Mimicry in butterflies: co-option and a bag of magnificent developmental genetic tricks

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Butterfly wing patterns are key adaptations that are controlled by remarkable developmental and genetic mechanisms that facilitate rapid evolutionary change. With swift advancements in the fields of genomics and genetic manipulations, identifying the regulators of wing development and mimetic wing patterns has become feasible even in nonmodel organisms such as butterflies. Recent mapping and gene expression studies have identified single switch loci of major effects such as transcription factors and supergenes as the main drivers of adaptive evolution of mimetic and polymorphic butterfly wing patterns. We highlight several of these examples, with emphasis on *doublesex*, *optix*, *WntA* and other dynamic, yet essential, master regulators that control critical color variation and sex-specific traits. Co-option emerges as a predominant theme, where typically embryonic and other early-stage developmental genes and networks have been rewired to regulate polymorphic and sex-limited mimetic wing patterns in iconic butterfly adaptations. Drawing comparisons from our knowledge of wing development in *Drosophila*, we illustrate the functional space of genes that have been recruited to regulate butterfly wing patterns. We also propose a developmental pathway that potentially results in dorsoventral mismatch in butterfly wing patterns. Such dorsoventrally mismatched color patterns modulate signal components of butterfly wings that are used in intra- and inter-specific communication. Recent advances—fuelled by RNAi-mediated knockdowns and CRISPR/Cas9-based genomic edits—in the developmental genetics of butterfly wing patterns, and the underlying biological diversity and complexity of wing coloration, are pushing butterflies as an emerging model system in ecological genetics and evolutionary developmental biology. © 2017 Wiley Periodicals, Inc.

MIMICRY IN BUTTERFLIES: A SINGULAR ADAPTATION

Few adaptations in nature are as striking and widely appreciated as bright, diverse wing color patterns of butterflies. These color patterns have evolved to serve diverse and crucial functions in sexual selection, predator avoidance, and thermoregulation. Of these, aposematism and mimicry[1,2] (Box 1) are phylogenetically widespread and exhibit considerable diversification with respect to polymorphism[3] and sex-limitation[4,5] (Figure 1), whereby one or both sexes may have morphological variants that are strongly regulated by allelic variants.[7,8] This morphological diversity reflects diverse ecological regimes, intense selection pressures and molecular mechanisms that have shaped the evolutionary and genomic histories of butterflies. Density- and frequency-dependent

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selection pressures,[2] working together with the functional (reproductive) roles as well as sexual selection acting differentially on the sexes,[5] influence this diversity of wing patterns.

WHAT IS MIMICRY?

Butterflies employ several survival strategies to escape predators. One is aposematism, which involves chemical defence and associated conspicuous wing color patterns to warn predators. Another is mimicry, where multiple species share a warning signal. However, there is honesty and deceit in mimicry. In 'Müllerian mimicry,' two or more chemically defended species resemble each other. This is an honest, mutualistic relationship where the shared wing patterns of several toxic species may reduce the net predation pressure on each toxic species,[9] and this may also help naïve predators learn the aposematic patterns more quickly. Müllerian mimicry is thus under positive density- and frequency-dependent selection.[10]

There is a mutually beneficial relationship between educated predators and the aposematic species that they have learnt to avoid eating. Occasionally, this relationship is exploited by some unrelated, chemically undefended—and thus palatable—species. These palatable mimics are called Batesian mimics, which may be considered to be parasites of the aposematic signals, which are protected by their mere visual and behavioral resemblance of aposematic species.[11] This is under negative frequency-dependent selection, the success of which depends on the inability of predators to distinguish between the honest aposematic species and the parasitic Batesian mimics, and the relative rarity of Batesian mimics.[4,10–12] Increase in the frequency of Batesian mimics will jeopardize mimicry systems as predators begin to encounter a greater proportion of conspicuously patterned but easy to capture palatable butterflies.

A region may have a community of butterflies exhibiting both Müllerian and Batesian mimicry.[13] These mimetic communities are known as 'mimicry rings' (Figure in Box 1). A region may have multiple mimicry rings, each with different wing coloration.

Such rich biological detail and the diversity of butterfly wing color patterns themselves provide some unusual advantages as study systems in evolutionary biology and developmental genetics. Butterflies are at a golden point where, like birds, they are large and conspicuous enough to follow in the field to understand their biology, and like *Drosophila*, small enough and easy to raise in captivity with a short lifecycle to be a good lab-based model system for genetic and other manipulations. A broad understanding of the functional basis of their wing color
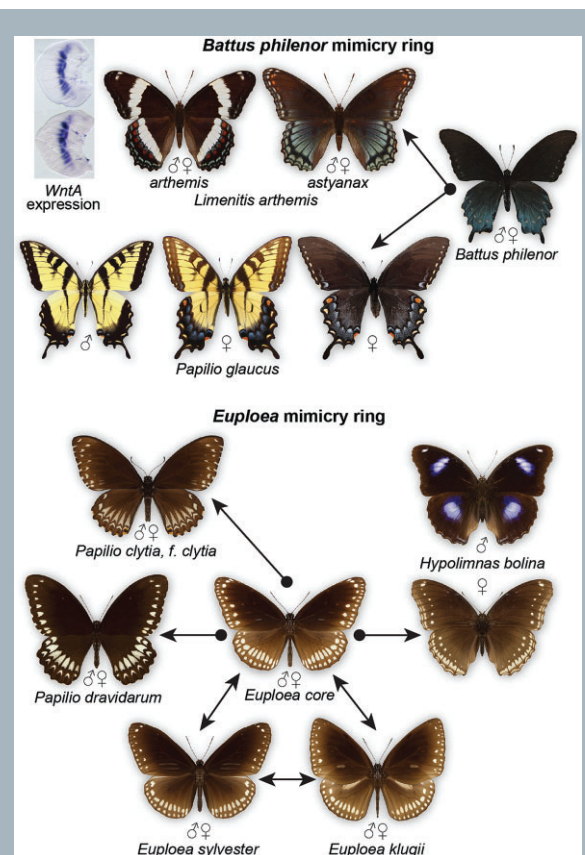
Batesian and Müllerian mimicry in butterfly mimicry rings. The two mimicry rings are driven by the aposematic species, *B. philenor* of North America and *Euploea* of S. Asia, which are mimicked by multiple Batesian mimics. The *Euploea* mimicry ring also has multiple aposematic species, which are Müllerian mimics of each other. Two-sided arrows point out the mutual benefit of Müllerian mimicry, whereas one-sided arrow-and-ballheads indicate unidirectional benefit of Batesian mimicry. Male of *H. bolina*, male and male-like female of *P. glaucus*, and white-banded form of *L. arthemis* are outside the mimicry rings. Wing patterns and mimicry in *L. arthemis* are controlled by the expression of *WntA*[14] (images of *in situ* hybridization: courtesy of Arnaud Martin).

patterns has emerged in the past 150 years. Here, we review the substantial progress that has recently been made in the molecular and developmental genetics of mimetic wing color patterning in butterflies—a fast-moving field.

GENETIC ARCHITECTURE OF MIMICRY

The genetic architecture of adaptations may have a significant impact on the evolutionary contingency with respect to selection pressures and local fitness landscapes. Wing patterns of butterflies show the following distinct types of underlying genetic architecture. Whether this diversity of genetic architecture modulates the evolutionary tempo and mode of wing pattern diversity with respect to specific selection pressures remains to be seen.

Supergenes as Single-Locus Architecture

Mimicry phenotypes are considerably complex, typically with several color patterns confined to specific wing areas and along dorsal and ventral surfaces. This complexity is further compounded by extensive mimetic polymorphism, that is, multiple co-occurring forms within a population, in many species (Figures 1, 3 and 4). When polymorphic mimetic forms mimic distinct models (Figures 1 and 4), the intermediate mimetic forms are maladapted since predators do not recognize them as unpalatable prey. Thus, the genetic architecture of polymorphic mimicry that is expected under such negative selection
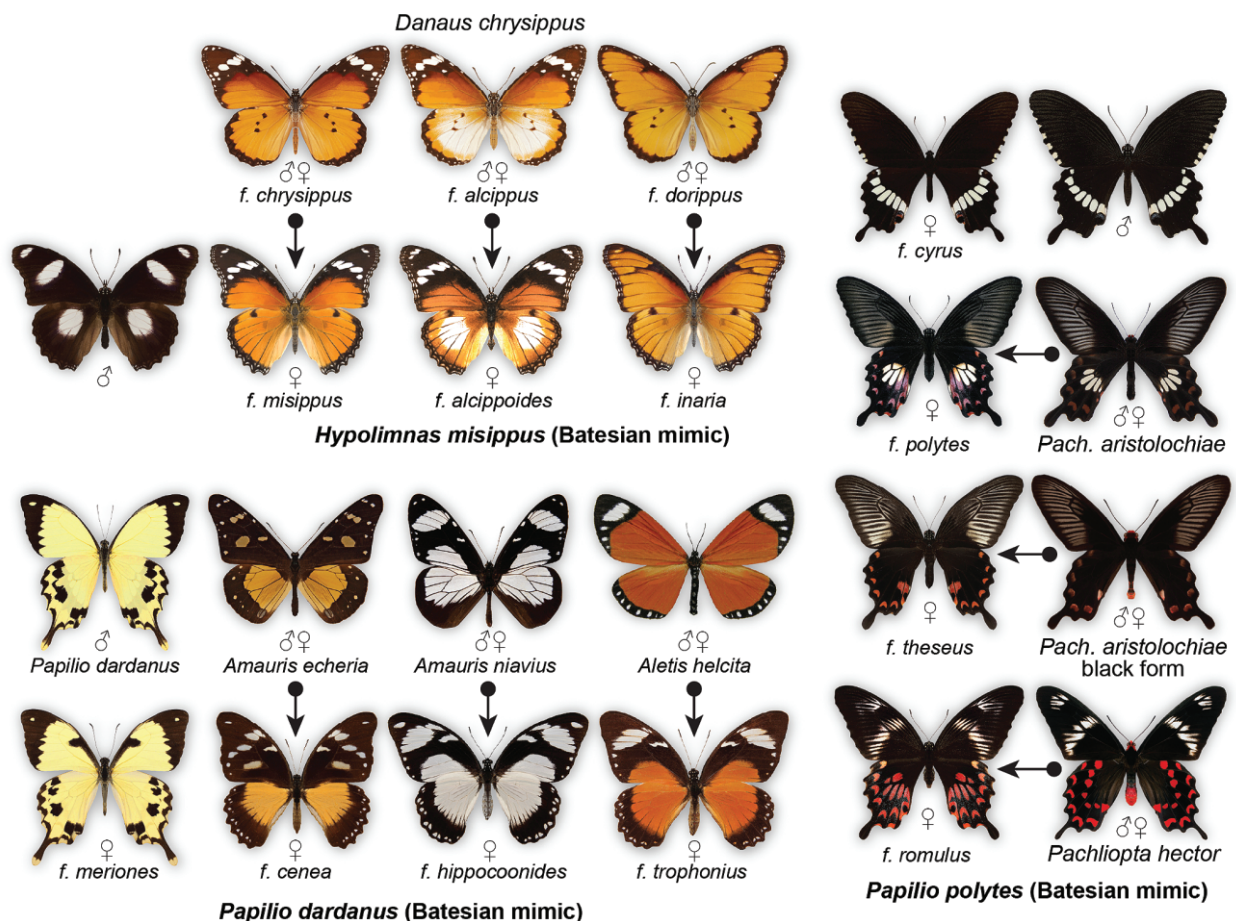

FIGURE 1 | Sex-limited mimicry and polymorphism in butterflies.[6] The *Danaus-Hypolimnas* mimicry ring illustrates a rare example where both the Batesian model and the mimic are polymorphic, female forms of the Batesian mimic H. missipus mimicking *D. chrysippus* in a form-specific manner. On the other hand, in *P. dardanus* and *P. polytes*, multiple female forms mimic distinct species of models. In the last two species, the male and male-like female forms are nonmimetic, which in *P. polytes* also represents the ancestral phenotype. These species represent some of the best examples of the degree to which natural selection—through predation—may drive nearly perfect and polymorphic wing pattern resemblance between Batesian models and mimics. Mimicry is controlled by co-option of major developmental genes in both the *Papilio*, whereas the molecular genetic basis in *Hypolimnas* is still unknown. See Figure in Box 1 for the explanation of arrows.

against the intermediates is classically believed to be supergenes. In its original formulation, a supergene refers to tightly linked functional genes that regulate a switch between complex polymorphic phenotypes.[28–32] The supergene architecture is thought to initially translocate functionally related genes in such a tight cluster at a single locus that there is little recombination between the individual genes contained therein. Thus, the mutations that accumulate in different alleles of supergenes regulate alternative polymorphic phenotypes.[29,33–35] In a series of breeding experiments that spanned decades and a diversity of species, Clarke and Sheppard studied inheritance of many polymorphic forms, which repeatedly showed a lack of recombinants (phenotypic intermediates) in female-limited polymorphic mimics: *Papilio polytes*,[36] *Papilio dardanus*,[37] and *Papilio memnon*.[38] Based on this, Clarke and Sheppard inferred the supergene architecture of these polymorphisms, but none of the supergenes implicated was characterized at a molecular level.

Heliconius numata—a superbly polymorphic Müllerian mimic that constitutes large mimicry rings with other *Heliconius* and ithomiine butterflies, and moths—provided the first molecular characterization of a supergene. Polymorphism in *H. numata* is governed by the P locus.[39] This is a 400 kb block, located on linkage group 15 (LG15), containing at least two large chromosomal rearrangements that have given rise to three supergene alleles, combinations of which are responsible for distinct forms.[40] The P locus corresponds to the *Yb-Sb-N* complex that is also on the LG15 in *Heliconius melpomene*. This supergene has arisen from a multilocus architecture that controls wing patterns in many *Heliconius* species (see the *Multilocus Architecture* section below), where translocations of a few wing patterning genes to LG15 followed by an inversion locking these genes in a single linkage group has secured this nonrecombining supergene.[39,40] The phenotypic polymorphism resulting from the P supergene is maintained by opposing forces of frequency dependent selection imposed by predation pressure and mate choice.[41]

The second molecular characterization of a supergene was provided by recent work on another fascinating polymorphic but in this case a Batesian mimic, *P. polytes*[15,16] (Figure 1 and Figure in Box 2). Based on the nonoverlapping wing patterns and presence of tails in specific female forms, Clarke and Sheppard had already inferred supergene architecture in this species, which now turns out to deviate from the original formulation of the idea.[28] In *P. polytes*, instead of multiple tightly linked genes, a single but complex autosomal gene, *doublesex* (*dsx*), controls mimetic polymorphism.[15] *dsx* is an important transcription factor known for its role as a terminal 'double switch' in the somatic sexual differentiation cascade in insects.[42] The pre-mRNA of *dsx* is sex-specifically spliced to encode male- or female-specific transcription factors that unleash a genetic cascade that channels development of embryos into male and female bodies.[42–44] In *P. polytes*, this early developmental transcription factor has been co-opted later during pupal development when wing color patterns are laid down, to produce distinct nonmimetic and mimetic wing patterns. *dsx* does this using its conventional bag of developmental tricks: (1) alternative splicing and (2) sex- and tissue-specific expression during critical developmental stages.[15,16] Apart from revealing a new function in polymorphic wing color patterning for this conserved gene, these studies also added a new trick to the bag of known tricks for *dsx*: tissue-specific expression across sexes and forms.[15] *dsx* not only splices into male- and female-specific forms, it also splices differentially in the abdomens and developing wings in the female pupae[15] (Figure 2(c)). Finally, the mimetic female form is produced by over-expression of wing-specific *dsx* splice variants[15,16] (Figure 2(d)), which may be partially suppressed by RNAi manipulation[16] (Figure (d) in Box 2). Similar to the *H. numata* supergene, the mimicry supergene in *P. polytes* is protected by an inversion covering ~130 kb in the mimetic form. Interestingly, *dsx* is a hotspot of adaptive molecular evolution: the mimetic *dsx* allele has accumulated a number of fixed nonsynonymous mutations, in addition to many more fixed synonymous mutations, that change the protein structure and presumably function.[15,16] Exons, their alternative splicing and molecular divergence of *dsx* in the Lepidoptera are summarized in Figure 2.

The concept of supergenes was conceived[4,28] and it developed considerably[8,32] in the 1960s and 1970s when structures, forms, and actions of genes were poorly understood. Recent molecular characterizations of supergenes suggest that we may classify supergenes into two modern classes: (1) classical supergenes—*sensu* Clarke and Sheppard, with tightly linked genes with or without inversions protecting them—as now shown in the mimetic polymorphism of *H. numata*,[39,40] the male polymorphism of the ruff,[33,34] and the pin and thrum flower polymorphism of *Primula*[45]; and (2) master regulator supergenes, as now shown in *P. polytes*. Master regulator supergenes will differ from other master regulators such as transcription factors (discussed in the next section) in controlling a broad range of phenotypes
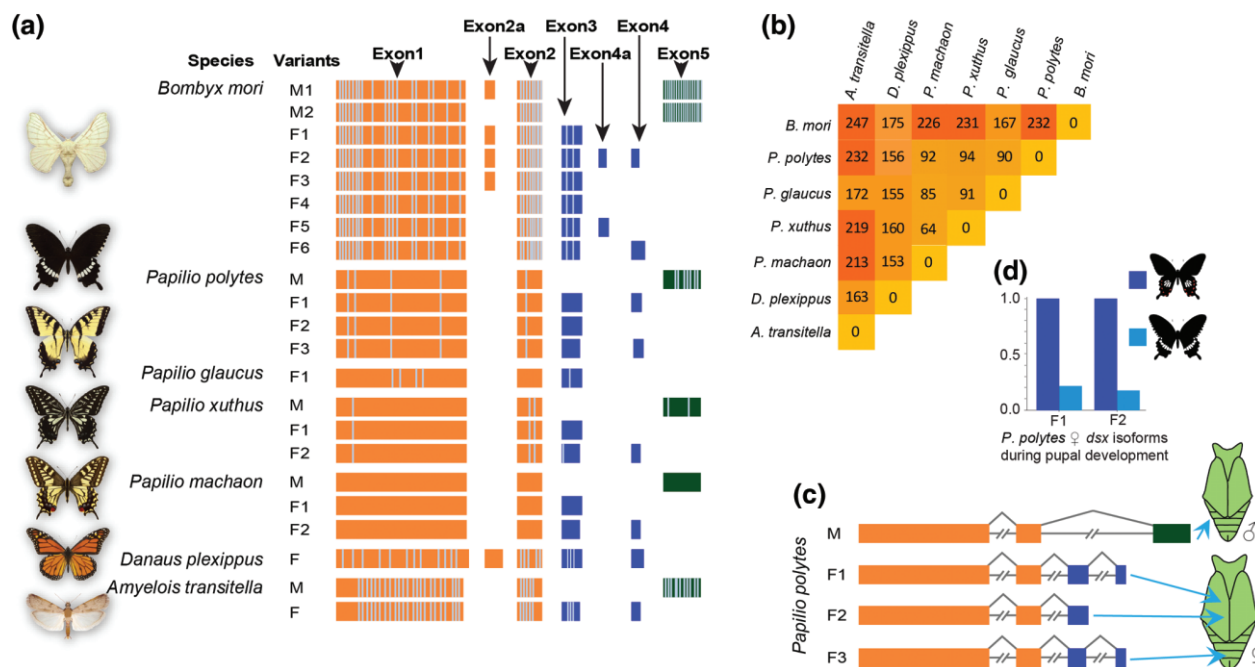
FIGURE 2 | The CDS regions of doublesex (dsx) show hundreds of SNPs in lepidopteran genomes, some of which are nonsynonymous, indicating that dsx may be a hotspot of adaptive molecular evolution in some regions but highly conserved in others. (a) Exon usage in the sex-specific isoforms and other variants are shown along with the SNPs (vertical gray bars) unique to each sequenced genome. It is yet unknown whether nonuniversal exon usage and the species-specific SNPs across different exons are adaptive, and how they might relate to sex-limited mimicry and other adaptive traits in butterflies. (b) Species pairwise comparison of the total number of SNPs of dsx, which shows some correlation between phylogenetic relatedness and the number of genetic differences. (c–d) dsx splice variants (a and c) and their tissue- (c) and form-specific (d) expression that controls female-limited mimetic polymorphism in P. polytes. Data are from GenBank and LepBase (panels a–b), and Kunte et al. (panels c–d). The mimetic female form is produced by upregulation of dsx (d).[16]

that are functionally linked. A good illustration of this is the action of *dsx* in the regulation of polymorphic mimicry in *P. polytes*. In this species, very different wing color patterns and the presence/absence of tails are coadapted as distinct nonmimetic and mimetic forms (Figure 1). Accounting for accompanying differences in the behavior and flight of mimetic and nonmimetic butterflies,[46,47] *dsx* potentially controls coadapted suites of diverse traits such as wing color patterns, presence of tails, flight, and other behaviors in alternative female forms of *P. polytes*, acting as a single—and singular—master regulator supergene. All these traits are otherwise unrelated in butterflies, suggesting that *dsx* has brought them under its control specifically in the context of coadapted traits as required for polymorphic mimicry in the presence of selection against phenotypic intermediates. It is obvious from this specific example of *dsx* that a single gene, according to this idea, may have multiple distinct functions but it may be treated as a master regulator supergene only in contexts where it controls a broad range of functionally linked traits (e.g., in polymorphic mimicry in *P. polytes*). The same gene may simultaneously be

treated as a gene with pleiotropic effects in contexts where it may regulate functionally unrelated traits (e.g., somatic sexual differentiation in early development and wing pattern elements[26] or caste differentiation in later development in the social Hymenoptera[48]). To our knowledge, the only other known example of a master regulator supergene may be the K locus that controls both alternative white/yellow wing banding patterns and assortative mate preference for those banding patterns in the dimorphic *Heliconius cydno*.[49] Whether the distinction between classical supergenes and master regulator supergenes proposed above is useful or not remains to be seen as examples of supergenes are characterized at a molecular level in the future, and as complexities of gene action and function are elucidated.

Multilocus Architecture

Master Regulators in the Neotropics
Supergenes are rarer examples of single loci of large effect controlling major adaptations. Most wing patterning in butterflies is instead regulated by multiple
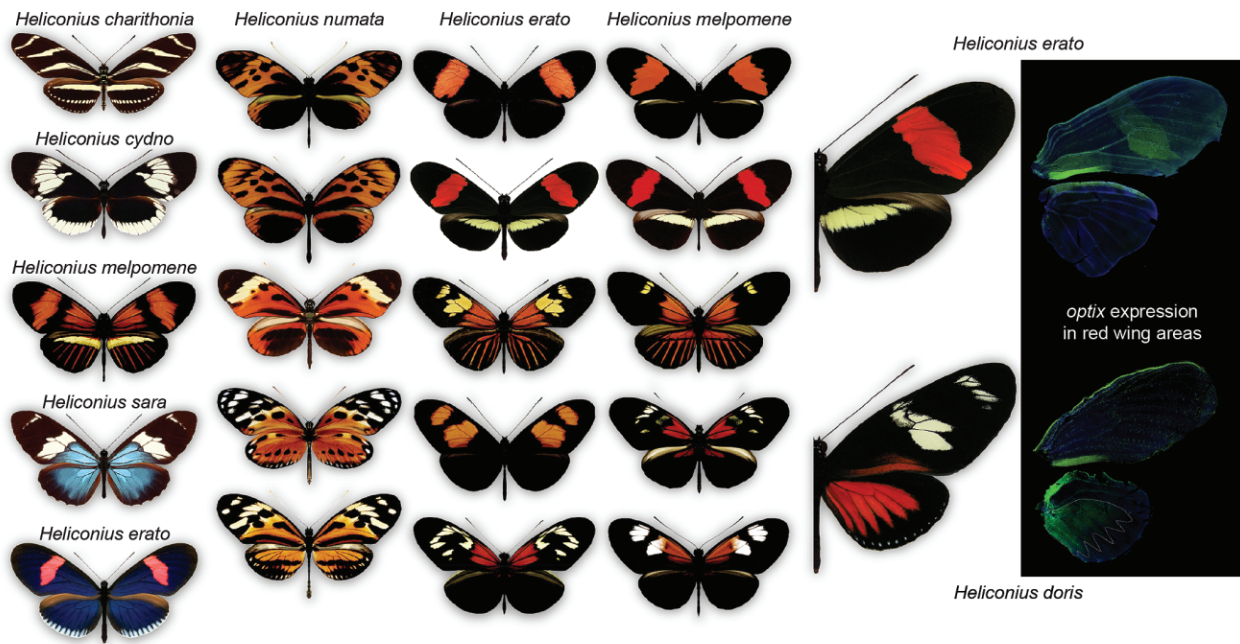
FIGURE 3 | The striking wing patterns of the Neotropical *Heliconius* butterflies are products of their aposematism, taxonomic diversity and loose reproductive isolation between species. Wing patterning alleles must have initially evolved under genetic drift and selection for aposematism as well as for Müllerian mimicry, and have subsequently been widely introgressed across species. *H. numata* has a supergene architecture that controls polymorphic mimicry. *H. erato* and *H. melpomene* show parallel diversification in wing patterning for Müllerian mimicry (top three forms), but also show hybrid and intermediate forms. Gene expression of *optix*, which controls red wing areas in *Heliconius* is shown on the right[26] (antibody staining: image courtesy of Arnaud Martin).

independent loci. The majority of the molecular and developmental genetic work on butterfly wing patterning in the past two decades has concentrated on the Neotropical *Heliconius* butterflies, popularly known as 'longwing butterflies.' Well known for their Müllerian mimicry, certain *Heliconius* species pairs have diversified into multiple co-occurring forms with similar phenotypes (e.g., the *H. erato–H. melpomene* (Figure 3) and the *H. cydno–H. sara/ H. sapho* clades). Breeding experiments since the 1970s[50–53] had identified dozens of wing patterning loci in *Heliconius*, indicating that a multilocus architecture with several large-effect loci govern the incredibly diverse and in some cases highly polymorphic wing patterns in these butterflies. The concepts of 'windows' and 'shutters' in wing color patterning recently provided a framework to study permutations of various wing pattern elements that comprise wing coloration across the entire *Heliconius* clade.[50]

Recent genotype–phenotype mapping[54–56] and gene expression[57–59] studies have shown that only a handful of large-effect loci control most of the wing pattern diversity in *Heliconius*. Most of the *Heliconius* and other tropical mimetic butterflies have black background colors on their wings. This melanic

coloration is controlled by the master regulator *WntA*—a Wnt signaling ligand and morphogen—in *H. erato*, *H. melpomene*, *H. cydno*,[58] and other nymphalid relatives such as *Limenitis arthemis*.[14] The expression of *WntA* outlines the boundaries of conspicuous patterns of visual importance (e.g., the white band in *L. arthemis* (Figure in Box 1) or forewing bands on *Heliconius* wings). *WntA* appears conserved for the protein sequence across the *Heliconius* and *Limenitis* clades, pointing towards *cis*-regulatory changes as the major governing factor for differential regulation of the wing phenotypes.[58]

Various color patches appear before this melanic wing color background is apparent during pupal development. The transcription factor *optix* (loci D, B, and G in classical *Heliconius* literature) regulates the red patterns and bands on the wings based on the variation upstream of its coding region.[26,57] The *cis*-regulatory diversity flanked by *optix* and *kinesin* (a previously identified target that showed a correlation with red forewing bands) appears to control *optix* expression in the wings and regulate different patterns of red[60–62] (Figure 2 and Figure in Box 3).

The locus *Yb* was known as the major-effect locus that controlled scale structure, and white and

BOX 2

THE TOOLBOX OF BUTTERFLY DEVELOPMENTAL GENETICISTS

Butterflies are very diverse and their biology in the field is much better known compared to that for model organisms, so experiments on butterflies are often illuminating with respect to the life of organisms in nature. This makes butterfly wing patterns attractive systems to study the genetics and evo-devo of adaptations. The field of developmental genetics of butterfly wing patterning is advancing rapidly because of modern technological innovations in next-generation sequencing and genome editing, which makes it possible to bring nonmodel organisms to the lab and under the purview of the latest genomic, molecular, and bioinformatic methods. However, butterflies—being nonmodel organisms—have few genetic and developmental resources such as pure-breeding mutational lines, fine-tuned transgenesis protocols, and commercially available laboratory stocks. Nonetheless, significant progress on the developmental genetics of butterfly wing patterning has been possible because of the methods and protocols described below. Genetic manipulations—the golden standard of developmental genetics—was until recently exclusively available in model organisms with decades of intensive molecular work. The advent of the CRISPR/Cas9 technology has already made such genetic manipulations feasible in nonmodel and rapidly emerging new model organisms such as butterflies.
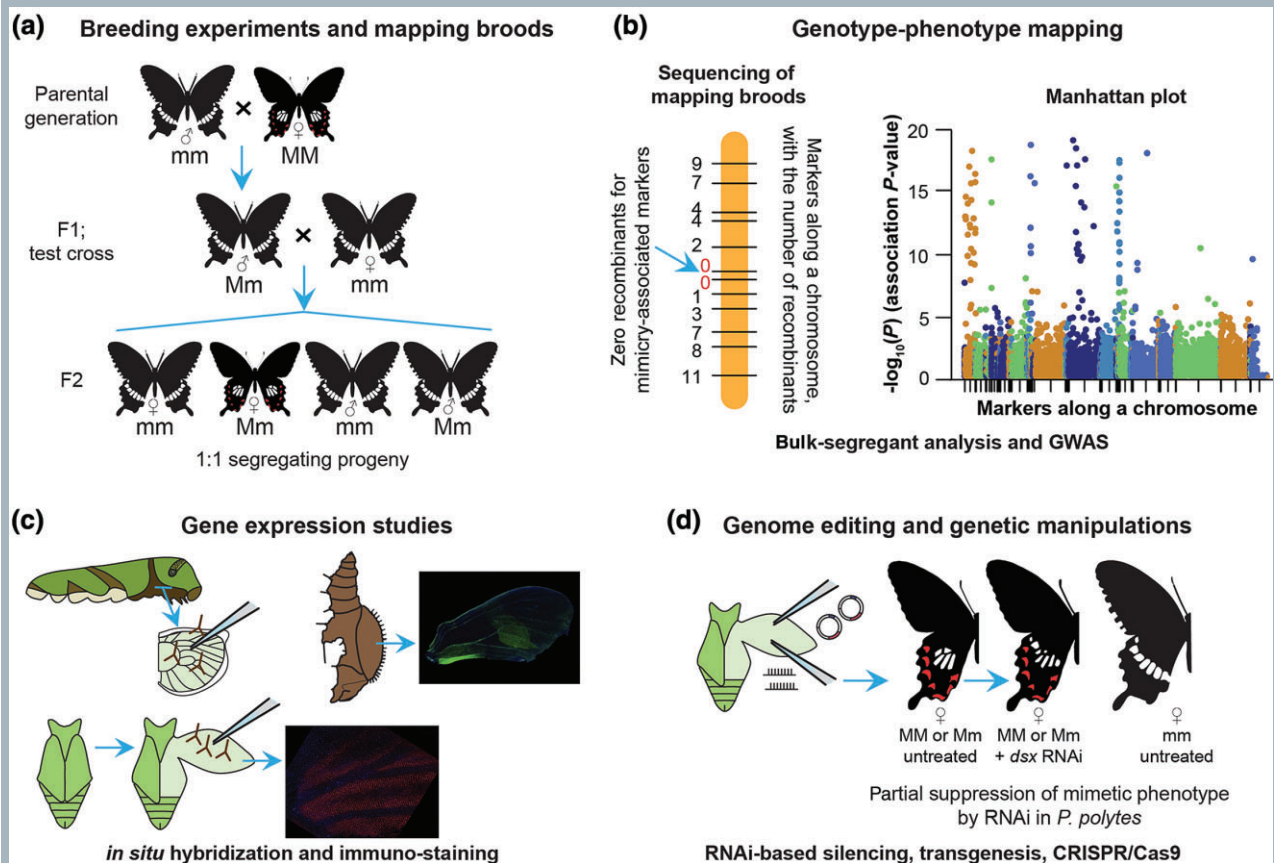
Commonly used methods and protocols to study butterfly wing patterning. (a) Crossing designs vary slightly in species with complete dominance (e.g., *Papilio polytes*, illustrated in Ref 15) versus co-dominance (many *Heliconius*), following the usual Mendelian inheritance patterns. It is important to use males—rather than females—that are heterozygous for mimicry alleles to generate mapping broods since butterflies show female heterogamety and achiasmatic oogenesis. (b) In absence of more advanced developmental genetic resources, most studies on butterfly wing patterning still rely largely on genotype–phenotype association methods such as bulk segregant analysis and genome-wide association studies (GWAS).[15] (c) However, gene expression studies are becoming popular among evolutionary geneticists venturing into developmental genetics. This has resulted in better insight into the developmental genetics of key mimetic butterflies such as *P. polytes*,[15,16] and *P. glaucus*,[17–19] *Heliconius erato*,[20] *H. melpomene*,[20] and *H. cydno*[20] groups, and *Limenitis arthemis*.[14] (d) Transgenesis[21,22] and CRISPR/Cas9-led manipulations[23–25] have already been used in butterfly wing patterning and vision, and their use in mimicry genetics is promising. RNAi-based silencing has already been successfully demonstrated in the mimetic *P. polytes*[15,16] (antibody staining: image courtesy of Arnaud Martin).

yellow color patterns, in *H. erato*, *H. melpomene*, *H. timareta*, and *H. elevatus*.[53,63,64] *Yb* has now been identified to be *cortex*, a highly conserved fizzy gene family member and annotated as a cell-cycle regulator,[65,66] associated with white and yellow pattern elements in *Heliconius* and melanization in peppered moths.[59,67] The differentially expressed *cortex* isoforms show variations in the exonic as well as intronic regions, which recently led to the belief that *cortex* regulates pigmentation and patterning by influencing scale cell development and thereby indirectly affecting melanisation.[59]

This recent work on wing patterning genes in *Heliconius* has repeatedly underscored the fact that different wing color backgrounds and patterns are controlled by unlinked master regulators perhaps working independently of each other at different developmental stages. However, the generality here appears to be that each one of them is a transcription factor, that is, a master regulator, which has been co-opted from its traditional function in early as well as pupal development to produce novel wing color patterns in butterflies. The list of genes that control specific wing colors and patterns in *Heliconius* as an outcome of co-option is given in Figure 5, and Figure S1 and Table S1 (Supporting Information). The specific actions of these genes in specific wing areas, forms, and subspecies of all *Heliconius* species are reviewed elsewhere.[20]

A Mimetic Marvel on the African Plains

Similar multilocus architecture of mimetic wing color patterns occurs in other butterfly species as well. *Hypolimnas misippus* is a pantropical polymorphic butterfly that shows female-limited mimetic resemblance to the aposematic *Danaus chrysippus* (Figure 1), both of which have been particularly intensively studied in Africa. *H. misippus* has four commonly identified female forms: *misippus*, *inaria*, *alcippoides*, and *inaria-alcippoides*, each exhibiting a different combination of black, brown, and orange pigments. These phenotypes show complex inheritance patterns that are genetically regulated by three interacting loci: (1) *M*, which controls forewing color; (2) *A*, which controls the presence or absence of white in the hindwing; and (3) *S*, which suppresses the output of *A*.[120,121] Incidentally, the wing patterns in the model, *D. chrysippus*, are also governed by three loci: (1) *C*, which produces all-orange forewings when dominant; (2) *B*, which produces brown ground color and is tightly linked with *C*; and (3) *A*, which produces orange hindwings when dominant.[122] The color pattern genetics of these two species differs in several characteristics, indicating potential multiple origins of the loci that govern remarkable and polymorphic similarity in this Batesian model-mimic species pair.[120,121] However, the molecular characterization of mimicry genes in these species is still missing.

Evolutionary Genetic Enigmas of the Old World Tropics

The molecular basis of wing pattern diversity is slightly better known in another African species, *P. dardanus* (Figure 1), in which the highly polymorphic Batesian mimicry corresponds to the *engrailed–invected* gene region.[123,124] Specific variation within the different regions of the engrailed–invected locus, which potentially contains a duplication, appears to associate with different female forms.[65] The engrailed–invected loci are, of course, important in anterior–posterior compartment and boundary formation in *Drosophila*.[125,126] This is yet another example of co-option of major developmental genes that have been recruited in butterfly wing pattern development (Figure 5). Although association studies have shown the engrailed–invected region to be associated with the *P. dardanus* mimicry, the exact action of this locus remains to be explored. This is especially relevant because crosses between specific populations appear to show differential dominance hierarchies and roles of *engrailed* and *invected*, indicating that mere association studies may be insufficient in this highly complex—and therefore challenging and instructive—species.[37,124,127]

Another *Papilio* from the Oriental region offers another difficult evolutionary genetic riddle, still awaiting molecular characterization. *P. memnon* is one of the most polymorphic mimics in the world, with nearly a dozen male forms and over two dozen female forms distributed across numerous populations that are splintered in a broad mainland–island mosaic of the Indo-Australian region. Unlike the very specific, adaptive mimetic polymorphism seen in *H. misippus* and *P. polytes*, polymorphism in *P. memnon* appears to have gone wild, perhaps in absence of strong selection[38,128] (Figure 4). Although many well-defined female forms are good mimics, many others do not appear to have any specific Batesian models in their ranges, and some of their wing pattern elements recombine to produce diverse non-mimetic phenotypes, including tailed and nontailed forms. As expected, the genetic basis of this polymorphism is excruciatingly complex, with mimetic forms in certain populations controlled by a single, complex, autosomal locus with at least 11 alleles that are expressed only in females.[38,128] The properties of this

locus appear congruent with the supergene architecture, which might have been assembled due to disruptive selection favoring linkage between coadapted loci.[38] Although sympatric forms often exhibit complete genetic dominance hierarchy, inter-population hybrid offspring show lesser mimetic resemblance compared to the progeny of intra-population polymorphic forms.[38] This indicates that *P. memnon* has genetic modifiers elsewhere in the genome, or an effect of the genomic background, that influence the action of the main mimicry supergene. It is possible that additional quantitative trait loci (QTL) have

small additive effect on these diverse wing pattern elements. *P. memnon* is closely related to *P. polytes*, hence two of its *doublesex* alleles (one in a mimetic and another in a nonmimetic form) were recently characterized in the hopes that the molecular basis of their mimetic polymorphism is also related.[129] However, *doublesex* alone is unlikely to explain the phenotypic details summarized above. Whatever their exact molecular genetic bases, mimetic polymorphism in *P. dardanus* and *P. memnon* strengthens the pattern of multilocus and master regulator architecture of adaptations. Considering the difficulties of
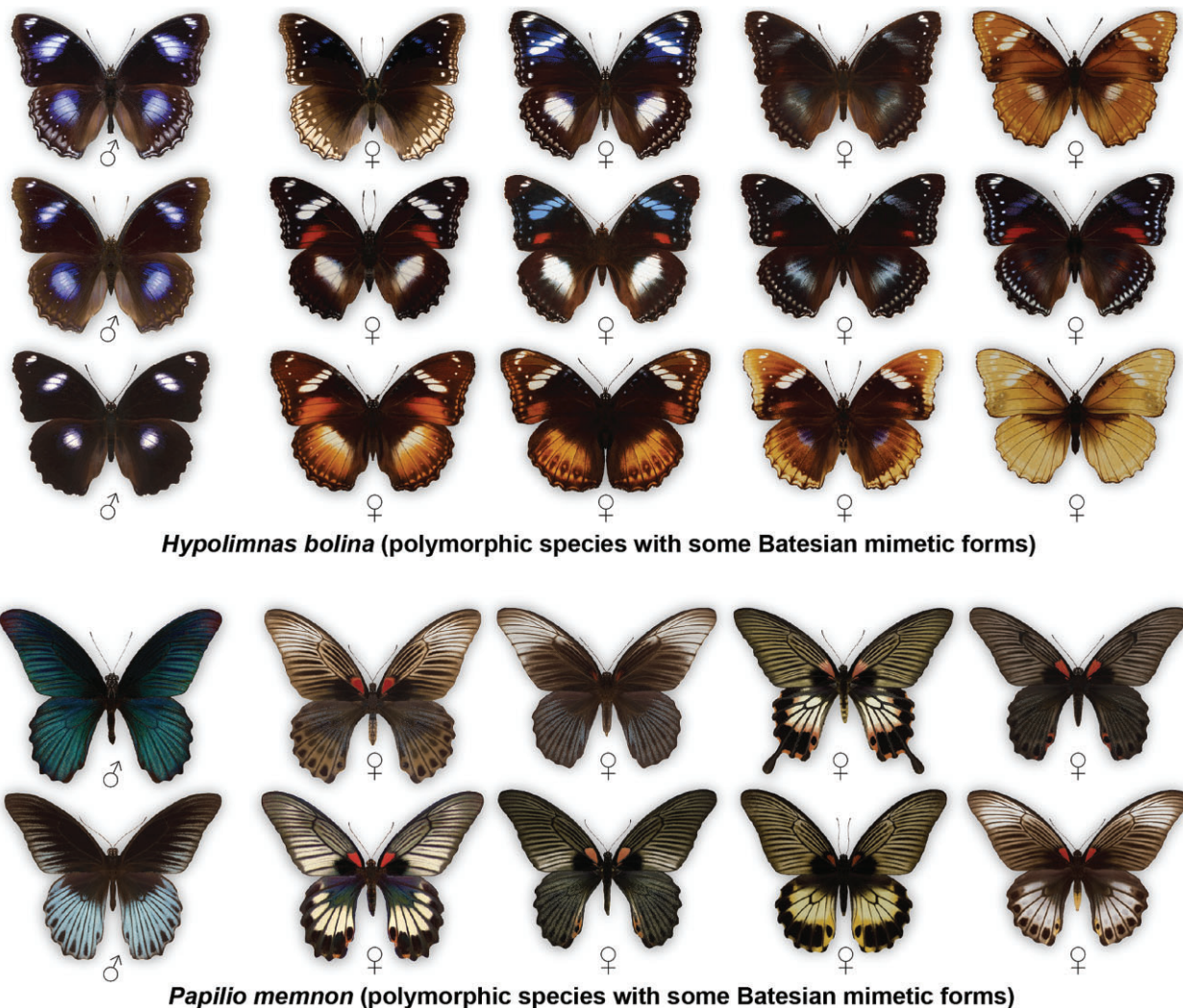

Hypolimnas bolina (polymorphic species with some Batesian mimetic forms)

Papilio memnon (polymorphic species with some Batesian mimetic forms)

FIGURE 4 | Polymorphism gone wild in mimetic butterfly species. *H. bolina* and *P. memnon* exhibit multiple female forms, many of which are neither male-like and ancestral nor mimetic, and their wing pattern elements appear to recombine frequently to produce a wide array of color pattern forms. In case of *H. bolina*, the wing pattern diversity may have been produced by relaxed predation pressures on islands, that is, under neutral processes—as many island populations are prominently variable with novel, nonmimetic wing patterns.[27] Males, on the other hand, show limited diversity of wing patterns within and across populations. In *P. memnon*, wing pattern elements and tails appear to switch occasionally between female forms, creating almost all possible permutations. The resultant morphological diversity is probably either selectively neutral or mildly deleterious. Shown here is only a selection of form diversity in these species. Genetics and development of these wing patterns are largely unknown, and likely differ from the genetic architectures so far known in other polymorphic, mimetic butterflies such as *P. polytes* and *Heliconius*.

access to various polymorphic populations and of captive breeding, the incredible mimetic polymorphism in other tropical species such as *Hypolimnas bolina* is likely to remain an evolutionary genetic enigma for some time.

New World Developmental Heterochrony

It may be readily appreciated from the examples discussed above that most of the known genes for mimicry and wing patterning in butterflies are autosomal. The North American *Papilio glaucus* offers a

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BOX 3

#### CO-OPTION, AND THE GENETICS AND EVOLUTION OF NOVEL PHENOTYPES

Functional portions of the genome are finite, and the apparent increase in morphological and functional complexity is not accompanied by a corresponding increase in gene number. One way in which this is achieved is by co-option, where spatio-temporal modulation of gene expression may fundamentally change the phenotypic effects of the individual genes and/or gene networks. Thus, co-option of genes in different developmental contexts may give rise to novel functions at the molecular level and account for complexity at the organismal level.

Several distinct mechanisms, which may sometimes work synergistically, facilitate co-option and novel functions, most of which have been demonstrated in butterflies:

1. Changes in regulatory regions:
   a Changes in the *cis*-regulatory region of genes leading to expression in either novel tissues or developmental context, and/or novel phenotypes, for example, *optix* in *Heliconius*.[26,57]
   b Changes in *trans*-regulatory genetic elements leading to acquisition of novel targets and/or atypical function, for example, DDC (dopa decarboxylase) and BAS of the melanin pathway in *P. glaucus*,[17] and possibly *doublesex* in *P. polytes*.[15,16]

2. Changes in functional regions:

   a Acquisition of novel domains via exon shuffling and translocation. This is well-known from the anti-freeze abilities of Antarctic fish,[139] but currently there are no examples in butterflies.
   b Gene duplication leading to relaxed selection and accumulation of mutations, with eventual sub-functionalization. For example, sub-functionalization of a new UVRh opsin gene paralog towards lower light wavelength, and its female-specific expression, has led to a novel sexual signal and mimetic color pattern element in *Heliconius erato*.[140]
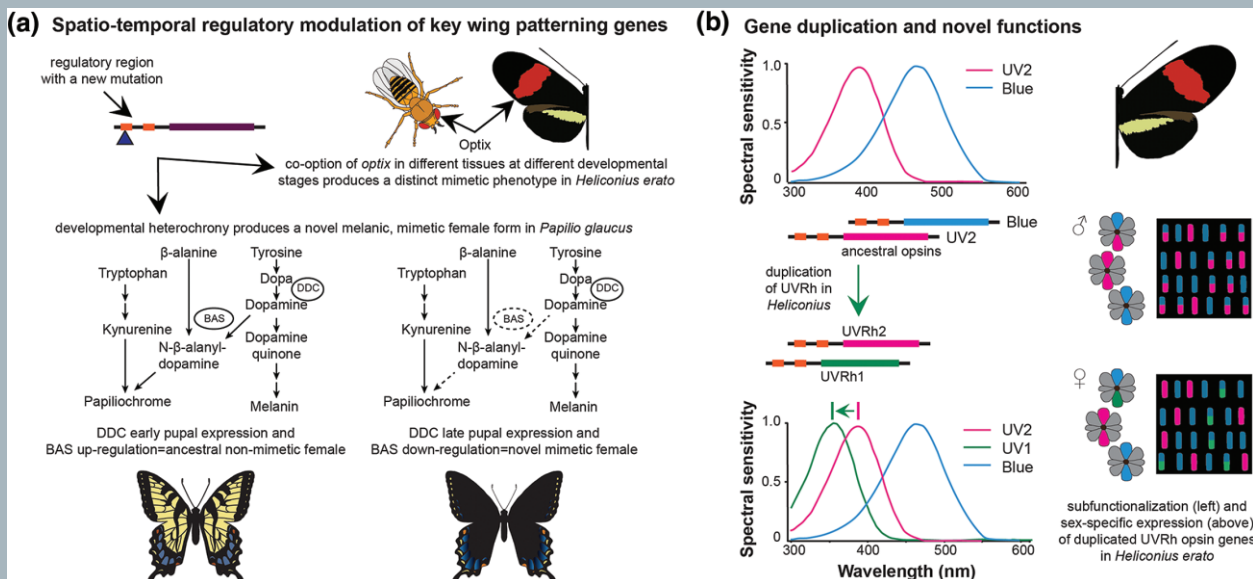
Mechanisms and some prominent examples of co-option in mimetic butterflies.

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contrast. It has two female forms—a male-like, tiger-striped, nonmimetic form, and a melanic form that mimics the aposematic *Battus philenor* (Figure in Box 1). These two female forms are matrilineally inherited through a large-effect W-linked locus such that the alternative copies of the W chromosome and the two female forms are largely co-inherited.[130,131] There is some uncertainty regarding mitochondrial leakage and existence of a Z-linked modifier—and therefore patterns of inheritance of the two female forms—in this species,[131] and there is so far no molecular genetic characterization of the mimicry gene(s). Nonetheless, developmental genetic basis of wing pattern dimorphism in this species has illuminated a fascinating but largely under-appreciated mechanism of developmental heterochrony.[132] The alternative tiger-striped and melanic forms are regulated by two partially (one-way) linked gene pathways that either produce melanin or papiliochrome—the pigment that is responsible for creamy white and yellow coloration in *Papilio* swallowtails (Figure (a) in Box 3).[17–19] The normal male and male-like female form of *P. glaucus* are produced by upregulation of a key enzyme, N-β-alanyl-dopamine-synthase (BAS), which subsequently lays down yellow background color of these nonmimetic butterflies a few days early in pupal development. Black stripes appear on these largely yellow wings in areas where melanin is deposited a few days later.[17–19] The evolution of the mimetic melanic female form was accompanied by the downregulation of BAS, which now delays deposition of the yellow scales, resulting in more widespread deposition of melanin pigment on the wings of the mimetic females. This elegant developmental heterochrony with respect to deposition of early-yellow versus late-melanic scales has brought a fundamental shift in the appearance of the mimetic female form.[17–19]

Mimetic wing patterns constitute a complex phenotype that involves alterations in the ancestral phenotype with respect to wing characteristics and behaviors. Recruitment of master regulators such as transcription factors may lead to co-option of the entire gene network or downstream signaling cascade in the context of wing patterning. The complex mimetic phenotypes may therefore be a manifestation of the combined effects of each branch of the cascading network that is set in motion by the master regulators.

CO-OPTION AND MIMICRY

The examples enumerated above show that evolutionary change often involves the use of old genetic tricks in new developmental contexts. Co-opting a preexisting enzyme, regulator, and/or genetic pathway in a different spatio-temporal context may give rise to novel, distinctly unrelated and sometimes unexpected phenotypes[133] (Figure in Box 3, Figure 5 and Table S1). Co-option may occur at different levels of a gene regulatory network: (1) at the apex of the network[134]; (2) at the terminus of the network[17,135]; (3) at an intermediate level of hierarchy[136]; and (4) integration of components from one network with those of another (Refs 47 and 133, and *P. glaucus* in Figure (a) in Box 3). Butterfly wing pattern adaptations usually involve mutations in regulatory and functional portions of critical genes that lead to distinct developmental and genetic mechanisms (Box 3), although their relative placements in gene regulatory networks and their downstream targets are still unknown.

Co-opted master regulators and gene regulatory networks have strongly influenced the evolution and developmental genetics of mimicry in butterflies, apart from other wing patterns such as eye-spots. Spatio-temporal switches in gene action leading to novel wing patterns in butterflies include co-option of a disparate set of genes involved in somatic sex determination during embryonic stages (*doublesex* in *P. polytes*[15,16]), body axis and boundary formation as well as limb development (*distal-less*,[137] *spalt*,[138] *engrailed/invected*[138] in nymphalid butterflies, and *P. dardanus*[124]), and eye morphogenesis and pigmentation (*optix* in *Heliconius*[26,57]). The inventory of known co-options involved in butterfly wing patterning is substantially longer (Figure 5, and Figure S1 and Table S1), suggesting that co-option may be the main driver of phenotypic novelty in butterfly wing color patterns. These co-options include a diversity of pathways and developmental stages as well as functions ranging from predator avoidance and mate attraction to thermoregulation (Figure 5(b) and (c)).

DORSOVENTRAL MISMATCH IN MIMETIC WING COLOR PATTERNS

A greatly under-appreciated feature of butterfly wing patterns is that they are in most cases dorsoventrally mismatched, that is, dorsal and ventral wing surfaces have different colors and color intensities (Figure 6(a)). This mismatch is a result of conflicting selection pressures that shape the evolution of butterfly wing color patterns: wings must be attractive to the potential mates and simultaneously inconspicuous or otherwise protectively patterned to evade predators. The
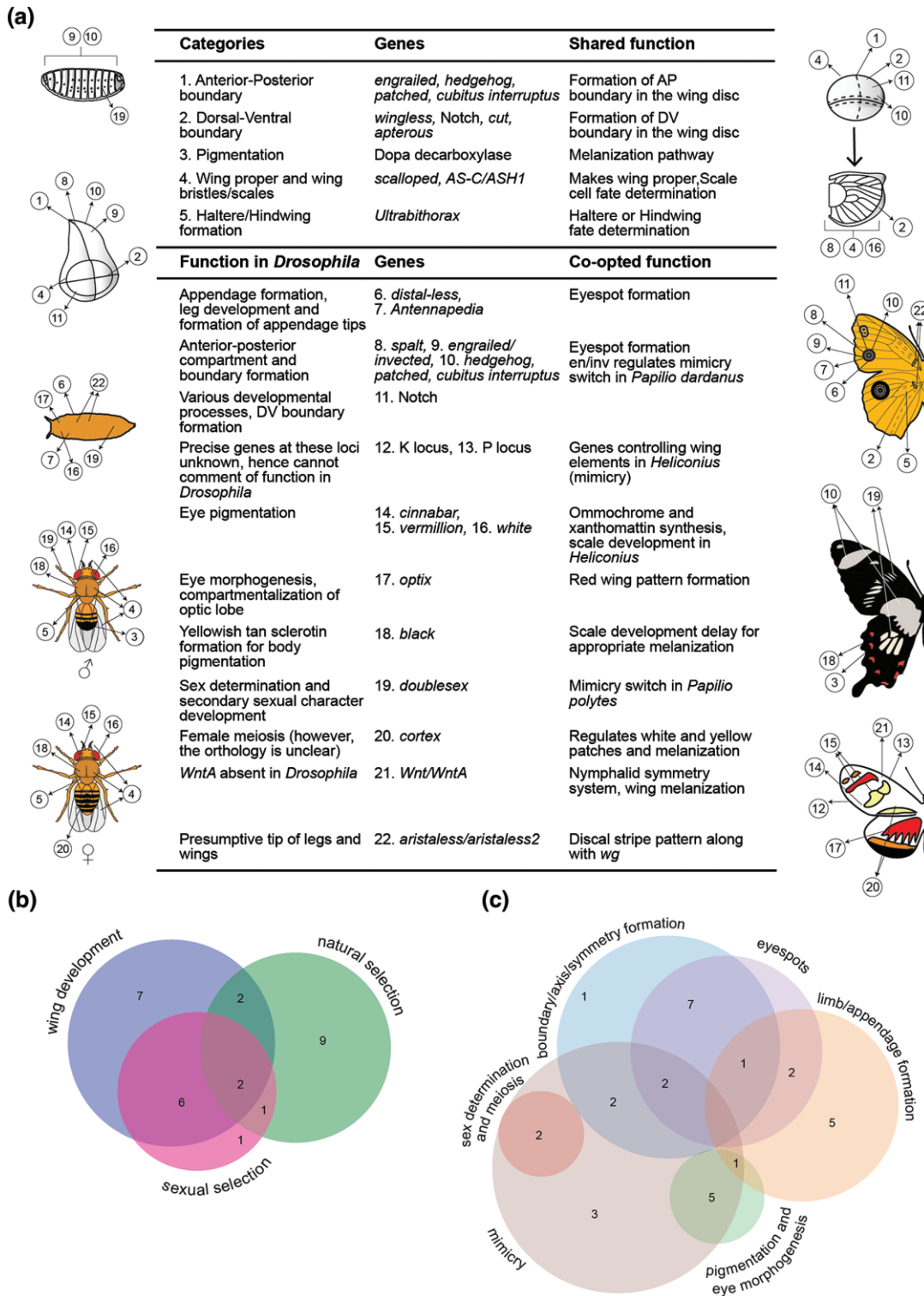
(a)

Categories	Genes	Shared function
1. Anterior-Posterior boundary	*engrailed, hedgehog, patched, cubitus interruptus*	Formation of AP boundary in the wing disc
2. Dorsal-Ventral boundary	*wingless*, Notch, *cut, apterous*	Formation of DV boundary in the wing disc
3. Pigmentation	Dopa decarboxylase	Melanization pathway
4. Wing proper and wing bristles/scales	*scalloped, AS-C/ASH1*	Makes wing proper, Scale cell fate determination
5. Haltere/Hindwing formation	*Ultrabithorax*	Haltere or Hindwing fate determination

Function in *Drosophila*	Genes	Co-opted function
Appendage formation, leg development and formation of appendage tips	6. *distal-less*, 7. *Antennapedia*	Eyespot formation
Anterior-posterior compartment and boundary formation	8. *spalt*, 9. *engrailed/ invected*, 10. *hedgehog, patched, cubitus interruptus*	Eyespot formation en/inv regulates mimicry switch in *Papilio dardanus*
Various developmental processes, DV boundary formation	11. Notch	
Precise genes at these loci unknown, hence cannot comment of function in *Drosophila*	12. K locus, 13. P locus	Genes controlling wing elements in *Heliconius* (mimicry)
Eye pigmentation	14. *cinnabar*, 15. *vermillion*, 16. *white*	Ommochrome and xanthomattin synthesis, scale development in *Heliconius*
Eye morphogenesis, compartmentalization of optic lobe	17. *optix*	Red wing pattern formation
Yellowish tan sclerotin formation for body pigmentation	18. *black*	Scale development delay for appropriate melanization
Sex determination and secondary sexual character development	19. *doublesex*	Mimicry switch in *Papilio polytes*
Female meiosis (however, the orthology is unclear)	20. *cortex*	Regulates white and yellow patches and melanization
WntA absent in *Drosophila*	21. *Wnt/WntA*	Nymphalid symmetry system, wing melanization
Presumptive tip of legs and wings	22. *aristaless/aristaless2*	Discal stripe pattern along with *wg*

(b) **(c)**

FIGURE 5 | Mimetic wing patterns in butterflies are controlled by co-option of major developmental genes. Co-opted genes and their distinct functions in embryonic development, metamorphosis, wing development, and color patterning in *Drosophila* and butterflies are listed. (a) Gene sets 1–5 have shared functions between *Drosophila* and butterflies, whereas 6–22 have known and possible co-options in butterflies. (b) Wing patterning genes in butterflies may serve multiple functions in response to selection for mimicry (natural selection) and mate choice (sexual selection). The assigned functions are based on published literature, although it is possible that additional functions for these genes will be discovered in the future. (c) Functional space of genes involved in wing development and color patterning in *Drosophila* and butterflies also illustrates co-option of genes at distinct developmental stages. Refs 68–119 are cited in Table S1.

dorsoventrally mismatched color patterns likely evolve in response to these selection pressures, presumably compartmentalizing signals encoded in wing coloration in sex-, surface-, and wing-specific manner. This signal compartmentalization evolves such that attractive coloration is restricted to the wing surfaces (usually on the dorsal surface) that are visible when butterflies are flying and better able to escape predators. On the other hand, inconspicuous coloration is usually restricted to the ventral surface, which is usually exposed when butterflies are resting.[141] This is adapted in a different way in mimetic butterflies: mimics may be well-matched to their models on the dorsal surfaces to warn (in case of Müllerian mimics) or fool (in case of Batesian mimics) their potential predators, but they are duller on the ventral surfaces[142] to escape notice when that is not beneficial. Moreover, females are better mimics compared to males,[142] because they benefit more from mimicry.[6,143]

What is the developmental genetic basis of this dorsoventral mismatch? The available (albeit disconnected) developmental genetic literature on *Drosophila* wing development provides a plausible model for holometabolous insects, which we develop and present here for butterfly wing patterns (Figure 6(b)). The adult dorsoventral mismatch may be traced to the D/V axis formation, which sets the dorsal and ventral wing surfaces on different tracks of color patterning. Several genes in the pathway are differentially expressed in the two wing compartments and one or more of these could regulate differential dorsoventral patterning and pigmentation in butterfly wings. Apterous may be the prime candidate that brings about this mismatch.[137] Being the first gene product to be exclusively expressed in just one compartment (dorsal), it sets in motion several downstream regulators that define and sort cells into dorsal and ventral compartments. PS1 and PS2 are position-specific integrins that are, respectively, expressed in the dorsal and ventral compartments of the wings, and are required for adhesion of the two compartments on evagination and folding of the wing epithelium.[137,144–147] PS integrins play a regulatory role in early wing morphogenesis[148] and integrins in general are known to affect morphogen gradients, cytoskeleton organization, cell polarity, migration, differentiation, and proliferation.[149,150]
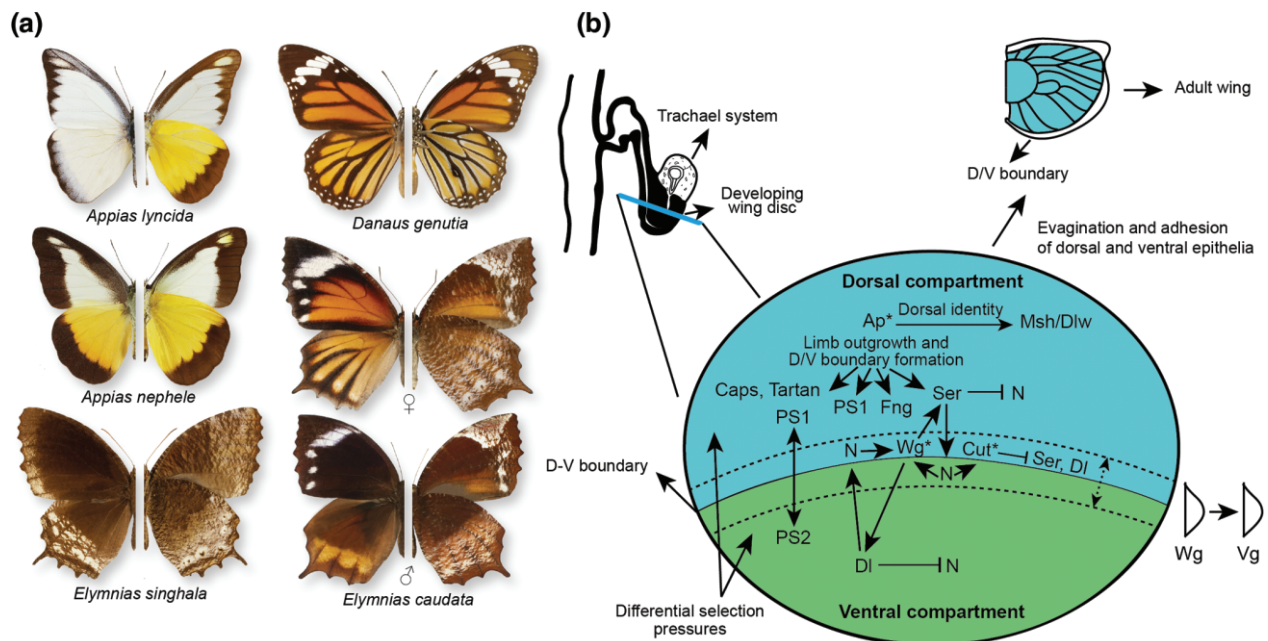

FIGURE 6 | Dorsoventral mismatch of wing color patterns is widespread in butterflies, irrespective of mimicry. (a) Dorsoventral mismatch is illustrated by distinct dorsal (on the left) and ventral (on the right) wing coloration in *A. lyncida*, whereas the two wing surfaces have matching patterns in *A. nephele*. Such dorsoventral mismatch has a special significance in mimetic butterflies where selection for sex-limited mimicry and efficacy of the conflicting signal components of predator avoidance and sexual attraction, have led to sex- and surface-specific wing patterns. For example, while the nonmimetic *Elymnias singhala* has somewhat similar wing color patterns on both wing surfaces, in *E. caudata* the ventral wing surface has remained inconspicuous to aid in crypsis, whereas the dorsal surface has diverged into novel male and female patterns, the female producing a superb mimetic resemblance to its toxic model *D. genutia*. (b) Determination of the dorsoventral boundary in insect wings. The gene network is known in *Drosophila* wing imaginal discs, based on which we present a developmental genetic hypothesis for butterflies. Asterisks denote genes that are expressed at the corresponding sites in both *Drosophila* and butterfly wing discs (see Table S1).

Thus, expression of PS1 and PS2 might have been modified under selection to give rise to different dorsoventral wing surfaces on which color patterns are laid down in a mismatching manner. Once the D/V boundary is formed, the master regulators from this stage subsequently control expression of pigment-producing genes in a wing surface-specific manner.

If this hypothesis is valid, disruptions in the developmental action of Apterous and PS1–PS2 should in turn disrupt the dorsoventral match (or mismatch) of butterfly wing patterns. These manipulations should set in motion a downstream cascade of transcription factors that will ultimately control specific wing pattern colors and elements in specific butterfly species. However, all of them must show similar or parallel effects on dorsoventral matching of wing patterns irrespective of the identity of wing coloration and their intermediate regulators. We hope that this hypothesis will be tested in mimetic and other butterflies soon.

CONCLUSIONS

The developmental genetics of butterfly wing patterns, especially that of mimetic species, is a fast-moving field that has seen prominent progress in the past two decades. The Neotropical *Heliconius* longwing butterflies and the Old World *Papilio* swallowtails are two emerging model systems, both of which are particularly promising because of their considerable species diversity and remarkable phenotypic diversification in wing color patterns within and across species as well as with respect to polymorphism and sexual dimorphism. This phylogenetically well-characterized variation complements excellent knowledge of the biological relevance of wing color patterns in the lives of butterflies, making these clades particularly attractive for the developmental genetics of adaptation. Building on these strengths, butterfly biologists have recently generated valuable genetic and developmental resources on these butterflies: genomes, transcriptomes, linkage maps, transgenesis and RNAi protocols, and more recently the use of CRISPR/Cas9 systems. Studies taking advantage of these resources, tools, and methods have shown that the molecular genetic and developmental bases of butterfly wing color patterns are equally diverse and complex. In *Heliconius* butterflies, there are no specific 'mimicry genes'; instead, a collection of genes that control specific wing colors act presumably independently, forming the whole wing color phenotypes of different forms and species based on the available allelic variation, which itself has been influenced by an evolutionary history of widespread introgression. Moreover, co-dominance of colors and patterns in different parts of the wing give rise to a broader variety of color patterns in nature. However, all the color patterning genes discovered so far are master gene regulators such as transcription factors *optix*, *cortex*, and *WntA*, which shows that these adaptive color patterns are regulated largely by *trans*-regulatory elements.

On the other hand, in *Papilio*, mimicry genes are often master regulators such as transcription factors *doublesex* and the *engrailed/invected* complex that switch entire wing color patterns in polymorphic species. These mimicry genes tightly control all the different wing color patterns, presence of tails and flight behaviors as coadapted suites of otherwise independent traits, producing alternative mimetic phenotypes in the manner of supergene alleles under a dominance hierarchy. The only *Heliconius* that appears to control wing pattern polymorphism in this manner is *H. numata*, in which dominance-based genetic architecture of wing pattern polymorphism has evolved independently.

Although the identity of specific wing patterning and mimicry genes varies in different butterfly species, a common thread binding all the known examples is that butterfly wing patterns are usually not controlled by *cis*-regulatory mutations within the pigment-producing genes. Instead, the master regulator transcription factors, which may be considered genes with pleiotropic effects, regulate butterfly wing color patterns. Although the genotype–phenotype links of these transcription factors with the wing patterns that they control are well-established in many species, the molecular links between these genes and their final phenotypic products are still elusive. For example, it is unknown how the transcription factors connect to and regulate expression of the pigment-producing genes, whether directly or through intermediate steps. Genetic pathways and gene networks that produce the specific pigments that give butterflies their color are also largely unknown.

The candidate genes that regulate mimetic wing color patterns have been co-opted from various early developmental time points and nonwing tissues into wing patterning networks. This raises several questions: (1) Are the same set of genes repeatedly co-opted into wing patterning and mimicry? (2) Do these genes (transcription factors) have specific properties or defined developmental roles that might make their co-option easier to employ in wing patterning switches? (3) Are similar developmental genetic processes and factors involved in modulating specific selection pressures on evolutionary adaptations? The

study of nonmodel or emerging model systems such as butterflies will be important in addressing these questions. In fact, without studies on nonmodel organisms, most of the knowledge gained recently about the co-option and other developmental and evolutionary actions of many critical genes would not have been possible. Comparisons with diverse nonmodel systems offer the advantage of exploring new dimensions at the interface of evolutionary biology, developmental genetics and genomics, and discovering possible functional spaces and niches that genes could occupy.

ACKNOWLEDGMENTS

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