to correspond to the gene *optix,* a transcription factor that upregulates the ommochrome biosynthetic pathway responsible for the bright red wing pigments xanthommatin and dihydroxyxanthommatin (Reed et al. 2011). In most insects, the expression of *optix* occurs primarily in the cells giving rise to the eye, and the subsequent deployment of these pigments is to insulate the photoreceptors in each ommatidium from stray photons of light escaping from adjacent ommatidia in the adult compound eye, enhancing visual acuity. Expression of these pigments in the insect wing appears to have occurred after the divergence of the Lepidoptera from their sister taxon, the caddisflies (Order Trichoptera), which display only a very limited repertoire of patterning on their wings (Marcus 2018). Finally, *Heliconius* locus *K* has been shown to correspond to the gene *aristaless1,* a
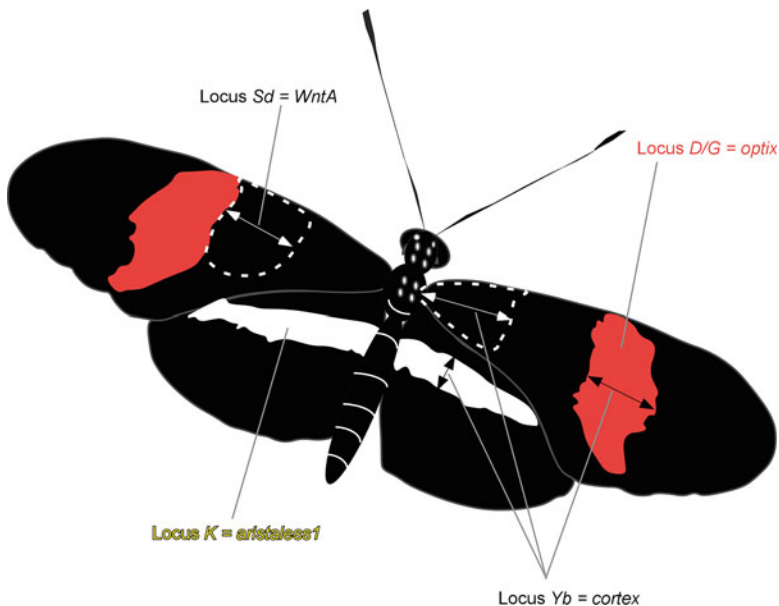

Fig. 6 Butterflies in the genus *Heliconius* have derived color patterns that represent extensive modification of the Nymphalid ground plan. These patterns vary substantially within and between species and are a classic example of Müllerian mimicry where multiple species have converged on similar aposematic coloration to deter predation. Depicted is *H. erato peterivina* showing the color patterns affected by the four loci with the greatest phenotypic effects on *Heliconius* aposematism: locus *Sd* (mapped to *WntA,* which encodes a secreted ligand that regulates the proximal-distal width of Discalis I (Martin et al. 2012)), locus *Yb* (*cortex,* a cell-cycle regulator that regulates the proximal-distal width of several wing bands (Nadeau et al. 2016)), locus *D/G* (*optix,* a transcription factor that upregulates the ommochrome biosynthetic pathway responsible for the red wing pigments xanthommatin and dihydroxyxanthommatin, known as locus *D* in *H. erato* and *H. melpomene* and known as locus *G* in *H. cydno* (Reed et al. 2011)), and locus *K* (*aristaless1,* a transcription factor that suppresses the portion of the ommochrome pathway responsible for synthesizing the yellow pigment 3-hydroxy-L-kynurenine (Westerman et al. 2018))

transcription factor that suppresses the portion of the ommochrome pathway responsible for synthesizing the yellow pigment 3-hydroxy-L-kynurenine (Westerman et al. 2018).

Conclusions

Experimental approaches involving classical genetics, comparative genomics, developmental biology, computational modeling, insect endocrinology, evolutionary comparisons of morphology, gene editing, and gene expression studies have each contributed to advancing our knowledge of the evolution and development of butterfly color patterns. This wide array of approaches, in combination with a diversity of model lepidopteran species under study, has provided us with an increasingly nuanced mechanistic understanding, as well as an increasing appreciation for what portions of the developmental machinery responsible for butterfly color patterns that have been most responsive to evolutionary pressures. Integration of mechanistic and evolutionary approaches with ecological studies that measure the effects of phenotypic change on trait function in the natural environment are expected to launch the field of butterfly evo-devo into additional new and exciting directions in the future.

Cross-References

▶ Developmental Plasticity and Evolution
▶ Evo-Devo and Niche Construction
▶ Evo-Devo and Phylogenetics
▶ Mechanisms of Pattern Formation, Morphogenesis, and Evolution
▶ Modeling Evolution of Developmental Gene Regulatory Networks

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