Patterns on the insect wing

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The evolution of wings and the adaptive advantage they provide have allowed insects to become one of the most evolutionarily successful groups on earth. The incredible diversity of their shape, size, and color patterns is a direct reflection of the important role wings have played in the radiation of insects. In this review, we highlight recent studies on both butterflies and *Drosophila* that have begun to uncover the types of genetic variations and developmental mechanisms that control diversity in wing color patterns. In butterflies, these analyses are now possible because of the recent development of a suite of genomic and functional tools, such as detailed linkage maps and transgenesis. In one such study, extensive linkage mapping in *Heliconius* butterflies has shown that surprisingly few, and potentially homologous, loci are responsible for several major pattern variations on the wings of these butterflies. Parallel work on a clade of *Drosophila* has uncovered how *cis*-regulatory changes of the same gene correlate with the repeated gain and loss of pigmented wing spots. Collectively, our understanding of formation and evolution of color pattern in insect wings is rapidly advancing because of these recent breakthroughs in several different fields.

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Introduction

The wings of insects are believed to be a monophyletic adaptation that allowed insects to exploit new niches, resulting in rapid diversification. Wings have evolved a range of sizes, shapes, colors, and patterns of venation to meet a variety of functional requirements. They not only function in flight but have also evolved to become flight-stabilizing structures (e.g. halteres of flies), highly sclerotized shields to protect the body (e.g. elytra of beetles), or in some cases altogether lost (e.g. in stick insects). Additionally, wing color patterns are important in sexual selection, mimicry, and predator avoidance, and these functions have been the topic of several recent studies [1–4]. Although many insects have spectacularly patterned wings (e.g. beetles and dragonflies), the butterflies and moths (Lepidoptera), with well over 100 000 species, have long been a favorite of those studying color and patterning in nature.

In contrast to the membranous, sclerotized wings of most insects, pigmented scales cover the wings of butterflies and moths. These evolutionarily novel scale structures are believed to be derived from sensory bristles found on many insect wings [5]. Each scale is an outgrowth of an individual cell, and is positioned in evenly spaced rows to form a grid of scales across the wing. Some evidence suggests this grid may be established via a lateral inhibition mechanism similar to that underlying *Drosophila* neurogenesis [6]. Similar to a low-resolution digital photograph, the pattern of the wing is a mosaic of individually structured and pigmented scales.

Drosophilid flies lack scales and the colorful wing patterns found in butterflies and moths, but they nevertheless exhibit an impressive variety of wing melanin patterns. Just as in the Lepidoptera, fly wing patterns reflect aspects of their life history and are intimately linked to the ecological niches within which they are found, but the process and nature of color and pattern formation on the wings of *Drosophila* appears to be much simpler. For example, there are no scales, color appears to be limited to shades of black and brown, and there are no examples of highly organized pattern elements such as the symmetric bands and concentric rings of color found in Lepidoptera. Wing spots in flies (Diptera) rely on melanin production, which, as a well-studied enzymatic process, has allowed investigators to quickly identify and test specific genes and regulatory changes underlying the evolution and development of melanic wing patterns in a group of Drosophilid flies [7,8].

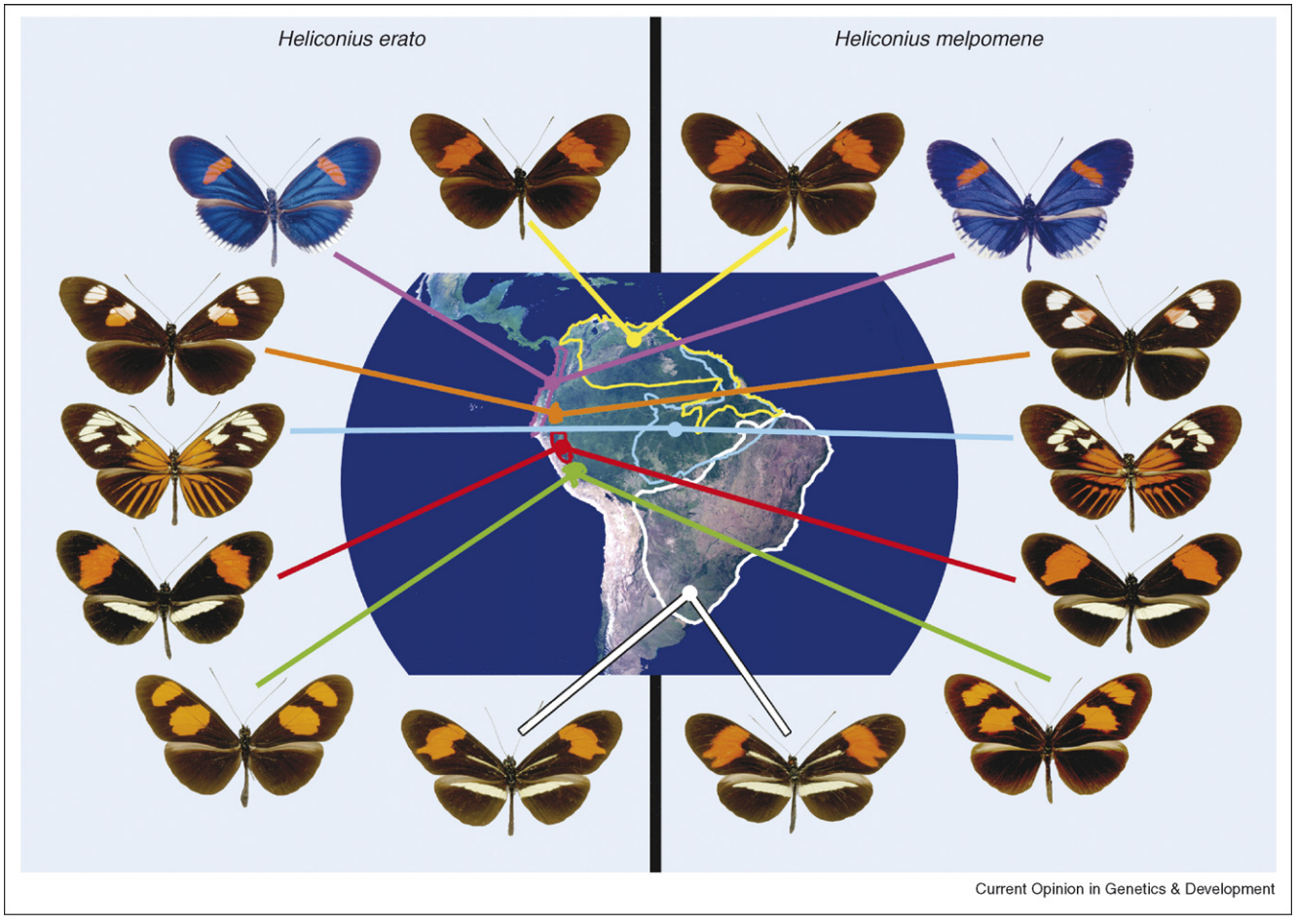
Müllerian mimicry: the story of *Heliconius* butterflies

The butterfly genus *Heliconius* consists of over 50 Neotropical species that have colorful wing patterns, which warn predators of their unpalatability. These *Heliconius* butterflies provide a classic example of Müllerian mimicry and are one of the best studied and most spectacular displays of this phenomenon. Different species of *Heliconius*, all of which are poisonous, have evolved shared color and pattern forms to co-operatively reduce the burden of warning naïve predators. Within a particular

geographic area, there may be several distinct mimetic forms that multiple species might have converged upon; when each form is represented by multiple species, it is termed a mimicry ring [9]. Selection, acting on members of these mimicry rings, has caused species of *Heliconius* to radiate into forms (termed races or subspecies) with many different wing patterns across their range. In regions where members of different races meet and interbreed (hybrid zones), the mimetic patterns of hybrid offspring often appear quite different from that of their parents. In at least one instance it seems that such hybridization may have led to speciation [10], but in most cases, selection against these novel non-mimetic hybrid patterns is high, helping to stabilize different races into discrete populations [1,9].

One particularly well-studied example of Müllerian mimicry involves the two *Heliconius* species, *H. melpomene* and *H. erato*, which live throughout South America (Figure 1). These two species have evolved into forms with many different wing patterns across their range [11,12]. Surprisingly, these two species appear to be part of the same mimicry rings (co-mimics) wherever their ranges overlap (Figure 1). Evolution in *H. erato* and *H. melpomene* is particularly interesting because it is an example of both rapid convergence of color pattern (among species) as well as rapid radiation (within species). This raises a series of interesting questions concerning how color patterns are generated at the genetic level in *H. erato* and *H. melpomene*. Are similar patterns in the different species generated using similar genetic mechanisms? What is the genetic basis of pattern underlying the rapid radiation in both species? These questions have recently been the focus of major efforts to map and identify the genetic changes underlying *Heliconius* radiation [13•,14•,15••,16] (reviewed in [17•]).

Figure 1



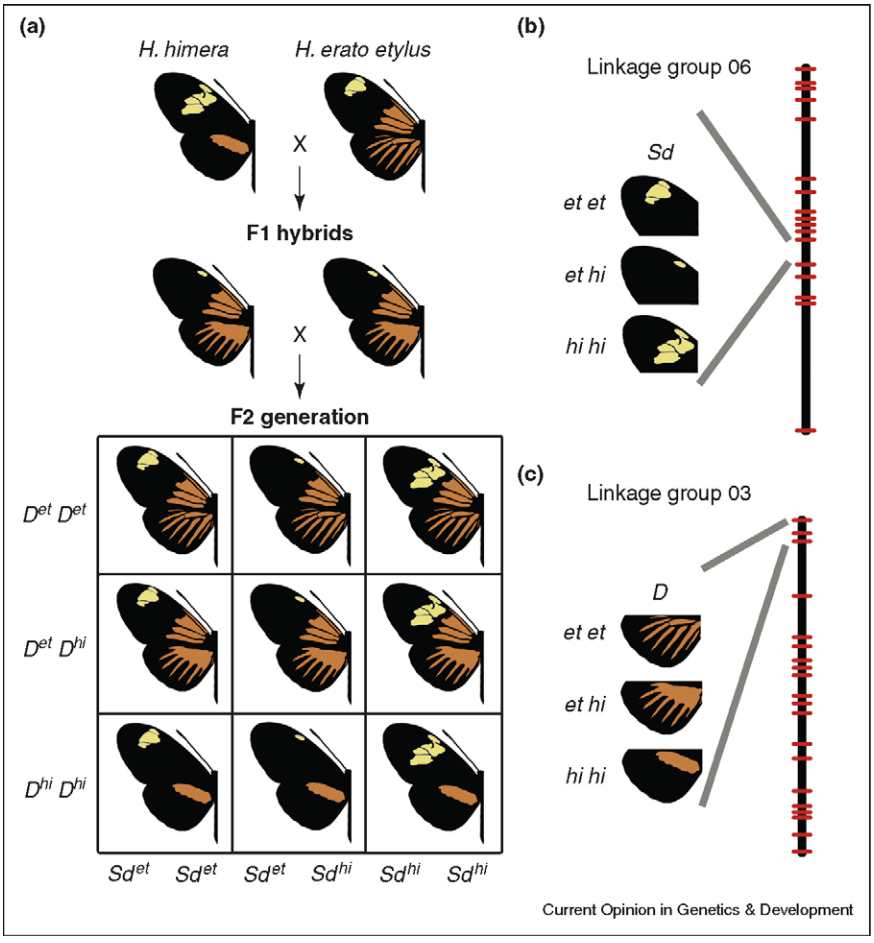
Map of South America showing distributions and forms of *H. erato* and *H. melpomene*. On the left are shown the various forms of the species *H. erato*, which overlap with a co-mimetic form of *H. melpomene*, shown on the right. Each species contains a diverse array of pattern forms, and each form is generally partitioned by geographic region. In a given area, the forms from each species often match one another with remarkable precision. Ranges were taken from [51] and represent the combined distribution of the pairs of forms. The form whose distribution is shown in red is also more broadly distributed in discontinuous areas not shown here. The map background image was provided from the Blue Marble data set by Reto Stöckli, NASA Earth Observatory.

In one of the earliest and largest studies, Sheppard *et al.* [18] used intraspecies crosses between 8 races of *H. melpomene* to identify 11 loci and 8 races of *H. erato* to identify 15 loci that affect wing patterns. Since then, other investigators have performed similar studies using other species such as *H. cydno*, *H. himera*, and *H. numata* (reviewed in [17•]). Most interestingly, these studies have shown that the basis of variation in wing patterns across a species' geographic range appears to be quite simple, consisting of only a few loci of major effect. A comparison of how these loci function in different species has highlighted several similarities in the genetics of wing patterning, and it has been hypothesized that variation at homologous loci (i.e. homologous genes at homologous chromosomal positions) may have been responsible for the radiation of different species of *Heliconius*. Indeed, analyses of crosses between closely related species of *Heliconius* have found that the genetic changes occurring among some species are the same as those responsible for inter-racial differences within a species [15,19].

Mapping of *Heliconius* patterning loci

Investigators of *Heliconius* have now begun to combine genetic linkage analysis with molecular markers to assemble a detailed high-density linkage map (Figure 2) [13•,14•,20]. A variant of restriction fragment length polymorphism (RFLP) analysis, the amplified fragment length polymorphism (AFLP) technique is becoming a common tool to study the genetics of animals with unsequenced genomes. This technique uses variation of genomic restriction sites and primer design to generate a unique set of amplified polymerase chain reaction (PCR) products and to segregate markers for each haplotype. Specific bands of interest can then be cloned, sequenced, and used to design primers for amplifying more anchor loci. In addition, the electrophoretic mobi-

Figure 2



One example of the types of crosses used in developing linkage maps in the *Heliconius*. This interspecies cross between *H. himera* and *H. erato etylus* allowed the mapping of two pattern loci, *Sd* and *D*, which control the yellow bar forewing patterns and the red ray hindwing patterns, respectively. The bars on the right represent linkage groups; red marks represent various molecular markers. *Sd* and *D* have been mapped to specific intervals using the crossing schemes outlined. Figure redrawn from [17•].

lity of allozymes and a generous set of microsatellites from several species of *Heliconius* have been used to complement the AFLP technique by adding additional segregating markers to generate a more detailed linkage map [13•,14•,20].

An additional source of informative molecular markers has come from the use of single copy nuclear loci that are candidate genes in wing patterning on the basis of data from studies on *Drosophila* and other species of butterflies. These genes can be PCR amplified and used as anchor loci, which are then used as a reference point for other markers. These candidate 'wing-patterning' genes have included important signaling pathway components known from *Drosophila*, such as *Decapentaplegic* (*dpp*), *wingless* (*wg*), and *hedgehog*, as well as genes involved in pigment biosynthesis known from previous biochemical and genetic studies on various insects. Another important source of molecular markers has come from EST projects in various other Lepidoptera [21–23].

Detailed linkage maps have now been published for *H. melpomene* and *H. erato*, both of which include 21 linkage groups corresponding to the 21 chromosomes [13•,14•]. The average distance between markers is relatively low (7 cM in H. *melpomene* and 5.1 cM in *H. erato*), and, in each case, several anchor loci are identified in close genetic association with *Heliconius* patterning loci. Mapping of the candidate genes mentioned above has not yet implicated any locus as being directly responsible for changes in pattern, with one exception. In a study of the relationship between male mating preference and female wing pattern in *H. cydno*, preliminary mapping indicated potential linkage between a genomic location near the signaling molecule *wingless* and a forewing color pattern phenotype [24•]. In other studies using *Heliconius* linkage maps, it was first shown that *H. erato* and *H. melpomene* have the same number of chromosomes, and evidence suggested conserved gene order [14]. Further work narrowed the position of one particular locus controlling wing patterning in both *H. melpomene* and *H. erato* to a homologous 1 cM interval, a region of approximately 180 kb, potentially containing a dozen genes [15]. Incredibly, this region was also found to be homologous to an important polymorphic locus (termed 'supergene') from *H. numata*, which acts by itself to generate several of the wing color morphs found within the species [15]. The many forms of *H. numata* look phenotypically very different from the forms of *H. melpomene* and *H. erato*, and instead mimic unrelated ithomiine butterflies. This finding supports not only the theory that homologous loci have contributed to the phenotypic radiation in multiple species, but also the theory that variation in a single or small number of loci has tremendous flexibility to generate a variety of wing patterns. To date, mapping efforts have not narrowed the focus to specific genes or genetic changes that might control wing patterning in *Heliconius*, but increasingly fine-scale mapping and sequencing of the genetic loci will begin to provide answers to these questions.

Eyespot patterns

As in *Heliconius*, there are several other efforts underway to try to understand wing color patterns and phenotypic variation in Lepidoptera. One well-studied element is the eyespot, a pattern of concentric color rings, visually resembling an animal eye, which is used to deflect attacks or startle predators. The signaling underlying eyespot patterning was initially uncovered via transplantation experiments demonstrating that the prospective center of the eyespot (the focus) was capable of inducing concentric eyespot patterns in surrounding tissue when moved to other regions of the wing during pupal development [25,26]. Two species, *Junonia coenia* and *Bicyclus anynana*, have been particularly well studied, with the latter being the source of several major recent studies [22,27]. In *Junonia*, a candidate gene approach identified several molecules whose expression presages the formation of eyespots [28]. Several transcription factors such as *Distal-less* (*Dll*), *engrailed* (*en*), and *spalt* are expressed in patterns coincident with concentric color rings in eyespots of both *Junonia* and *Bicyclus* [29]. In addition to these transcription factors, signaling molecules such as *Notch*, *wingless*, and *dpp* have been found to be expressed between veins at the distal margin of developing imaginal discs, which may be correlated with patterns found in these regions [28,30]. Attempts to assign function to these genes in controlling pattern elements have mostly been correlative. For example, the size and location of eyespots in *Bicyclus* directly correlate with both En and Dll protein expression patterns in the imaginal disc, and co-segregation of sequence polymorphisms in *Dll* with eyespot size suggests that these genes may function in eyespot patterning [27,31]. As in *Heliconius*, co-ordinated efforts are ongoing to understand the nature of pattern formation in *Bicyclus* through the generation of EST libraries, linkage maps, molecular markers, and other genomic tools [22]. Also of note is the recent publication of the first Lepidopteran genome sequence, the silkworm moth *Bombyx mori* [32,33], and the establishment of transgenesis in both *Bombyx* and *Bicyclus* [34–37].

Structural color

Thus far, we have discussed work aimed at understanding how the wing is patterned, but not how colors themselves are generated. Much of the variation in pattern is because of changes in pigmentation of scales in specific regions of the wing. An interesting example of work on how pigment is produced includes [38], where both the developmental timing and the regulation of the pigmentation pathway are shown to be important. Often, even more striking are the effects of changes in fine-scale microstructure, which can affect the way light is reflected by wing scales. This 'structural color' is perhaps best known for producing the effect we call iridescence, such as the brilliant metallic

blue of Morpho butterflies, as well as subtle changes in UV reflectivity [39,40]. As these structures affect the visual appearance of wings, they are important features in long-distance signaling, camouflage, and thermoregulation.

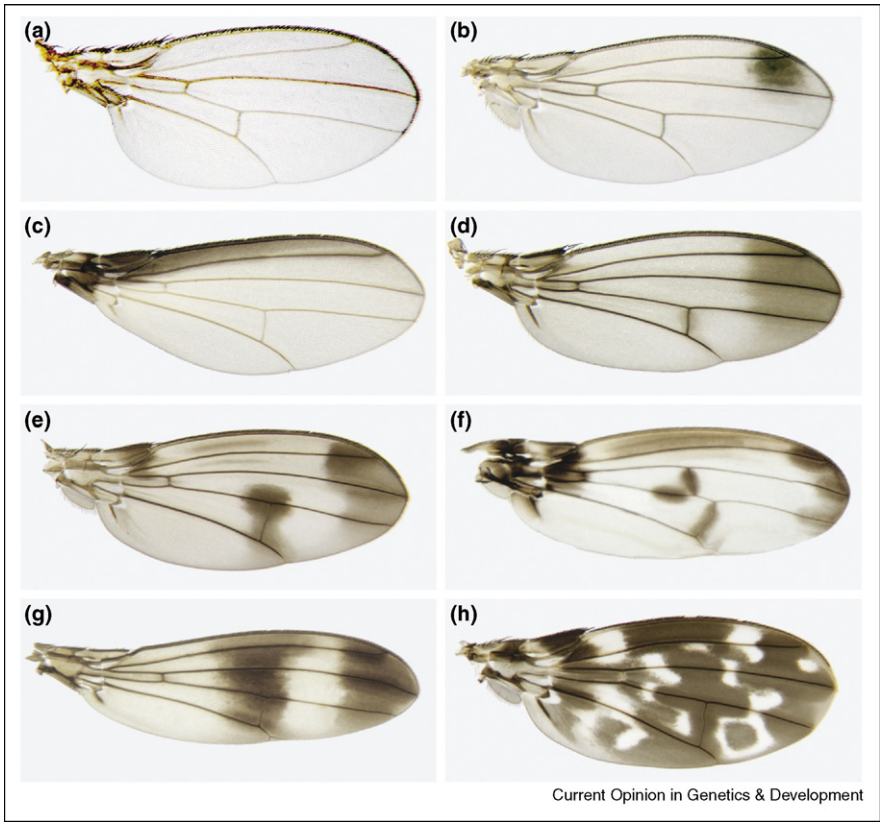
Interests of material scientists, physicists, and theoretical biologists have contributed to our understanding of how individual Lepidopteran scale effects are produced and suggest that this information can be applied to the engineering of human-designed materials [41–46]. In one example, it has been found that scales on swallowtail wings function to efficiently extract fluorescent light, in a process similar to high-emission light-emitting diodes (LEDs) using two-dimensional photonic crystals [47]; this LED technology was developed for human use only a few years previously. Currently, little is known about how scale morphology is encoded genetically and what cellular mechanisms produce these different structures. These structural studies highlight the complex nature of butterfly wing patterning and will hopefully stimulate further study on the interplay of structural color and pigment deposition.

Drosophila wing pattern evolution

Although fly wing patterns cannot match the incredible diversity of butterfly wing patterns, they do exhibit an impressive array of pigment patterns that serve many of the same purposes (Figure 3). Selection in mate recognition, camouflage, mimicry, and thermoregulation all drive phenotypic change within this group. As in the study of butterfly color patterns, understanding the evolution of phenotypic diversity is a major goal of some *Drosophila* biologists, who have made use of the tools developed during the long history of *Drosophila* as a genetic model system. In addition, the biochemical basis of melanization and the process of wing development and patterning are well understood, allowing for a variety of unique and informative experiments. One such study found that melanization of *D. melanogaster* abdominal cuticle was controlled by the reciprocal functions of Yellow and Ebony proteins [48]. Interestingly, complementary expression of these genes is also found in the wings, leading to the hypothesis that changes in their expression patterns may drive novel patterns of wing pigmentation in different species of *Drosophila*.

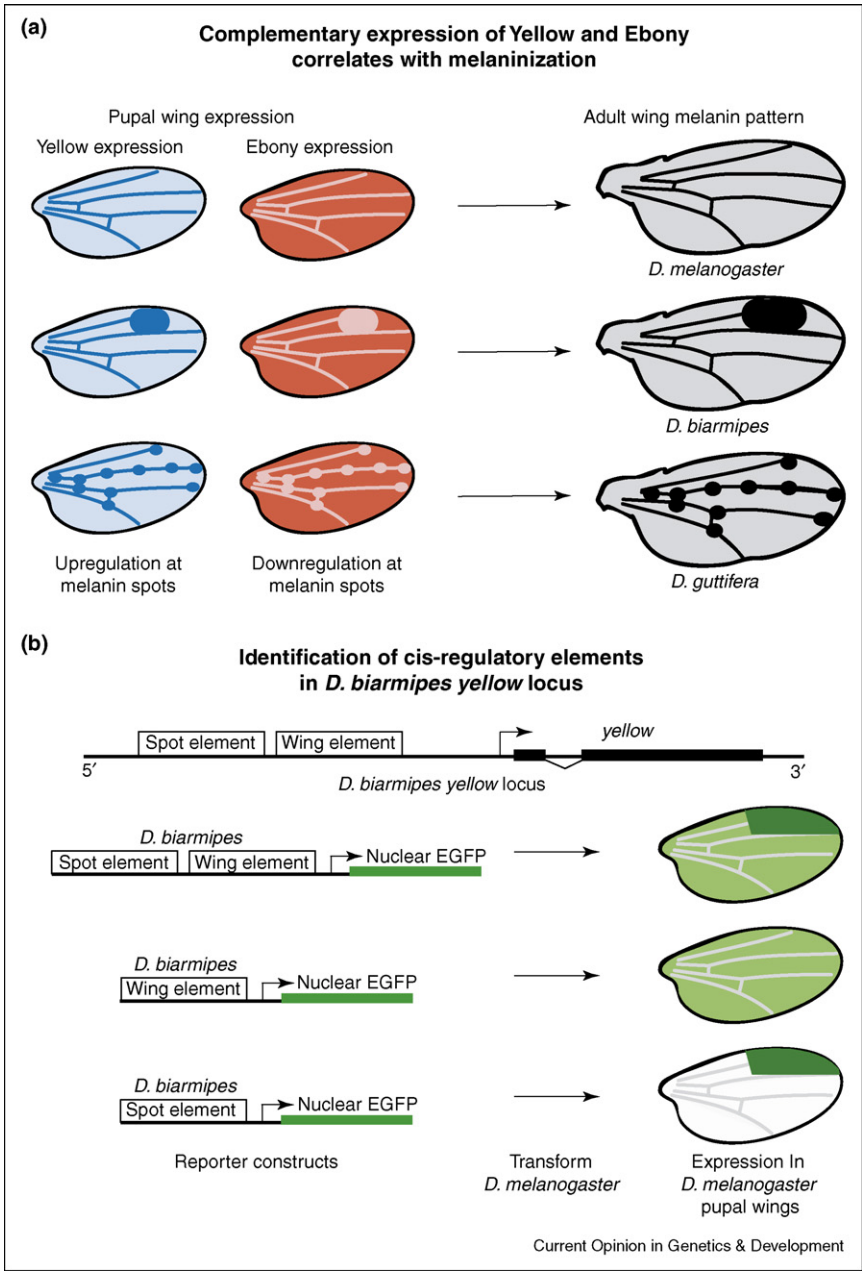
For example, in *Drosophila biarmipes*, the wings of male flies contain a single distal melanic spot. This spot has been shown to be coincident with an upregulated patch of Yellow expression during *D. biarmipes* pupal wing development (Figure 4) [49••].

Figure 3



Examples of the diversity of pigmentation patterns found on the wings of *Drosophila*. **(a)** *D. melanogaster*, **(b)** *D. suzukii*, **(c)** *D. eurypeza*, **(d)** *D. imparisetae*, **(e)** *D. micromyia*, **(f)** *D. heteroneura*, **(g)** *D. cilifemorata*, and **(h)** *D. craddockae*.

Figure 4



(a) Complementary expression of Yellow and Ebony in pupal wings correlates with melanization in adult wings of several species of Drosophilid flies. Low levels of Yellow expression (light blue) are found throughout the pupal wings. High levels of Yellow expression (dark blue) are found in pigmented areas such as along veins or in species-specific patterns. The expression of Ebony is complementary to that of Yellow. Ebony is homogeneously expressed (dark red) except in regions of the wing that will become pigmented, where expression of Ebony is downregulated (light red). The melanin pattern found in the adult wing reflects the complementary expression pattern of these two proteins during pupal development. Black represents areas of the wing with high melanin deposition in contrast to the low levels (light gray) seen in the rest of the wing. **(b)** The *D. biarmipes yellow* locus was functionally dissected by testing the ability of putative enhancer regions to drive reporter gene expression in *D. melanogaster*. An 8-kb region upstream of the *D. biarmipes yellow* locus was capable of driving nuclear GFP in low levels (light green) throughout the wing and high levels (dark green) in an anterior distal spot, thus in a pattern similar to that seen for native *D. biarmipes* Yellow. The upstream region was found to contain two functional elements. One was capable of driving ubiquitous low-level nuclear GFP (light green) wing expression. The other was able to drive the spot-like expression of nuclear GFP (dark green) in the distal part of the pupal wing.

Because the evolution of *cis*-regulatory elements is believed to be an important mechanism driving phenotypic change, such a change could account for the novel expression of *yellow*. In support of this hypothesis, it has been shown that a region upstream of the *D. biarmipes yellow* gene is capable of driving transgene expression in a spot-like pattern when transformed into *D. melanogaster* (Figure 4). In addition, transcription factor binding sites for Engrailed protein (a gene with highly conserved patterns of expression in the posterior region of insect wings) are present within the upstream 'spot' element and are functionally important in spatial control of transgene expression (preventing its expression in the posterior region of the wing). However, positive regulators necessary for upregulating *yellow* expression in the *D. biarmipes* spot pattern have not been identified.

To further understand the evolution of melanic wing spots and the correlated changes in *yellow* expression, Prud'homme *et al*. [50•] identified a clade of *Drosophila* in which some members had a melanic wing spot. By mapping this wing spot character onto a phylogeny of Drosophilid flies, it appears that two independent gains and five losses of the wing spot have occurred. A comparison of regulatory sequences within the *yellow* locus of Drosophilid flies was then used to highlight potential *cis*-regulatory changes that could account for the evolution of *yellow* expression and melanic spot formation. Genomic regions of interest from several species of Drosophilids were subsequently tested within *D. melanogaster* to assay their ability to drive transgene expression in pupal wings. Using this approach, it appears that the same *cis*-regulatory 'spot' element was disrupted during each phylogenetic loss of the spot. In contrast, independent cooption of distinct *cis*-regulatory 'spot' elements is correlated with the repeated gain of the 'spot' wing pattern. However, although *cis*-regulatory changes at the *yellow* locus have allowed its novel expression, ectopic expression of Yellow in the wing of *D. melanogaster* is not sufficient to generate an actual pigmented wing spot (even when *ebony* is downregulated), indicating that changes at other loci are likely to be involved in the evolution of this pattern element [49••]. The complementary expression of Yellow and Ebony might normally be considered as evidence for upstream trans-evolution accounting for changes in the expression of both genes. Surprisingly, identification of regulatory changes at the *yellow* locus may indicate that either *cis*-level evolution may have occurred in concert for both *yellow* and *ebony* to establish the complementary patterns of their expression, or Yellow has a still unknown indirect ability to repress *ebony* expression. Because of the advanced nature of *Drosophila* genetics, continued work on their diverse wing pattern elements will allow cooperative interactions with other projects such as those in butterflies, with the goal of understanding the evolution of phenotypic diversity.

Conclusions

Color patterns on insect wings are an outward, and in many cases obvious, reflection of the complex mechanisms used to generate phenotypic diversity. Because of the long history of ecological and evolutionary investigation into the role wing patterns play in life history, we have come to appreciate how quickly and significantly selection may drive morphological change in several groups of insects. The development of new techniques in butterflies is allowing us to determine the mechanisms of morphological divergence and convergence in this incredible group of winged insects. Perhaps one of the most important evolutionary findings in wing patterning studies is that morphological changes at the species or genus level may be controlled by changes in just a few genes. The detailed studies of wing spot evolution in *Drosophila* parallel the efforts of the butterfly research community to understand morphological change and pattern formation. As research in these fields moves forward, there remain several other unanswered questions. It is still not known whether the genes controlling wing patterning in Lepidoptera and *Drosophila* are the same and to what degree novel genes may contribute to each process. In addition, it is unknown how genomic organization (e.g. clustering of wing patterning genes) may play a role in wing pattern evolution. Undoubtedly, the identification and characterization of the changes driving *Heliconius* evolution will help answer these types of questions. Owing, in part, to the rapid progress of several research groups, insect wing patterning is becoming a paradigm in the area of phenotypic diversity.

Acknowledgements

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This is the first high-resolution linkage map developed for *H. melpomene*. It is made up of AFLPs, microsatellites, SNPs and two color pattern switch genes; it has 21 linkage groups corresponding to each of the 21 chromosomes. Concurrent development with a map of *H. erato* has allowed for comparison between the these two species that so often mimic one another.

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This effort expands on a 'first generation' linkage mapping effort [20] and increases the density of the map, as well as the resolution of the location of two color pattern loci. This work allowed for comparisons between *H. erato* and *H. melpomene*.

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In this paper, the authors compare pattern loci within and among three *Heliconius* species. Perhaps one of the most surprising findings of linkage mapping and crossing efforts in the *Heliconius* has been that multiple loci that are involved in producing specific color patterns seem to map to nearly the same region. The authors call this region in *Heliconius* a 'supergene', and have narrowed the location to a specific genomic region, perhaps containing as few as 12–14 genes. Also interesting is that none of the loci map to locations near any of the potential candidate genes found in butterfly 'eyespot' patterning work.

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This project used quantitative trait mapping to place a mate preference choice (a patch of color on the forewing) along with the preference cue itself. Surprisingly, each was found to map to the same location, and this location is also linked to the patterning candidate gene *wingless*. This is the first and to date the only study to link a signaling molecule to a *Heliconius* color pattern locus.

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