

Multiple approaches to study color pattern evolution in butterflies

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Abstract

The myriad colors and patterns on butterfly wings have caught the attention of biologists for well over a century. Today, with the advent of more sophisticated genetic and developmental tools, it is possible to identify and study the evolution of genes, gene networks, and the effect of the environment on the networks underlying wing color patterning. In addition, using molecular phylogenies and the comparative approach, it is possible to infer ancestral wing patterns, direction of evolutionary change, and occurrence of parallelism and convergence. Finally, the driving forces behind wing pattern evolution can be estimated using bioassay studies such as predator-prey and mate choice experiments. Here we review the different approaches to answer both proximate and ultimate questions about butterfly wing pattern evolution, and we highlight future research directions in a field that has the potential to become truly integrative.

Introduction

The diversity of colors and patterns on the wings of butterflies has caught the attention of evolutionary biologists for more than a century. Why do butterflies have such diverse wing patterns? What are the developmental mechanisms that generate such diversity? What are the evolutionary forces that drive and maintain this diversity? Because of the sustained efforts of many biologists interested in these questions, butterfly wing patterns have become exceptional model systems for integrating knowledge about the developmental and genetic processes that produce morphological variation, as well as the selection forces that drive these processes.

In contrast to reviews that focus on scientific questions while highlighting gaps in the literature, here we provide a synthesis of current approaches, covering an array of diverse tools and techniques, to answer both mechanistic and functional questions. This review is divided into two sections. The first section addresses mechanistic approaches to studying wing pattern evolution, such as identifying genes

and developmental mechanisms responsible for generating the different wing patterns. The second section addresses the functional significance of butterfly wing patterns, such as what information is conveyed, how it is conveyed, how the environment influences this interaction, and how patterns affect reproductive success. At the end of each section, we propose how new approaches may answer unresolved questions in understanding butterfly wing pattern evolution.

Experimental approaches to examine proximate mechanisms of wing pattern evolution

Butterfly wing patterns result from the way particular genes are organized into gene networks, which in turn regulate patterns of cell growth and differentiation throughout development. The goal of studying butterfly wing pattern evolution at the mechanistic level is to identify how an ancestral developmental program can be modified to produce the diversity of butterfly wing patterns observed today. This task includes not only identifying the genes belonging to the developmental networks but also documenting changes in network organization and regulation, changes in the sensitivity of these networks to environmental influences, and the resultant changes in the physiological and developmental responses that produce the final phenotype.

The network conceptual approach is able to unify both micro- as well as macro-evolutionary mechanistic processes of wing pattern evolution. For instance, changes to a network element, for example to a gene's cis-regulatory element, could either affect the expression levels of that gene and produce a quantitative micro-evolutionary change, or lead to the co-option of an entire set of pre-wired downstream genes into a novel developmental context and perhaps pave the way for a macro-evolutionary novelty.

Below we present several experimental approaches that currently are being used to address the mechanistic basis of wing pattern evolution. While we still lack understanding of the complete set of genes involved in producing any wing pattern, researchers nevertheless are making headway in identifying those genes and patterning mechanisms. The approaches include: i) methods to discover genetic variants that explain wing pattern differences within and between closely related species (i.e. QTL mapping); ii) techniques to describe spatial, temporal, or quantitative changes in patterns of gene expression in a developing wing (e.g. immunohistochemistry,

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in situ hybridizations, microarrays, or q-PCR); iii) methods to investigate the role of insect hormones and environmental change in altering wing patterns; and iv) histological approaches to test the function of organizer groups of cells during pattern development.

At the end of this section, we describe new tools and approaches currently being developed for butterflies that will allow: i) functional tests of candidate genes in the network; (ii) the identification of genetic regulatory elements; iii) tests of whether macro-evolutionary events such as gene network co-option underlie the evolution of new wing patterns; and iv) the modeling of gene networks *in silico*.

QTL mapping studies

QTL mapping is an unbiased genomic search for loci carrying mutations that alter wing patterns across individuals, populations, or species. QTL mapping identifies genomic regions genetically linked to traits of interest by comparing the pattern of co-segregation of polymorphic genetic markers positioned across the genome with the observed wing pattern traits.¹ This powerful method has one

major limitation: it can only be applied to investigate differences in wing patterns among individuals that can be crossed with each other. This technique has been used to identify the loci underlying the different race patterns in several species of *Heliconius* butterflies² and the locus that explains the multiple female-limited color forms in *Papilio dardanus*.³ Usually these loci are termed “switch” loci because different alleles cause the presence or absence of particular pattern elements or change their color. QTL mapping can also be used to identify loci that cause more quantitative changes in wing patterns between populations.

In addition, the QTL mapping approach can determine a candidate genes' involvement in causing trait differences. Candidates are ruled out when a polymorphic marker at the candidate gene and a particular wing pattern trait are not found to be genetically associated. A genetic association, however, only implies that the causative mutation is close to the marker but it does not directly implicate the candidate gene. For example, polymorphisms at the transcription factor *Distal-less* were associated with differences in eyespot size in *Bicyclus anynana*,⁴ and polymorphisms at another transcription factor, *invected*, perfectly associated with the *H* locus in *Papilio dardanus*.³ Once an association is found, subsequent positional cloning efforts are needed using genomic information or sequenced bacterial artificial chromosomes (BACs) from the region of interest, as well as more closely spaced markers in these regions, to further elucidate the candidate loci.⁵

In situ localization of candidate wing pattern genes

The *in situ* protein or mRNA localization approaches can be a first step in identifying candidate wing patterning genes. These genes are tested later for differential expression across species, and for function. The *in situ* approach generally establishes whether there is a visible correlation between the spatial expression of a candidate gene (at the mRNA or protein level) and the adult wing pattern. Both techniques identify possible candidate genes in the differentiation of wing patterns but they do not establish causation.

Labeled antibodies or labeled complementary mRNA are used to detect the expression of a known gene in a developing wing. For *in situ* protein localization it is important to have antibodies targeting butterfly proteins. With the exception of the antibody targeting *Distal-less*,⁶ most other antibodies that cross-react in butterflies were developed to target proteins in flies, grasshoppers, crustaceans, and even humans (see examples in Figure 1a). As proteins become more divergent with evolutionary distance, the absence of a signal does not

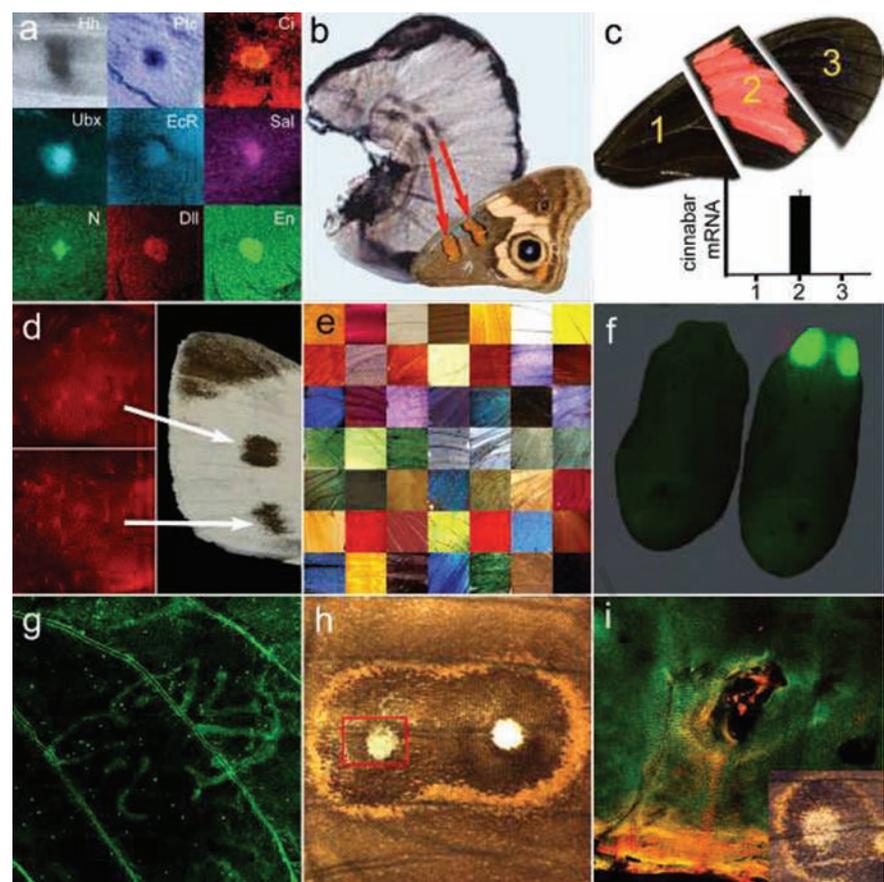


Figure 1. Approaches that address the mechanistic basis of wing pattern development. (a) Genes known to be expressed in the eyespot foci of *Bicyclus anynana* (*Cubitus interruptus*, *Ci*, *Ultrabithorax*, *Ubx*, *Ecdysone Receptor*, *EcR*, *Spalt*, *Sal*, *Notch*, *N*, *Distal-less*, *Dll*, *Engrailed*, *En*) and *Junonia coenia* (*Hedgehog*, *Hb*, *Patched*, *Ptc*) during the larval stages of wing development, suggesting that a complex network of gene interactions are involved in differentiating eyespots.^{8–10,14} (b) The gene *wingless* is expressed in two stripes of cells during the larval stage that later map to the two orange bands on the forewing of *J. coenia*.¹⁰ (c) The gene *cinnabar* is expressed in a section of the wing of *Heliconius erato* that contains the colored band, but not in the more proximal or distal areas.¹¹ (d) The transcription factor *spalt* is expressed in the black patches of scales on the wings of *Pieris rapae*.⁹ (e) The developmental regulation of pigment biosynthetic pathways and structural colors are only just beginning to be understood. This mosaic displays the astonishing diversity of color on butterfly wings (original butterfly images courtesy of T. Larsen). (f) Wildtype (left) and transgenic *B. anynana* butterfly pupae (right) where EGFP, up-regulated by the 3xP3 eye specific promoter, is used as a marker for transgenesis.¹⁵ (g) Laser heat-shocks can be used to activate transgenes (in this case EGFP) in precise patterns during pupal wing development in order to test gene function.¹⁶ (h) Grafting of focal cells (red square) shows the organizing properties of these cells in eyespot development. (i) Wing damage followed by wound healing activates many of the same genes involved in eyespot patterning, and results in changes in adult wing pigmentation (inset).⁹

necessarily rule out the presence of the orthologous protein in butterflies, and the presence of a signal could represent the targeting of a non-orthologous protein. Positive antibody stainings should, when possible, be validated with a more specific technique such as mRNA localization using a species-specific probe. This latter technique, however, is more time consuming and can be applied only when partial cDNA sequences for the candidate gene are available (Figure 1b). In the case of recently duplicated genes, mRNA localizations may not be able to tell whether each paralogue has

diverged in expression.

The correlation between the expression of a gene and a wing pattern indicate that the evolution of that pattern likely involved the direct or indirect recruitment of that gene into the wing-patterning network. Candidate genes for the differentiation of eyespot and chevron patterns have been identified in *Junonia coenia*, *Bicyclus anynana*, *Vanessa cardui* and *Lycaeides melissa*^{7–9} (Figure 1a). The candidate genes *wingless* (Figure 1b) and *cinnabar* (Figure 1c) correlate with bands across the wing in *Junonia coenia* and *Heliconius erato*,

respectively.^{10,11} The transcription factor *spalt* correlates with the black spots and tips of the wing in *Pieris rapae* (Figure 1d),⁹ and pigment biosynthesis genes have been associated with particular areas of color in *Papilio glaucus*, *Vanessa cardui*, and *Heliconius erato*.¹¹⁻¹³

Microarrays and quantitative PCR

Differential gene expression techniques, such as microarrays and quantitative PCR (q-PCR), can help discover genes differentially expressed in two or more tissue samples, and thus help identify candidate wing patterning genes. The tissue samples can be wings from different species, polymorphic forms, seasonal forms, or sections from a wing displaying different color patterns. If gene expression differences are found across samples, this indicates that those genes are being differentially regulated in the two samples. A single mutation in a gene at the beginning of a gene regulatory network can sometimes be responsible for divergent gene expression patterns at multiple loci.¹⁷ Microarrays, however, will not pinpoint the genomic location of the mutation, just the identity of the affected downstream target genes. Combining microarray technology with QTL mapping (see previous section), however, overcomes these shortcomings.

A recent study applied microarrays and q-PCR to establish a correlation between gene expression patterns and particular wing colors in *Heliconius erato*.¹¹ Total mRNA was isolated from sections of wings that displayed different colors, the correspondent cDNA was hybridized to a microarray containing all of the genes previously known to be expressed in wing tissue, and differences in expression levels of particular genes between the different wing sections were confirmed and quantified using q-PCR (Figure 1c). Some of the genes isolated in this experiment coded for enzymes in pigment biosynthetic pathways that were being spatially regulated. Other enzymes, part of the same biosynthetic pathway, were more broadly expressed on the wing,¹¹ and were not associated with particular color patterns. The differential spatial regulation of enzymes within a biosynthetic module suggests that the genes in those modules may not be tightly integrated and co-expressed by the same top gene regulator. Future work should try to link the action of early patterning genes such as transcription factors with the pigment enzymes for a more complete picture of the wing patterning process. Work on enzyme regulation in *Drosophila* may lead the way for similar work on butterflies,¹⁸ although butterflies are clearly using a larger set of pigment biosynthetic modules to color their wings (Figure 1e).

Hormone assays

Hormones are known to play an important role in mediating butterfly wing pattern sea-

sonal polyphenisms: adaptive changes in wing pattern in response to predictable seasonally fluctuating environments such as temperature, photoperiod, or humidity.¹⁹

Wing epidermal cells are only sensitive to hormone levels if they express hormone receptors. Thus, detailing both the hormonal profiles and the precise spatial and temporal regulation of the receptors will determine which wing patterns will be sensitive to hormones and which will not. The general approach in implicating hormones in wing pattern variation is first to document changes in hormone titers between animals reared in different environments via either analytical chemistry techniques such as high performance liquid chromatography (HPLC), or biochemistry techniques such as ELISA or Radioimmuno assays. Then one can either rescue a certain wing pattern by injecting purified hormones or hormone analogues into the seasonal form with the lowest titer,²⁰ or rescue the alternative wing pattern by injecting hormone antagonists into the seasonal form with the highest titers.

How environmental variables lead to hormonal changes and how these interact with wing patterning genes is still not fully understood, especially in observed cases of wing pattern seasonal polyphenisms. Rearing temperature, for instance, alters ecdysterone profiles and the expression pattern of *Distal-less* in the eyespot field during the larval and pupal stages,²¹ but how ecdysone and *Distal-less* functionally connect in the wing patterning gene network is still unclear. The expression pattern of *Distal-less* and that of the ecdysone receptor overlap spatially in at least two nymphalid species. In *Junonia coenia*, the ecdysone receptor is expressed in a dynamic pattern in the eyespot focus and in the eyespot field during the pupal stage of wing development,²² whereas in *Bicyclus anynana* the receptor is expressed in the eyespot centers at an earlier time point during the larval stages (Figure 1a). The next step is to determine how the receptor is wired into the color pattern gene network in these species.

Ecdysterones are perhaps the best studied hormones underlying butterfly seasonal polyphenisms,¹⁹ but other hormones should also be investigated. Juvenile hormones (JHs), for instance, have recently been implicated in regulating larval color patterns in *Papilio* butterflies²³ and polyphenic switches in larval coloration in *Manduca* moths.²⁴ Detailed examination of which ecdysteroid (ecdysone versus 20-hydroxyecdysone), and which JH hormone (there are several JHs in Lepidoptera^{25,26}) is involved in regulating wing patterns is still lacking for most systems.

Surgical manipulations

Surgical manipulation investigates proper-

ties of signaling cells, or receiving cells, in differentiating wing patterns. When similar epithelial perturbation experiments yield different results in different species, then either the perturbed cells or the cells responding to those cells have evolved different properties. Some of the earliest investigations into wing pattern development in Lepidoptera involved either damaging or transplanting wing epidermal tissue in an attempt to find clusters of cells with “pattern organizing” properties.²⁷ These experiments can be informative on several levels. First, by damaging the cluster of cells at different times during development, it is possible to infer when the cells are signaling to the surrounding cells.²⁸ Similarly, by transplanting these focal cells to new locations on the wing, it is possible to infer how they are responsible for specifying the wing pattern (reviewed by Beldade and Brakefield²⁹). For instance, transplanting cells that are localized at the center of a future eyespot pattern, the focus, between selected lines with different eyespot color patterns revealed which features of the color pattern were controlled by properties of these signaling cells versus properties of the surrounding cells (Figure 1h) (reviewed by McMillan *et al.*³⁰). When these perturbation experiments were applied to non-eyespot color patterns, as in *Pieris rapae* black wing tips and *Heliconius* banding patterns, they generally have yielded limited results.^{31,32} One possibility is that bands of color are not produced by signaling cells in that region of the wing, but perhaps instead from signals from the wing margin or hinge.^{9,33} Future experiments targeting these regions should provide further insight into the diversity and evolution of wing pattern organizers in a variety of species.

New directions

Functional genetic studies

Functional genetic studies are important to test whether candidate genes that are genetically linked to certain adult color patterns, or expressed in suggestive patterns during the larval or pupal stages of wing development, actually are involved in the development of those patterns. These experiments normally involve manipulating expression levels of the candidate gene and then observing the effect on the adult wing pattern. Knock-down, over-expression, or ectopic expression of genes are all useful approaches for determining gene function.

Common knock-down experiments use RNA interference (RNAi).³⁴ If knocking down levels of a gene at the relevant developmental time alters the wing pattern, then the gene is likely required for normal pattern development. RNAi is accomplished by injecting double-stranded RNA (dsRNA) into the body cavity of the animal at particular times in development.

Currently only genes expressed in lepidopteran tissues other than the wing have been successfully targeted with dsRNA injections at very high mRNA concentrations after post-embryonic development (around 10–50 µg total mRNA per individual) (reviewed by Ramos and Monteiro³⁵). To date, injections of dsRNA in the developing wings of *Bicyclus anynana*, *Danaus plexippus*, and *Junonia coenia* butterflies at the larval and pupal stages have yet to yield results (A. Monteiro, P. Beldade, S. Reppert, and B. Reed, unpublished results and personal communications).

A different knock-down approach involves synthesizing dsRNA molecules inside the target cells using transgenic methods (Figure 1f).³⁵ If the candidate gene is placed downstream of an inducible promoter, the dsRNA molecule will only be produced at the right stage in development, and thus will not disrupt earlier development. The inducible heat-shock promoter from *Hsp70* from *Drosophila* can drive gene expression after a short heat-shock in *Bicyclus anynana* (Figure 1g),¹⁶ and hence can be used to knock-down candidate genes at controlled times during development.

Ectopic or over-expression experiments are another approach to functionally implicate genes in wing pattern development. Ectopic gene expression, where a gene is activated in a novel location, tests gene sufficiency in inducing a color pattern, whereas gene over-expression tests whether the pattern is altered in the opposite direction relative to the knock-down experiments (e.g. trait gets larger, smaller, etc.). Ectopic expression has been accomplished successfully in *Junonia coenia* by injecting a viral vector containing the homeotic *Ultrabithorax* gene into larval and pupal wings and observing a homeotic transformation of part of the forewing color patterns into those of the hindwing.³⁶ Transgenic tools, on the other hand, are being used currently in *Bicyclus anynana* to up-regulate levels of several candidate wing patterning genes (B. Chen, D. Ramos, and A. Monteiro, in preparation). A laser tool has also been developed to perform controlled heat-shocks in pupal wings, and activate genes in small clusters of cells to test their sufficiency in controlling colors or patterns (Figure 1g).¹⁶

Enhancer detection

It is likely that some wing pattern evolution results from changes in gene regulation rather than from changes in protein coding sequences.³⁷ These regulatory regions, however, are difficult to isolate and identify. Transgenic experiments could be used to discover cis-regulatory regions that control the expression of genes in particular patterns on the wing. Ongoing work in *Bombyx mori*,³⁸ a model lepidopteran, has had success using the Gal4-UAS system³⁹ to “trap enhancers” with random inser-

tions of the Gal4 yeast transcription factor driven by a basal actin promoter. When Gal4 falls next to an enhancer in the *Bombyx* genome, it is transcribed in a specific spatial-temporal pattern during development. Those patterns can be visualized by crossing the Gal4 lines with a transgenic line carrying the UAS (upstream activating sequence that binds Gal4) driving a reporter gene such as green fluorescent protein (GFP). If some of the GFP patterns are expressed in specific areas of the wing, then identifying the genes flanking the Gal4 insertion may identify potential new candidate wing patterning genes. Alternatively, the enhancer line could drive other candidate genes (instead of GFP) in those patterns to test gene function and/or sufficiency. Once enhancer sequences that drive genes in specific wing patterns are identified, it will be possible to trace the evolution and modification of these sequences across species and correlate their modifications to alterations in wing patterns.

Network co-option

A fundamental pursuit is to identify how the gene networks underlying the differentiation of butterfly wing patterns originated. The large number of transcription factors and signaling pathways implicated in color patterning of eyespots (Figure 1a) suggests that complex networks of interacting genes are involved in differentiating these patterns. Two competing hypotheses for the origin of such networks are that they evolved *de novo*, one gene at a time, or that the genes were pre-wired to coordinate the development of a different trait in a different location, and this network was later co-opted to the wing.¹¹ Arguing for the latter hypothesis, the leg/antennae network,¹⁰ a circuit that patterns the anterior-posterior axis of wings,¹⁴ and a wound healing network (Figure 1i)⁹ have all been proposed as possible ancestral networks redeployed in wing eyespot development. Pigment biosynthetic modules deployed in eyes may also have been recruited as pre-wired networks to color wings.¹³ Future work should aim at testing network recruitment ideas as well as distinguishing them from *de novo* network evolution scenarios.⁴⁰ One proposed avenue to distinguish co-option from *de novo* network evolution involves isolating cis-regulatory elements of genes predicted to be in the middle of both the ancestral and the derived networks and testing whether they are pleiotropic; that is, able to drive a reporter gene in both developmental contexts.⁴⁰

Modeling

When complex gene networks underlie the differentiation of butterfly wing patterns it becomes difficult to predict how perturbations (or mutations) to any of the network components affect the final phenotype. The dynamics (and evolvability) of these systems can only be

fully understood with the help of modeling. Traditionally, butterfly wing pattern development has been modeled using simple physical principles, such as gradients of diffusion or reaction-diffusion, and without identifying specific molecules.^{41–43} Recently, models have taken a more gene-centered approach, modeling the physical principles of specific molecules belonging to conserved gene regulatory circuits.⁴⁴ Here complex regulation of multiple gene products over time, starting with diffusion from the wing veins, converges on the stable expression of genes in the future eyespot centers. All of the proposed gene connections in these early models need confirmation with empirical experiments. These models, nevertheless, can serve as a starting point for predicting the outcome of network perturbation experiments in the future. Modeling approaches are bound to become more and more central to the field of wing pattern evo-devo as the complexity of gene expression patterns and gene regulation continues to unfold.

Developmental genetics of structural color

While research that addresses the functional significance of structural colors has yielded some insights, for example UV and iridescent colors being used in sexual communication,^{45–48} and having different thermoregulatory properties,⁴⁹ little attention has been paid to the developmental underpinning of structural colors in butterflies. Some of the more spectacular iridescent, blue, violet, and green colors in scales are a result of the way light interacts with the nano-structures present on the surface of wing scales. These morphological structures vary tremendously across butterfly species^{50,51} but little is known about the genes and developmental processes involved in building these nano-morphologies, which makes this area of evo-devo wide-open for research.

Experimental approaches to examine ultimate mechanisms of butterfly wing pattern evolution

Another way to approach the evolution of butterfly wing patterns is to study the functional attributes of these traits. Here the focus is on understanding the ecological and physiological traits within and between species and how variation in these traits relates to differential reproduction. An additional focus is to evaluate how wing patterns evolve in relation to other ecological conditions including both intra- and interspecific interactions. Ultimately, understanding both the current and past natural and sexual selection events will illuminate why and when wing patterns

diversified.

Butterfly wing patterns are the keystone to visual communication within and between species and thus form the basis of decision making for both potential predators and potential mates (Figure 2). Wing pattern evolution is biased and constrained by how the signal is generated by the signaler, emitted through the environment, and sensed by the receiver. In addition, wing patterns may be important in other activities besides communication such as thermoregulation. A complete understanding of wing pattern evolution has to be made in the context of where, when, and with whom the species evolved.

Here we review the major experimental approaches used in recent studies addressing the function of particular wing patterns as well as their origin and evolution. The approaches include methods to estimate whether wing patterns are being used in: i) interactions with predators; ii) selecting and evaluating mates; and iii) thermoregulation. We also review approaches: iv) to discover whether novel wing patterns originate via hybridization among preexistent species; and v) to describe when and where wing patterns originate, and to test alternative hypotheses about modes of wing pattern evolution using phylogenetic approaches.

At the end of this section, we present a brief summary of exciting new directions and opportunities in the study of function and butterfly wing pattern evolution: i) color perception and wing pattern co-evolution; ii) environmental change and wing patterns; iii) wings patterns, learning, and mate behavior; iv) multi-modal signals and wing patterns.

Predator bioassays

Predator bioassays are the primary method for evaluating the role of butterfly wing patterns in protecting butterflies from attack. A variety of different variables can be quantified, such as butterfly survivorship, butterfly flight behavior, rate of predator detection, rate of predator learning, and rate of predator forgetting. Understanding these interactions provides context to most defensive adaptations in butterflies, such as camouflage, disruptive coloration, and warning coloration.

To identify the exact wing pattern element and its functional role in signaling between butterfly and predator, models or proxies of butterfly wings and a single avian predator are often used. These experiments answer questions about predator perception,⁶⁰ signal generation,⁵² and the effect of the environment on signal emission.⁶¹ For example, experiments using paper models of butterfly wings and unknown avian predators found that larger size and higher number of eyespots made better anti-predator wing markings⁶¹ (Figure 2a).

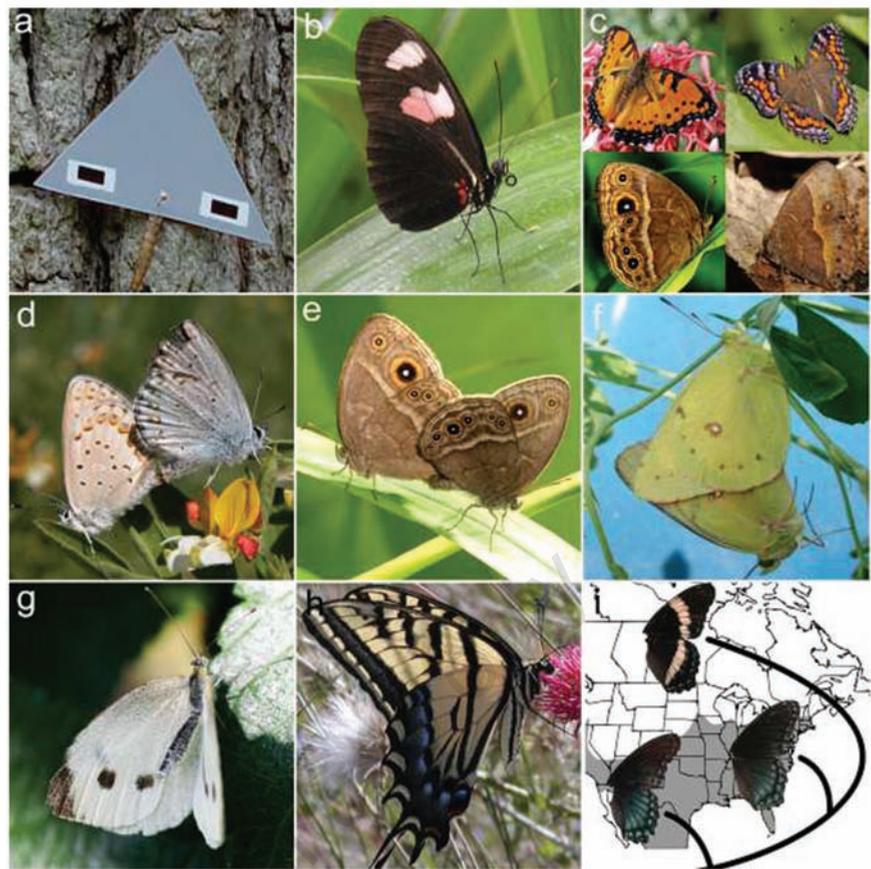


Figure 2. Visual representations of approaches that address ultimate questions in butterfly wing pattern evolution. (a) Predator bioassays using paper models of butterfly/moth wings⁵² (photo credit M. Stevens). (b) Predator bioassays using live butterflies such as *Heliconius erato*⁵³ (photo credit J. Oliver). (c) Mark recapture studies to evaluate predation over a larger geographic area, especially useful for investigating the adaptive advantage of seasonal plasticity in wing patterns as seen in *Precis octavia* (top row) and *Bicyclus anynana* (bottom row)⁵⁴ (photo credits T. Larsen, W. Piel, and A. Monteiro). (d) Mating studies evaluating male mate choice in *Lycides idas*⁵⁵ (photo credit J. Fordyce). (e) Mating studies evaluating female mate choice in *B. anynana*⁴⁶ (photo credit W. Piel). (f) Mating studies evaluating mate quality in *Colias spp.*⁵⁶ (photo credit R. Rutowski). (g) Behavioral and physiological assays to evaluate wing pattern and thermoregulation in *Pieris rapae*⁵⁷ (photo credit A. Stoehr). (h) Phylogenetic approaches using character mapping to understand mimicry evolution in the genus *Papilio*⁵⁸ (photo credit J. Oliver). (i) Phylogenetic approaches using hypothesis testing in the *Limenitis arthemis* complex⁵⁹ (photo credit J. Oliver).

These experiments are often effective at examining the ethology of components in the predator–prey interaction, but with the potential cost of missing information related to the actual predator–butterfly behavioral ecology and its resultant effect on the evolution of butterfly wing patterns.

Live butterflies are often used to explore behavioral interactions between predator and prey. One approach is to capture and release wild caught butterflies to wild, uncaged predators in a natural environment^{53,62} (Figure 2b). This type of experiment is useful in assessing the survival function of different wing patterns, but does not control the effects of predator experience. Another method is to use a single predator and butterfly species in a controlled laboratory setting.^{63–65} This is a powerful

approach for untangling how both the behavior of the butterfly and the morphology of the wing pattern affect butterfly survivorship. For example, the comma butterfly, *Polygonia c-album*, is a well-known leaf mimic that avoids predation by resembling a leaf. It does not flee even after being detected by a predator. Less than a third of the prey are ever detected in predation trials, but once detected they have a very low survival rate.⁶⁴ In addition, manipulating individual wing patterns allows for the identification of the exact elements that conferred the adaptive advantage for the butterfly.⁶⁵

Finally, mark-recapture studies in the field with live butterflies have been useful for understanding the geographic or temporal pattern of butterfly distributions, and how they relate to wing pattern evolution.^{54,66,67} These

experiments do not evaluate the predator–prey interaction directly, but instead measure predation indirectly by comparing the recapture rate of butterflies over time. For example, *Heliconius* butterflies that resembled the dominant co-mimic in a given geographic area were more likely to be recaptured than those that resembled a form not present in that geographic location.⁶⁷ This is likely because of differences in rates of predation: butterflies that share the cost of predator education with another species have higher survivorship. Furthermore, the dry season form of *Bicyclus anynana* appears to have a higher fitness advantage relative to the wet seasonal form when both are released in the dry season, and vice versa for the wet season form when both types are released in the wet season⁵⁴ (Figure 2c).

Mating bioassays

Mating bioassays are used to evaluate species recognition and mate quality.⁶⁸ These assess the signal function of both patterns and colors such as brightness, hue, saturation, shape, and size. Mating bioassays quantify parameters such as mate acceptance, mating frequency, mate choice, and mate preference. Understanding how and when butterfly wing patterns communicate information regarding mate identity and quality is needed to understand why these sexually selected visual signals change across space and time and how butterflies diversify.

There are two primary types of mating bioassays: no-choice and choice experiments. In no-choice experiments, one sex is presented with a single individual or a single type of wing pattern and the experimenters ask whether that wing pattern contains enough information for the opposite sex to recognize a potential mate.^{55,69} For example, using computer generated paper models of manipulated wings, male *Lycaeides idas* initiated courtship with *L. melissa* female models when either hindwing spots or orange chevrons were made to look more like female *L. idas*, indicating that the wing pattern elements used to define this group taxonomically are also effective mate recognition signals⁵⁵ (Figure 2d).

Choice tests, either paired or multiple, are often used to assess the order in which an individual ranks prospective mates. Usually, one sex is presented with a choice between two or more forms of the opposite sex. The choices can be alive or be model butterflies. For instance, when a variety of eyespot traits were manipulated in live *Bicyclus anynana* males, females preferred to mate with those displaying dorsal eyespot pupils, but did not discriminate against males with concealed ventral eyespot pupils⁴⁶ (Figure 2e). Experiments with model butterflies can help generate large behavioral data sets as long as the static behavior of the model does not interfere with mate choice or

recognition. For instance, male *Colias philodice* butterflies were found to prefer female models with less melanization on their wings even though more melanization affects thermoregulation and is correlated with greater female fitness especially at high elevations (Figure 2f).⁵⁶ When manipulating wing patterns or using models, it is important to use a spectrophotometer to ensure good color matches, and to work in a controlled, broad-spectrum lighted environment.

Wing patterns can also evolve owing to intra-specific competition, such as territory defense and/or mate guarding. Interactions between butterflies of the same sex involve conspicuous and potentially stereotyped aerial displays that are not well understood. Besides age and size of an individual, subtle differences in wing coloration such as brightness or hue may be important in these interactions (reviewed by Kemp and Wiklund⁷⁰). Much more research manipulating these wing patterns and their components is needed to understand the role of male-male or female-female competition in the evolution of butterfly wing patterns.

Thermo-physiological approaches

Butterfly wing patterns, especially patterns of wing melanization, are also important for thermoregulation because many butterfly species raise their body temperature by basking in the sun. Behavioral studies have shown that the correlation between melanization patches and basking behavior, such as wing position in relation to the sun and perch choice, is adaptive in Pierid butterfly species.⁷¹⁻⁷³ Some experiments have measured correlations between wing pattern variation and wing absorbance spectra, body temperature, or ambient temperature variation^{49,74} (Figure 2g). Other experiments study wing patterns by adding or subtracting pattern elements and then monitoring changes in body temperature using thermocouples on either live or model butterflies.^{75,76}

Hybridization assays

Hybridization between species and sub-species can generate novel wing patterns in a variety of butterfly taxa.⁷⁷⁻⁷⁹ Recreating the putative genetic crosses in the laboratory and comparing the morphologies of the laboratory hybrids with the putative natural hybrid can help confirm the natural hybridization event. Comparing the genomes of the putative parental species with the putative hybrid species provides separate confirmation. For example, using comparative genomics, an unnamed alpine-dwelling species of the butterfly genus *Lycaeides* was found to be a hybrid, between *L. idas* and *L. melissa*,⁷⁸ thus providing an example of speciation via hybridization. In addition, these studies can be combined with behavioral bioassays. Two recent studies with

Heliconius species suggest that an assortative mating mechanism, once hybridization takes place, facilitates the process of sexual isolation and speciation of the hybrid into a novel species.^{77,80}

Phylogenetic approaches

A phylogenetic framework is useful to document the direction and pattern of radiation of butterfly wing patterns over time. At the core of phylogenetic approaches are estimates of evolutionary history for a given group of taxa and subsequent analyses using these phylogenies to address specific questions regarding butterfly wing pattern evolution. While phylogenetic methods are employed many times merely to document when and where certain wing patterns evolved, in some instances they have also been used to test the specific hypothesis of which selection factors underlie the evolution of the trait.

A frequent phylogenetic approach is to evaluate the origin of certain wing patterns by reconstructing ancestral states on the phylogeny and estimating the number of times a wing pattern element has been gained or lost.^{58,59,81,82} For instance, in the swallowtail genus *Papilio*, mimetic forms have evolved multiple times⁵⁸ (Figure 2h). Additionally, phylogenies can be used to provide statistical support for alternative hypotheses. Model-based approaches, such as parametric bootstrapping,⁸³ assess the relative support for various hypotheses of wing pattern evolution. In the genus *Limenitis*, there are several species with a phenotype that mimics a toxic model, the pipevine swallowtail, *Battus philenor*. The mimetic phenotype may have evolved once and been subsequently lost in some lineages – a hypothesis congruent with an evolutionary tree in which the mimetic lineages are not sister taxa. Alternatively, mimicry evolved once if mimetic lineages of *L. arthemis* are each other's closest relatives. Using parametric bootstrapping to assess the likelihood of both hypotheses, the current available data support the first hypothesis that *L. arthemis* lost its mimetic phenotype and re-evolved a cryptic wing pattern in the absence of the toxic model⁵⁹ (Figure 2i). Future uses of model-based approaches in the study of wing pattern evolution should integrate population-level and other stochastic processes to assess relative likelihoods of alternative evolutionary models.

Phylogenetic approaches also investigate the relationship between biotic or abiotic variables and the evolution of particular wing patterns. For example, when controlling for phylogenetic relationships, Costa Rican nymphalid species occurring in forest habitats are more likely to exhibit polarized wing reflectance relative to species occurring in open habitats⁸⁴ Investigating rates of evolution in a phylogenetic framework provides another means to

investigate the selective forces driving wing pattern evolution. Within the genus *Bicyclus*, characters on different wing surfaces are evolving at different rates: ventral and hind-wing characters evolve more slowly and at equivalent rates between the sexes, suggesting stabilizing selection by natural enemies.⁸⁵ In contrast, dorsal and forewing characters evolve at higher rates and are more likely to demonstrate different rates between the sexes, a pattern consistent with the hypothesis that these characters are involved primarily in mate signaling and thus subject to sexual selection.

New directions

Color perception

Much research has focused on the signal aspect of wing pattern evolution. However, how the receiver perceives and responds to these signals is equally important in shaping wing pattern evolution. Current research has made great strides in understanding the molecular biology, the neurobiology, and the physiology of vision. In butterflies, color tuning has evolved by opsin gene duplication followed by positive selection on individual opsin loci,⁸⁶ and through the use of various filtering pigments.⁸⁷ In birds and other butterfly predators, color vision has diversified mainly by the tuning of photoreceptors.⁸⁸ The next step is to compare how the evolution of color vision in both butterflies and their predators relates to changes in wing patterns. The expectation is that these two traits may be co-evolving. Advances in this field will require the integration of molecular biology, neurobiology, color space modeling, and phylogenetic techniques.

Environmental change

Global changes in temperature and seasonality affect the distribution and phenology of many butterfly species. Now butterflies are found in new habitats with different community members (reviewed by Parmesan⁸⁹). These butterfly populations may provide an excellent opportunity for studying evolution in action in regard to butterfly wing patterns. As average temperatures increase, wing melanization may become reduced either by developmental or genetic change. Wing patterns related to mating and predator avoidance may also change depending on the new interactions in the community. Understanding wing pattern change will require monitoring diversity indices in the field as well as monitoring changes in thermoregulation and intra- and interspecific species interactions.

Learning and mating behavior

Mating behavior in insects including butterflies is quite complex and is not entirely a hard-wired, fixed action pattern. Recent find-

ings in *Drosophila* have demonstrated that learning through experience influences mating behavior, such as courtship and mate preference, and may help explain sympatric speciation.^{90,91} Similar results have been observed in crickets.⁹² Butterflies are well known for their ability to learn colors in both foraging and oviposition contexts.^{93,94} The next step is to evaluate the ability of butterflies to learn wing patterns in the context of mate selection. A learned preference could become a fixed preference via genetic accommodation⁹⁵ and may provide insight into how sympatric and hybrid speciation may occur in butterflies.

Multi-modal signals

Wing patterns are visual signals, but butterflies use many other modalities for communication, such as olfactory or auditory signals. Wing patterns could be combined with these other modalities to increase communication efficacy. Much fruitful research has explored the way scent and visual signals are used by pollinator-moths in foraging behavior (see Raguso and Willis⁹⁶). The combination of smell and color increases a moth's ability to detect flowers, as well as their ability to learn and to retain these flower's properties in memory. Future research should take this framework and apply it to butterfly wing pattern signals used in mating and predator interactions. Perhaps certain wing patterns are most effective when combined with certain pheromones or defensive volatiles. Combining signals may lead to the concerted evolution of both wing patterns and olfactory signals. Probing these modes of wing pattern evolution will involve the integrated use of analytical chemistry, behavior, and sensory biology techniques.

Experiments that distinguish proximate from ultimate

It is important to ask to what extent wing pattern diversity has been guided or constrained by developmental systems, and to what extent natural or sexual selection have shaped this diversity. One avenue is to use artificial selection to attempt to modify pattern elements in new directions, and test whether the mechanisms of generating wing patterns are constrained in some fundamental way. For example, artificial selection applied to eyespot characters in *B. anynana* showed that the relative sizes of eyespots on the dorsal forewing were probably maintained by natural and/or sexual selection,⁹⁷ whereas the constant proportions of the color rings in eyespots on the ventral hindwing are invariable owing to the lack of additive genetic variation in developmental systems.⁹⁸ A different approach involves creating novel wing patterns (using markers, scale

removals, paper models, and/or other manipulations), perhaps patterns that are seen in other species but not in the test species, and ask whether these novel patterns and/or colors fair better in sexual selection and/or natural selection experiments. The results of these experiments would bear on the role of genetic variation and developmental constraints in determining the evolution of wing patterns.

Conclusions

Currently the field of butterfly wing pattern evolution is being explored using many complementary and integrative approaches. Here we attempted to highlight a diversity of research approaches aimed at answering how, when, and why butterfly wing patterns have evolved and radiated in the particular way that they have, but many gaps remain. As new researchers decide to take on some of the remaining challenges, we will get closer to a satisfying understanding of the complex web of interactions, from ecology to gene regulation, that have shaped the evolution of these fascinating, beautiful, and complex organisms.

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