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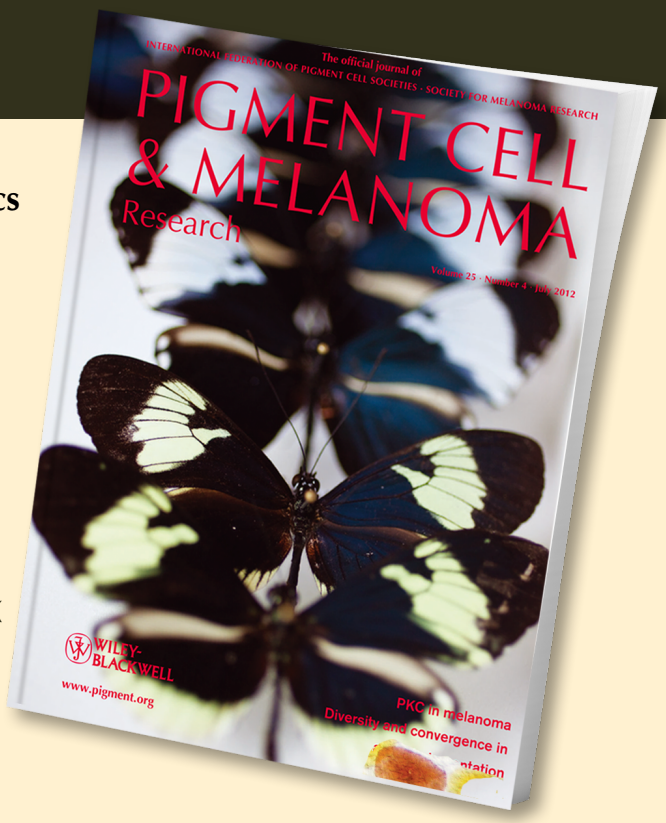

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DOI: 10.1111/j.1755-148X.2012.01014.x
Volume 25, Issue 4, Pages 411-433

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Unraveling the thread of nature's tapestry: the genetics of diversity and convergence in animal pigmentation

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KEYWORDS evolution/adaptation/convergence/*Drosophila*/butterfly/reptile

PUBLICATION DATA Received 7 March 2012, revised and accepted for publication 5 May 2012, pulished online 12 May 2012

doi: 10.1111/j.1755-148X.2012.01014.x

Summary

Animals display incredibly diverse color patterns yet little is known about the underlying genetic basis of these phenotypes. However, emerging results are reshaping our view of how the process of phenotypic evolution occurs. Here, we outline recent research from three particularly active areas of investigation: melanin pigmentation in *Drosophila,* wing patterning in butterflies, and pigment variation in lizards. For each system, we highlight (i) the function and evolution of color variation, (ii) various approaches that have been used to explore the genetic basis of pigment variation, and (iii) conclusions regarding the genetic basis of convergent evolution which have emerged from comparative analyses. Results from these studies indicate that natural variation in pigmentation is a particularly powerful tool to examine the molecular basis of evolution, especially with regard to convergent or parallel evolution. Comparison of these systems also reveals that the molecular basis of convergent evolution is heterogeneous, sometimes involving conserved mechanisms and sometimes not. In the near future, additional work in other emerging systems will substantially expand the scope of available comparisons.

Introduction

Living organisms display fantastic variation in coloration, a testament to evolution's seemingly endless innovation in shaping life. Among animals, coloration plays an essential role in the most basic pursuits of life – survival and reproduction. For instance, cryptic coloration allows some animals to escape predators by blending into the background. For others, survival is aided by bright and flashy coloration, which can serve to warn potential

Significance

The study of pigmentation has played a critical role in the fields of genetics, development and evolution. Today, work on a variety of non-model systems is complementing the rich history of research focused on mammalian pigmentation genetics. We review the latest research focused on color patterning in Drosophila, butterflies, and lizards, paying particular attention to the mechanisms responsible for convergent evolution. We also highlight additional emerging systems that promise to shed light on the genetics of diversity and convergence in animal pigmentation.

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predators of the distasteful compounds that await them should they attempt a bite. Some of the most striking examples of vibrant coloration in animals stem from the interplay between males and females in the pursuit of mates. In their quest to identify and reproduce with only the most attractive or healthiest partners, females, and sometimes males, of many species have driven the evolution of a myriad of color forms, from the vibrant blues and greens of the exaggerated peacock tail feathers to the tiniest white spots on the wings of butterflies. Furthermore, because local environments vary substantially across time and space, natural and sexual selection often push populations toward different ends of the color spectrum, resulting in dazzling color variation among even the closest of relatives.

Decades of research focused on natural history, ecology, and behavior have revealed the function of color patterns in a wide range of animals (Cott, 1940; Edmunds, 1974; Endler, 1978; Jones et al., 1977; Kaufman, 1974; Kettelwell, 1956). One aspect of animal coloration that remains largely unresolved, however, is the actual genetic material responsible for evolutionary change – the 'thread' in nature's tapestry. What are the genes whose RNA and protein products interact to produce animal color patterns? What is the functional DNA sequence variation that causes color pattern variation among individuals, populations, and species? How does the process of development translate this DNA sequence variation encoded in the genome into different color pattern phenotypes? Do independent evolutionary origins of similar color patterns occur via similar molecular mechanisms? With advances in genomics and molecular biology, researchers are now able to delve into the mechanisms underlying adaptive pigmentation in an unprecedented way.

Pulling the thread to reveal the genetic basis of pigmentation

Modern research on the genetic basis of animal pigmentation has been carried out in parallel in both vertebrates and invertebrates. Study of adaptive color variation in vertebrates has focused largely on mammals (Hoekstra, 2006), and recent progress has been made in the study of mice (Bennett and Lamoreux, 2003; Hoekstra et al., 2006; Manceau et al., 2011), wolves (Anderson et al., 2009), and sheep (Gratten et al., 2007, 2008, 2010). Other work is focused on vertebrates as diverse as birds, fish, and lizards (Hubbard et al., 2010). In invertebrates, special attention has been given to *Drosophila* fruit flies (Kopp et al., 2000; Wittkopp et al., 2003b, 2009), which are useful as a genetic model system, and butterflies, which display extreme wing pattern diversity (Joron et al., 2006a; McMillan et al., 2002; Monteiro and Prudic, 2010). In addition to employing a diverse group of study species, researchers use many different methods to unlock the genetic mechanisms of pigmentation, including approaches such as genetic mapping and positional cloning, association studies, candidate-gene analysis, study and manipulation of gene expression, transgenics, and experiments carried out in the wild (Hoekstra, 2006; Hubbard et al., 2010; Joron et al., 2006a; Manceau et al., 2010; McMillan et al., 2002; Monteiro and Prudic, 2010; Papa et al., 2008; Protas and Patel, 2008; True, 2003; Wittkopp et al., 2003a).

Much of the work in this field is ongoing; yet, the results that have emerged to date have already reshaped our view of how evolutionary adaptations are built. For instance, since Charles Darwin's *On the Origin of Species by Means of Natural Selection*, biologists have viewed adaptation as a gradual process, involving the combined action of many genes, each with a small impact on the overall phenotype. Work on animal pigmentation has revealed that this is not always the case. Color pattern variation across the animal kingdom, from the bright warning colors of tropical butterflies to the cryptic colors of beach mice, is now known to be controlled by a small number of genes, each with a large influence on color pattern phenotype (Papa et al., 2008; Steiner et al., 2007). While it remains to be seen whether these major-effect alleles are generally the result of one or more mutations, this result does suggest that evolution can occasionally take large leaps across phenotypic space. In addition, biologists are currently debating the question of whether regulatory or structural changes in genes are most important for morphological evolution (Hoekstra and Coyne, 2007; Stern and Orgogozo, 2008). In just the last few years, study of animal pigmentation has yielded unique insights into this debate by showing that both types of changes play significant roles, sometimes even in producing the same phenotype (Steiner et al., 2007). Analysis of pigment variation is also shaping our view of constraints in evolution (Gompel and Prud'homme, 2009). It is often assumed that evolutionary history and established connections in developmental cascades might constrain evolutionary potential, leading to a highly conserved genetic basis for similar phenotypes among related species. Work on animal coloration is beginning to reveal the real-world complexity of this scenario, with certain genes being used widely across distantly related organisms even while genetic heterogeneity exists in closely related organisms for the very same phenotype (Kingsley et al., 2009; Kopp, 2009; Manceau et al., 2010; Steiner et al., 2009).

Here, we review recent research focused on the evolution and genetics of pigmentation in invertebrates and vertebrates, focusing primarily on color patterning in *Drosophila*, butterflies, and lizards. This work complements the deep knowledge gained about pigmentation from studies of mammals and zebrafish, both of which have been reviewed elsewhere (Barsh, 1996; Bennett and Lamoreux, 2003; Hoekstra, 2006; Hofreiter and Schoeneberg, 2010; Hubbard et al., 2010; Kelsh, 2004; Kondo et al., 2009; Manceau et al., 2010; Parichy, 2003,

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2006, 2007; Protas and Patel, 2008). Because each of these systems involves multiple evolutionary origins of similar pigmentation phenotypes, they are all especially well-suited to address the intriguing question of how convergent phenotypic evolution occurs at a molecular level. Therefore, we pay special attention to the results focused on comparative analyses among independent origins of similar phenotypes. Additionally, we discuss other emerging biological systems that offer particular promise for examining the evolutionary genetics of convergent pigmentation and patterning.

Melanin pigmentation in *Drosophila*

In *Drosophila*, the only known cuticular pigments are catecholamine polymers that include dark melanins (black or dark brown) and light sclerotins (yellow, tan, or colorless). *Drosophila* has no specialized pigment cells or structures. Monomeric pigment precursors are secreted by epithelial cells and polymerized in the overlying cuticle, so that pigmentation is determined in a nearly cell-autonomous manner. Most *Drosophila* species have spatially patterned pigmentation, with alternating dark and light areas that reflect the different balance of pigments produced by the epithelial cells in those areas (Wittkopp et al., 2003a). Some species, however, are uniformly light or dark with little spatial variation in the color or intensity of pigment.

Compared to other animals such as vertebrates or butterflies, little is known about the ecological functions of *Drosophila* pigmentation, and the most obvious a priori hypotheses have little empirical support. In some species and populations, the intensity of pigmentation varies in latitudinal or altitudinal clines where darker flies are found in cooler areas, suggesting a role in thermoregulation (Gibert et al., 1999; Munjal et al., 1997). In other clades, however, darker species are found closer to the equator (Brisson et al., 2006; Hollocher et al., 2000). Similarly, although uniformly pale coloration of some flower-feeding species might be cryptic on their food sources, other species that feed on lightly colored flowers have dark pigmentation (Bock, 1976; Sultana et al., 1999).

An important clue to the function of *Drosophila* pigmentation may lie in the fact that catecholamine pigments are not simply decorations, but structural components of the insect cuticle that are crucial to its skeletal and barrier functions (Moussian, 2010). It is possible that some of the evolutionary changes in pigmentation reflect not selective pressures on pigmentation per se, but rather a pleiotropic response to selection on physiological traits. For example, the *ebony* mutants of *D. melanogaster*, which are darker than wild type, have weaker wing cuticle and lower UV tolerance, but increased resistance to desiccation (Jacobs, 1985; Kalmus, 1941). Desiccation tolerance provides a likely explanation for several instances of color variation within and between species. In the *Drosophila cardini* species group, darker species or intraspecific morphs are usually found in the more open areas while lighter flies occur in shaded habitats. Experimental analysis in *D. polymorpha* has shown that the darker morph is more resistant to desiccation when genetic background is accounted for (Brisson et al., 2005). A positive correlation between darker pigmentation and higher desiccation resistance is also seen in the Indian populations of *D. melanogaster* and *D. jambulina*, although here the causal relationship is not clear because genetic backgrounds were not controlled (Parkash et al., 2009a,b). Interestingly, an opposite pattern is observed in *D. ananassae*: dark pigmentation alleles introgressed into an otherwise light genetic background decrease, rather than increase, desiccation resistance in laboratory experiments (A. Kopp, unpublished data), while in *D. americana*, no association is seen between pigmentation and desiccation resistance (Wittkopp et al., 2011). Similarly, although the dark *ebony* mutants of *D. melanogaster* have reduced cuticle strength (Jacobs, 1985), selection for lighter pigmentation in *D. falleni* increases its susceptibility to nematode infection, suggesting that darker flies have stronger cuticles (Dombeck and Jaenike, 2004). These conflicting observations suggest that the links between pigmentation and cuticle physiology are complex and may be different in different species. The relationship between pigmentation and barrier function may depend not on the color as such, but on the specific polymers that are cross-linked to the cuticle, leading to selection on different genes in the pigmentation pathway.

The color patterns of many *Drosophila* species are sexually dimorphic, suggesting that they may play a role in mate choice. Although the wings of *Drosophila* are typically transparent, several species in the *melanogaster* group have male-specific black spots on their wings. This morphological pattern correlates with mating display behavior that takes advantage of the spots, suggesting that their origin was driven by sexual selection (Kopp and True, 2002; Prud'homme et al., 2006). Unfortunately, experimental evidence for the role of wing spots in mate choice is only tentative (Singh and Chatterjee, 1987). Abdominal pigmentation also shows dramatic sexual dimorphism in many *Drosophila* species (Kopp et al., 2000; Wittkopp et al., 2003a), but there is no evidence that male-specific color patterns contribute to mating success. Overall, it seems clear that no single selective force can provide a universal explanation for the evolution of *Drosophila* pigmentation, and different functional pressures may dominate in different species.

The most common type of evolutionary change in *Drosophila* pigmentation affects the spatial arrangement of light and dark areas. These changes are most pronounced in the abdomen, which has spatially patterned pigmentation in most *Drosophila* species. A typical pattern is a band of dark pigment located at the posterior

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edge of each abdominal segment (Wittkopp et al., 2003a). In most species, these bands are also modulated along the medio-lateral axis, sometimes to the point of being broken up into separate spots. Changes along the antero-posterior and medio-lateral axes in different species create a wide variety of spatial patterns, but these patterns are all composed of the same stripe and spot elements that appear repeatedly in different evolutionary lineages. The most common type of sexual dimorphism in abdominal pigmentation involves increased melanization of posterior segments in males but not females. This pattern has evolved independently in many distantly related clades. In other instances of convergent evolution, some species have lost spatial patterning and became uniformly dark or light.

In addition to frequent changes in abdominal pigmentation, many drosophilid lineages have evolved novel color patterns on other body parts, wing pigmentation being particularly common (Wittkopp et al., 2003a). The Hawaiian clade of picture-wing *Drosophila* is the most famous (Edwards et al., 2007), but wing pigmentation has evolved convergently in many other groups including some close relatives of *D. melanogaster* (Kopp and True, 2002). Other drosophilid species have unusual pigmentation on their thorax, ranging from bristle-associated speckles in the *repleta* species group to the dramatic silvery racing stripes that evolved, apparently independently, in *Zaprionus* and *Phortica* (Okada and Carson, 1983; Yassin et al., 2010). The prevalence of convergent color patterns among drosophilid species provides an excellent opportunity to test whether the same genes and molecular pathways are responsible for the repeated evolution of similar traits in different lineages.

Molecular basis of pigment patterning and synthesis in *Drosophila*

Recent work on the evolution of color patterns in *Drosophila* has benefited from decades of classical *Drosophila* genetics. As in other genetic model systems such as mouse and chicken, pigmentation mutants were among the first to be discovered in *Drosophila*. By the time the first catalog of *D. melanogaster* genes was compiled in 1968, it included dozens of pigmentation genes (Lindsley et al., 1968). Most of these genes affect various aspects of pigment synthesis and polymerization. The enzymatic functions of many of these genes were subsequently determined by integrated genetic and biochemical approaches, and the overall structure of the pigment metabolism pathway was well understood by the 1980s. This work was summarized in a detailed review by T. R. F. Wright, which still remains a definitive reference for *Drosophila* pigmentation genetics (Wright, 1987).

A simplified schematic of the pigment synthesis pathway is shown in Figure 1A. The synthesis of all pigments begins with the conversion of tyrosine to dihydroxyphenylalanine (Dopa) by the tyrosine hydroxylase encoded by the *pale* gene. Some dopa is then converted to black melanin by extracellular enzymes encoded by the *yellow* gene family (Han et al., 2002; Wittkopp et al., 2002b). In another branch of the pathway, dopa decarboxylase (Ddc) converts dopa to dopamine, which serves as a precursor for brown melanin. Alternatively, dopamine can

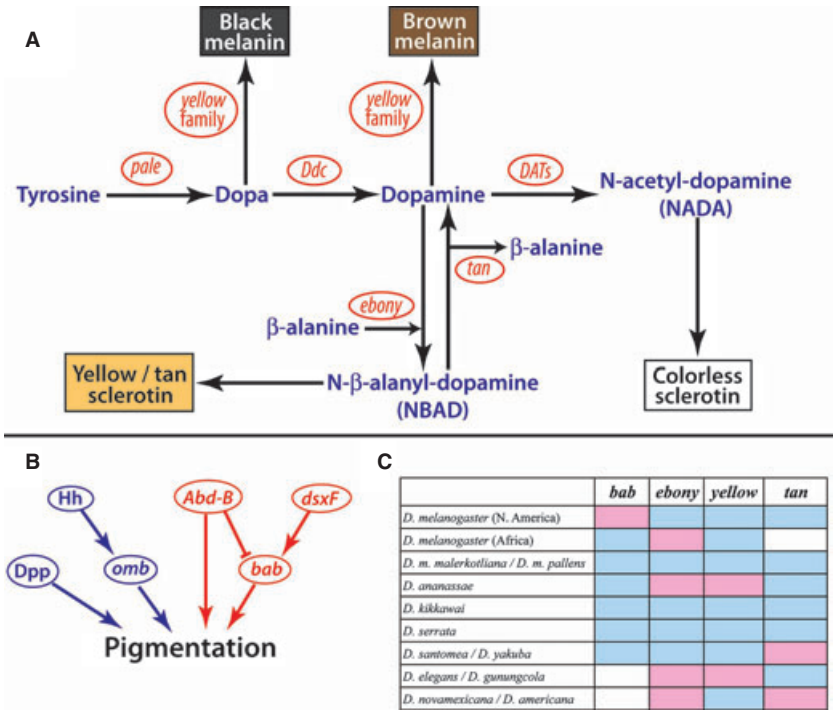

Figure 1. Genetic control and evolution of pigmentation in *Drosophila*. (A) A simplified scheme of the core biosynthetic pathway that produces cuticular pigments. Intermediate metabolites are shown in blue and the enzymes that produce them in red. Many enzymes that function in the late and peripheral branches of the pathway are not shown. (B) Spatial control of abdominal pigmentation in *D. melanogaster*. Genes that control sexually monomorphic striped pigmentation are shown in blue, and genes that control sex-specific pigmentation of posterior segments are in red. (C) Genes associated with natural variation in pigmentation in different *Drosophila* species. Pink and blue indicate that the gene contributes or does not contribute to color variation in that species, respectively; white indicates that no information is available.

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be shunted toward the production of light pigments. The product of the *ebony* gene converts dopamine to N-β-alanyldopamine (NBAD), the precursor of yellow sclerotin. NBAD synthesis is reversible, and some portion of it is converted back into dopamine by an NBAD hydrolase encoded by the *tan* gene (True et al., 2005). Finally, a family of dopamine-acetyl-transferases (DATs, also known as AANATs) converts dopamine to N-acetyl dopamine (NADA), which serves as a precursor for colorless sclerotins. The final polymerization of cuticular pigments is controlled by extracellular phenoloxidases (Kawabata et al., 1995; True et al., 2001; Wright, 1987). *Ddc, ebony, tan*, and *yellow* are transcribed and translated into the epidermis during the pupal stage (Kraminsky et al., 1980; Walter et al., 1996; Wittkopp et al., 2003a). However, the most upstream step in pigment synthesis – the conversion of tyrosine into dopa by the Ple enzyme – does not take place until eclosion, when Ple is activated post-transcriptionally by a hormonal cascade involving ecdysis-triggering hormone and eclosion hormone (Davis et al., 2007). This activation step explains why *Drosophila* cuticle becomes pigmented immediately after eclosion but no earlier.

The next advances came from developmental genetics. It has long been thought that the spatial color pattern is determined by the differential expression of enzymatic genes in different body regions, but the mechanisms controlling this expression were not understood. In the 1990s, work on *Drosophila* pattern formation has finally identified the regulatory pathways that establish the spatial coordinates in the *Drosophila* abdomen (Figure 1B). In *D. melanogaster,* most abdominal segments bear a posterior stripe of dark pigment. This pattern is regulated by the Hedgehog signaling pathway acting through the transcription factor *optomotor-blind* (*omb*) (Kopp and Duncan, 1997; Kopp et al., 1997). The variable width of the pigment bands along the medial-lateral axis is controlled by the decapentaplegic (Dpp, a member of the TGF-β family), wingless (Wg, a member of the Wnt family), and epidermal growth factor receptor signaling pathways (Kopp et al., 1999). Most members of the *melanogaster* species group have an additional sex- and segment-specific pattern: the last two abdominal segments in males have uniform black pigmentation that masks the usual pigment stripes. This pattern is repressed in females by the expression of two related transcription factors encoded by the *bric a brac* (*bab*) locus, which is regulated in turn by the HOX gene *Abdominal-B* (*Abd-B*) and the sex determination gene *doublesex* (*dsx*) (Kopp et al., 2000; Williams et al., 2008). In *Drosophila* species that have pigmented wings, at least some of the black pattern elements are controlled by the Wg signaling pathway (Werner et al., 2010), although other signals and transcription factors are almost certainly involved and are likely different in different species.

Today, our largest gap in understanding the development of *Drosophila* pigmentation is in the middle: the regulatory connections between the transcription factors that establish the spatial color patterns and the enzymes that translate this spatial information into a biochemical output are not yet clear. Although *Abd-B* is known to regulate *yellow* expression directly (Jeong et al., 2006), other direct transcriptional targets of *Abd-B, bab*, and *omb* remain unknown, and the existence of intermediate regulatory layers cannot be ruled out.

Examining the genetic basis of convergence in *Drosophila*

In light of the cell-autonomous development of *Drosophila* pigmentation, the evolution of color patterns must necessarily involve changes in the activity or spatial regulation of enzymes in the catecholamine metabolism pathway. However, the branched organization of this pathway offers multiple genetic paths to the same phenotype. Different metabolic reactions draw on a shared pool of soluble precursors to produce several distinct light and dark pigments (True, 2003; Wittkopp et al., 2003a; Wright, 1987). At least one of these reactions is reversible, with the opposing reactions catalyzed by the products of the *ebony* and *tan* loci (True et al., 2005). This nonlinear pathway structure means that changes in the output of different enzymatic reactions can produce similar phenotypes. For example, darker pigmentation can be caused either by increased expression or activity of enzymes required for the synthesis of dark pigments (e.g., *Ddc, yellow*, or *tan*), or by decreased expression or activity of enzymes involved in the synthesis of light pigments (such as *ebony, black,* or *Dat*). Increased or decreased expression of these enzymes can in turn be caused either by mutations in the regulatory regions of these loci, or by changes in the expression of their upstream regulators such as *bab, omb*, and *Abd-B*.

Correlations between pigmentation and gene expression patterns in different species and direct genetic analysis of inter- and intraspecific differences confirm these predictions. Many independent evolutionary changes in abdominal, thoracic, and wing pigmentation are associated with changes in the spatial expression of *yellow*, *ebony*, and *tan* (Jeong et al., 2008, 2006; Kopp and True, 2002; Prud'homme et al., 2006; Rebeiz et al., 2009a,b; Werner et al., 2010; Wittkopp et al., 2002a; Wittkopp et al., 2003b). However, a key conclusion emerging from recent work is that genetic changes at different loci are responsible for color pattern variation in different species (Kopp, 2009) (Figure 1C). For example, *D. santomea* differs from its sibling species *D. yakuba* and other members of the *D. melanogaster* species subgroup by the absence of dark male-specific abdominal pigmentation (Carbone et al., 2005; Llopart et al., 2002). This difference is controlled by several loci, among which *tan* plays a prominent role (Jeong et al., 2008; Rebeiz et al., 2009b). A similar difference in color patterns is found between *D. m. malerkotliana* and *D. m. pallens* in the *ananassae* subgroup. In this case, however, *tan* makes

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no contribution to the phenotypic change, nor do any other known pigmentation genes (Ng et al., 2008). Global differences in the intensity of pigmentation are controlled by *ebony* and *tan,* but not *yellow*, in *D. americana* and *D. novamexicana* (Wittkopp et al., 2003b, 2009), and by *ebony* and *yellow,* but not *tan,* in *D. elegans* and *D. gunungcola* (Yeh and True, pers. comm.). The genetic basis of color variation can also differ in different populations of the same species. Differences in abdominal pigmentation in *D. melanogaster* are associated with *bab* but not *ebony* in North America (Kopp et al., 2003), and with *ebony* but not *bab* in sub-Saharan Africa (Pool and Aquadro, 2007). The overall picture is that (i) in different species, variation in color patterns is controlled by overlapping but non-identical sets of genes, (ii) each gene contributes to pigmentation differences in some but not all species, and (iii) major evolutionary changes can be caused by loci that do not correspond to any of the known components of the *Drosophila* pigmentation pathway (Figure 1C).

Even when the same gene is implicated in parallel phenotypic evolution, the molecular nature of the underlying changes can be different. For example, *cis*-regulatory changes in *yellow* are associated with convergent evolution of male-specific wing spots in the *melanogaster* and *obscura* species groups. However, the causative changes have occurred in an upstream enhancer in the *melanogaster* group, but in an entirely different, intronic enhancer in the *obscura* group (Prud'homme et al., 2006). Similarly, *tan* has been implicated in pigmentation differences in both *D. santomea* / *D. yakuba* and *D. americana* / *D. novamexicana* species pairs, but the causative changes have occurred in a distant upstream region in the former case and in an intronic region in the latter (Jeong et al., 2008; Wittkopp et al., 2009). In fact, three independent *tan* alleles that evolved in *D. santomea* result in the same reduced pigmentation phenotype (Jeong et al., 2008).

Despite these differences in the genetic basis of evolutionary change, some broader generalities may yet emerge. First, with rare exceptions (Ohnishi and Watanabe, 1985), both inter- and intraspecific differences in *Drosophila* pigmentation are controlled by multiple loci (Carbone et al., 2005; Martinez and Cordeiro, 1970; Ng et al., 2008; Spicer, 1991; Wittkopp et al., 2003b). Second, in cases where the molecular basis of these differences has been identified, multiple mutations contribute to the overall effect of each locus. For instance, in the African *D. melanogaster*, at least five mutations in a *cis*-regulatory region of *ebony* contribute to its pigmentation phenotype (Rebeiz et al., 2009a). In the North American populations of the same species, *bab* explains over 60% of variation in abdominal pigmentation (Kopp et al., 2003), but this contribution reflects the cumulative effect of many mutations concentrated in three distinct functional regions including a tissue-specific enhancer, a basal promoter, and an element that controls chromatin structure; each individual mutation contributes no more than 1.3% of the overall genetic variation (Bickel et al., 2011). Together, these observations suggest that major-effect mutations are rare in the evolution of *Drosophila* pigmentation and that most differences within and between species are caused by the accumulation of many subtle mutations.

The third general pattern is that in all cases that have been dissected at the molecular level, the evolution of *Drosophila* pigmentation is associated with *cis*-regulatory changes. This is probably not surprising, because many intermediate metabolites in the catecholamine pathway also function as neurotransmitters (True, 2003; True et al., 2005; Wittkopp and Beldade, 2009). Changes in the activity of the enzymes that regulate their production not only affect pigmentation, but also have pleiotropic effects on nervous system function and behavior. This pleiotropic constraint may explain why no mutations in protein-coding sequences have been found to contribute to natural variation in *Drosophila* pigmentation so far.

Butterfly wing patterns

Butterfly wing patterns are extremely diverse, so much so that most of the approximately 18 000 species of butterflies can be distinguished based on wing pattern alone. Lepidopteran wing patterns consist of a two-dimensional grid of partially overlapping, colored scales that are attached to the wing cuticle. The color of a scale is determined both by the pigment deposited in it and by its morphological structure. Butterfly wing pigments primarily consist of melanins, ommochromes (including precursors and papiliochromes), pterins and flavonoids, with different clades utilizing different suites of compounds (Nijhout, 1991). Scale microstructure produces iridescence (Ghiradella, 1984, 1989). Many white colors are also structural, as are most blues and greens (Mason, 1926, 1927a,b).

Butterfly wing patterns generally have well-characterized ecological functions, making them an attractive system to examine the molecular genetic basis of adaptive color pattern variation. For many species, we understand the role the wing pattern plays in a variety of contexts including thermoregulation, crypsis, warning color, mimicry, and mate choice (Nijhout, 1991). Substantial progress has been made in elucidating the developmental and genetic basis of two distinct aspects of butterfly wing patterning, namely wing eyespots and wing pattern mimicry. Because both of these phenomena occur widely across the Lepidoptera, they are particularly good examples with which to explore the mechanisms behind convergent evolution.

Eyespots on butterfly wings

Several lineages of moths and butterflies display 'bullseye' patterns of contrasting color pigments on their

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wings that are apparently used for two separate functions – (i) startling, intimidating, or deflecting predator attacks, and (ii) attracting mates (Figure 2A) (Kodandaramaiah, 2011; Stevens et al., 2008a). For instance, peacock butterflies, *Inachis io*, when attacked by a bird will rapidly display the large hidden eyespots on the dorsal surface of their wings that startle the predator and help the butterfly escape (Vallin et al., 2005). Alternatively, at low-light conditions, when many insectivorous birds are searching for food, the smaller eyespots adorning the margins of the wings of many satyrid butterflies are sufficient to deflect bird attacks toward the wing margin and away from the body (Olofsson et al., 2010). Eyespot size and number can trade off with each other in equally effective ways for the purpose of predator deterrence (Stevens et al., 2008b). Experiments using paper models of moths with drawings of eyespots suggested that a single large eyespot is equally effective at deterring predation as multiple smaller eyespots with a similar total area (Stevens et al., 2008b). The eyespots on the hidden dorsal surface of the wings of some satyrid butterflies also have a role in sexual signaling. The wing scales at the center of these eyespots usually do not carry any pigment but, instead, possess fine nano-scale cuticular morphologies that reflect white and UV light. In *Bicyclus anynana*, both males and females respond to the presence of these reflective eyespot centers and are less likely to mate with the other sex if the centers are removed (Prudic et al., 2011; Robertson and Monteiro, 2005). This behavior suggests that dorsal eyespots play a role in mate selection and/or mate recognition.

Eyespots vary widely in number across the Lepidoptera, and within a species, eyespots often vary between dorsal and ventral surfaces. A study looking at the rates of evolution (presence and absence) of eyespots as well as simpler bands of pigmentation, showed that these wing pattern elements 'flick on and off' at different rates depending on whether they are present on the ventral exposed surfaces or on the dorsal hidden surfaces of the wings (Oliver et al., 2009). For instance, dorsal eyespots evolve rapidly across closely related *Bicyclus* species, and at different rates in males and females, whereas the exposed ventral eyespots evolve slowly and at equal rates across sexes (Oliver et al., 2009). These patterns of variation suggest that stabilizing natural selection and diversifying sexual selection are acting on the exposed and hidden wing surfaces, respectively (Oliver et al., 2009). When comparisons are made across more distantly related species, however, it is clear that ventral eyespots can also vary substantially in number. Overall, the experiments described above suggest that the combined actions of sexual and natural selection, as well as the potential existence of multiple adaptive peaks, are responsible for the evolution of eyespot size and number in the Lepidoptera.

Genetic basis of eyespot variation

A series of candidate-gene studies have identified several transcription factors, ligands, and receptors whose expression is associated with early eyespot differentiation in nymphalid butterflies. Some of these genes may determine which cells will produce which pigments but they are not 'pigmentation genes' per se. Several transcription factors and receptors are expressed in or flanking the eyespot centers during the larval stages of wing development (Figure 2B) (Carroll et al., 1994; Keys

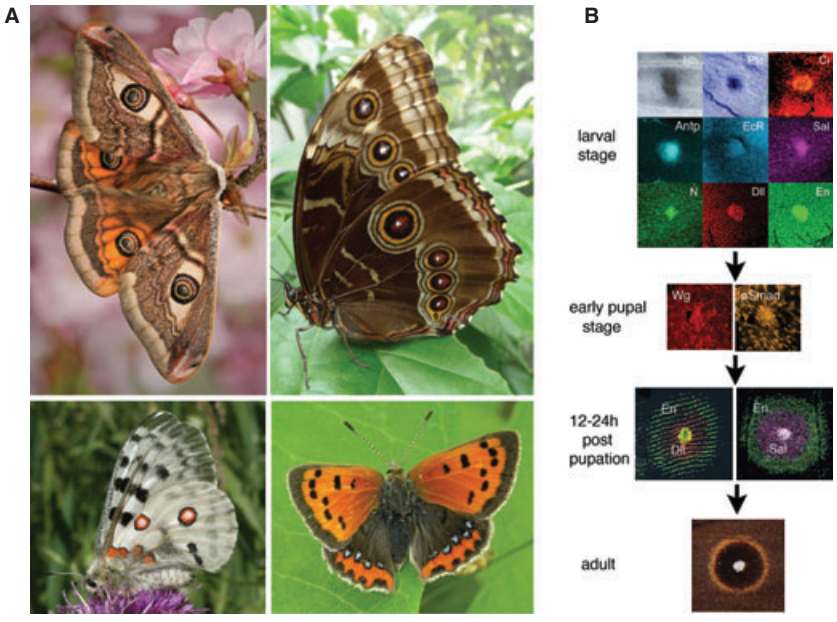

Figure 2. Butterfly and moth eyespots. (A) Clockwise: *Saturnia pavonia* (photo by Igor Siwanowicz), *Morpho peleides* (photo by A. Monteiro), *Lycaena phlaeas* (photo by Dale Rhoda), and *Parnassius appolo* (photo by Matt Rowlings) displaying variation in eyespot number and position on the wing – saturniid moths have single eyespots centered on their discal cross-vein, whereas butterflies center their eyespots between adjacent longitudinal veins. (B) Eyespot-associated genes in nymphalid butterflies at different stages of wing development, from the late fifth-instar larvae to the early pupal stage. Genetic data from *Bicyclus anynana* include the proteins Ci (Cubitus interruptus), Ubx (Ultrabithorax), EcR (Ecdysone Receptor B1), Sal (Spalt), N (Notch), Dll (Distal-less), En (Engrailed), Wg (Wingless), pSmad (phosphorylated Smad); and from *Junonia coenia* Hh (*hedgehog* mRNA), and Ptc (*patched* mRNA).

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et al., 1999; Monteiro et al., 2006; Reed and Serfas, 2004; Saenko et al., 2011). Then members of two candidate signaling pathways, Wingless and TGF-B, are expressed in the central cells shortly after pupation (Monteiro et al., 2006), and three other transcription factors, Distal-less, Spalt, and Engrailed, are later associated with the distinct rings of colored scales that make up an eyespot (Figure 2B) (Brunetti et al., 2001). *Distal-less* is the only gene, so far, that has been associated with eyespot size variation (Beldade et al., 2002).

Different nymphalid lineages display variation in the number of genes expressed in the eyespot centers during the larval stage (Saenko et al., 2011; Shirai et al., 2012) as well as variation in the way that the transcription factors expressed in the rings are associated with the colored scales and pigment biosynthetic modules (Brunetti et al., 2001). For instance, in *Bicyclus anynana* expression of the transcription factor Spalt is associated with black-colored scales, whereas in *J. coenia*, Spalt is associated with both black- and yellow-colored scales (Brunetti et al., 2001). This may indicate evolutionary lability in the linkages between wing pattern differentiation genes and the actual pigmentation genes that they regulate. In butterflies, the pigmentation genes that become expressed shortly before adult emergence appear to belong to conserved pigment biosynthetic modules. These modules include genes such as DDC, involved in melanin formation, vermillion and cinnabar, involved in the production of ommochrome pigments, henna involved in pteridine biosynthesis, and others (Beldade et al., 2006; Koch, 1991; Koch et al., 1998; Nijhout and Koch, 1991; Reed and Nagy, 2005; Wittkopp and Beldade, 2009).

In basal-branching butterflies from the family Pieridae, single patches of black scales, in place of eyespots with concentric rings, develop on the wings. These spots appear to share some developmental similarities with nymphalid eyespots, but they do not appear to share many of the same genes. None of the nymphalid focal marker proteins tested so far (N, Sal, En, Dll, Antp) are observed at the center of these spots (Monteiro et al., 2006; Shirai et al., 2012), whereas one of the 'central black disk' candidate selector proteins in *Bicyclus*, Spalt, is expressed in these spots right after pupation (Monteiro et al., 2006). It is still unknown what upstream signal is activating Sal in these spots, but early pupal ablations of the cells at the center of these spots suggest that a central signal is responsible for spot differentiation, as is the case for nymphalid eyespots (A. Stoehr and A. Monteiro, unpublished data). Currently, the gene expression data from *Pieris* spots and *Bicyclus* and *Junonia* eyespots suggest that if eyespots evolved from simpler spots, as those found in *Pieris*, the transition involved maintaining a conserved central signaling group of cells but elaborating both the mechanism of central cell differentiation as well as the mechanism that responds to the central signals to produce the multiple rings of color.

Examining the genetic basis of convergence in butterfly eyespots

Eyespots in papilionid and lycaenid butterflies (Figure 2A) do not share central marker genes with nymphalid eyespots (J.C. Oliver and A. Monteiro, unpublished data; Shirai et al., 2012), suggesting that eyespot development in these distinct families is convergent and uses distinct developmental mechanisms. Surprisingly, eyespots in the distantly related saturniid moths express at least two of the same eyespot focal marker genes as in nymphalid butterflies (En and Dll) (Monteiro et al., 2006). Saturniid eyespots, however, are not found in homologous regions of the wing relative to nymphalid eyespots (Figure 2A). Saturniid eyespots are usually found as single units in the center of the wing and straddling a cross-vein, whereas, nymphalid eyespots (and *Pieris* spots) are most often found deployed repeatedly across the wing, centered in the space between adjacent longitudinal veins.

The differences observed between the number and position of saturniid and nymphalid eyespots beg the question of whether these eyespots are homologous, that is, whether eyespots have persisted in these lineages because their most recent common ancestor and were merely lost (in multiple butterfly lineages) and shifted in position and duplicated in number (in Nymphalidae), or whether, instead, eyespots have evolved independently in different regions of the wing in these two lineages (Monteiro, 2008). Recent comparative work suggests that nymphalid eyespots evolved once within that clade (J.C. Oliver and A. Monteiro, unpublished data), and points, thus, for independent origins of saturniid and nymphalid eyespots. An explanation for the shared similarities in nymphalid and saturniid gene expression patterns could either be due to parallel recruitment of the same genes into each eyespot's gene regulatory network, one by one, or by the independent co-option of the same conserved gene regulatory network, that is, perhaps serving a separate function elsewhere (Monteiro, 2012; Monteiro and Podlaha, 2009).

The gene regulatory network co-option scenario appears to be supported for the origin of nymphalid eyespots. Comparative gene expression work in several subfamilies of the Nymphalidae (J.C. Oliver and A. Monteiro, unpublished data) suggests that there was a rapid, saltational-like event where multiple genes were co-opted to the eyespot centers in a short evolutionary period, coinciding with the origin of eyespots. After this rapid gene co-option event happened, many genes were lost from the eyespot centers without altering the appearance of the adult eyespots (J.C. Oliver and A. Monteiro, unpublished data). This suggests that eyespots may have originated via the co-option of an overly complex network of genes, functioning somewhere else in the body, but where not all genes functioned in the development of the novel trait. Many of these genes were, thus, subsequently lost from eyespots without affecting trait development. Future comparative gene

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expression data with saturniids, as well as other insect lineages carrying eyespot-like patterns, should further elucidate how many times eyespots have evolved into distinct insect lineages, and the multiple mechanisms used to build these exquisite functional traits.

Wing pattern mimicry in butterflies

In addition to eyespots, another common protective mechanism used by butterflies is wing pattern mimicry, in which one species evolves to look like another. There are two different classes of mimicry: Batesian mimicry (Bates, 1862), a parasitic relationship in which undefended 'mimic' species evolve to resemble toxic or otherwise protected 'model' species; and Müllerian mimicry (Müller, 1879), a mutualism in which two or more protected 'co-mimic' species evolve to resemble one another. While the evolutionary dynamics of these two types of mimicry differ dramatically, they have together generated many independent origins of nearly identical wing patterns across butterflies, either between mimics and models in Batesian systems or among co-mimics in Müllerian systems.

One butterfly mimicry system that has received substantial attention is the Neotropical butterfly genus *Heliconius* (Brown, 1981; Joron et al., 2006a; Papa et al., 2008). The genus consists of 45 species and hundreds of named subspecies and racial phenotypes (Brown, 1981). This diversity is distributed in two ways: (i) many *Heliconius* species exhibit geographic variation in wing patterning such that they shift phenotypes radically every few hundred kilometers (Turner and Mallet, 1996) and (ii) co-occurring species, while often closely related and hybridizing, are sorted into as many as five or six distinct mimicry rings, or groups of co-mimetic species (Mallet and Gilbert, 1995). In contrast to many natural systems, much is known about the agents and targets of selection in *Heliconius,* and there is experimental evidence for the role of wing patterning in warning coloration and mimicry (Benson, 1972; Kapan, 2001; Mallet and Barton, 1989). The bright color patterns of *Heliconius* butterflies are adaptations that warn butterfly-feeding bird predators of their unpalatability. Furthermore, natural selection for Müllerian mimicry has resulted in many instances of color pattern convergence among *Heliconius* species, as well as convergence with other chemically defended butterflies like ithomines. Wing coloration has also been shown to play an important role in *Heliconius* mate choice, with males preferring to court females that share their wing patterns (Chamberlain et al., 2009; Jiggins et al., 2001; Kronforst et al., 2006b).

Heliconius mimicry generally involves convergence between distantly related species within the genus. In fact, the genus consists of two subclades (Beltran et al., 2007; Brower, 1994) and most examples of mimicry consist of pairs of co-mimetic species, with one species coming from each of these two clades (Gilbert, 2003; Turner, 1976). A notable exception to this general rule is mimicry in the Amazon, where multiple *Heliconius* species, other butterflies, and even day-flying moths have converged on a shared 'rayed' color pattern. One of the best-studied examples of mimicry in *Heliconius* is between *H. erato* and *H. melpomene*. Each comes from one of the two within-*Heliconius* subclades, and both are widely distributed across much of Central and South America. *Heliconius erato* and *H. melpomene* mimic one another throughout their range but they have each radiated into over 20 diverse geographic wing pattern forms, resulting in a concordant, geographic mosaic of color pattern races (Flanagan et al., 2004; Quek et al., 2010; Sheppard et al., 1985; Turner and Mallet, 1996).

Some important aspects of the evolutionary history of the convergent radiations in *H. melpomene* and *H. erato* have recently been worked out. It is now known, for instance, that contrary to the previous speculation (Brown et al., 1974; Sheppard et al., 1985; Turner and Mallet, 1996), the two species did not diversify at the same time (Flanagan et al., 2004; Hines et al., 2011; Quek et al., 2010). Rather, estimates of DNA sequence divergence suggest that *H. erato* radiated first, starting approximately 2.8 million yrs ago, with the *H. melpomene* radiation dating to approximately 2.1 million yrs ago (Quek et al., 2010). Furthermore, the phylogeography of the two species differs, suggesting that they originated on opposite sides of South America (Quek et al., 2010). The emerging picture is of a scenario in which *H. erato* diversified first and was then tracked by *H. melpomene*. If this is true, one important question that remains is why did *H. erato* diversify into geographic races in the first place? Because predators are expected to exert strong stabilizing selection on warning patterns, it is not obvious how such extreme diversity could evolve. Potential explanations may include natural selection, such as divergent selection to maximize signal efficiency in different light environments, sexual selection, or even stochastic events that allowed novel color patterns to evolve (Mallet, 2010).

Genetic basis of wing pattern mimicry in butterflies

Regardless of the cause, the fact that many *Heliconius* species posses a diversity of wing patterns makes them an attractive model to explore the genetic basis of color patterning. A variety of classic crossing experiments have revealed that much of the intraspecific variation in *Heliconius* wing patterning is controlled by a small number of large-effect 'switch' loci, Mendelian traits that turn color pattern elements on and off, shift their position on the wing, or change their color (Sheppard et al., 1985; Turner, 1971; Turner and Crane, 1962). More recently, crosses between closely related species, such as between *H. melpomene* and *H. cydno*, *H. cydno* and *H. pachinus*, and *H. erato* and *H. himera*, have shown that the same switch loci that operate within species also appear to control the sometimes radical phenotypic divergence between species (Gilbert, 2003; Jiggins and

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Mcmillan, 1997; Kronforst et al., 2006a,b; Naisbit et al., 2003).

Over the past decade, there has been a major push to characterize *Heliconius* mimicry genes at a molecular level. Using a variety of approaches including genetic mapping, association mapping, genomic methods, and analyses of candidate-gene expression, genomic regions responsible for a number of the major mimicry genes in *Heliconius* have now been positionally cloned (Baxter et al., 2010; Counterman et al., 2010). Particular attention has been placed on the *B/D* and *Yb* loci of *H. melpomene* (Baxter et al., 2008, 2010; Jiggins et al., 2005), which control red patterning and the hindwing yellow band, respectively; the *D* and *Cr* loci in *H. erato* (Counterman et al., 2010; Kapan et al., 2006; Tobler et al., 2005), which control red and melanic patterning, respectively; and the *K* locus in *H. cydno* (Chamberlain et al., 2009; Kronforst et al., 2006b), which controls whether the wings are white or yellow. Identifying the genes and the specific nucleotide substitutions responsible for mimicry is important because it will reveal the molecular targets of natural selection and the raw material for adaptation. Beyond that however, *Heliconius* offers a unique opportunity to examine the molecular basis of convergent evolution because co-mimics, like *H. erato* and *H. melpomene*, have independently evolved near-identical wing patterns time and again.

Examining the genetic basis of convergence in mimetic butterfly wing patterns

While *Heliconius* co-mimics converge on even the smallest details of their color pattern, early crossing experiments revealed that matching pattern elements segregate rather differently in crosses, leading to the speculation that the underlying genetic basis may differ between the two clades (Mallet, 1989; Sheppard et al., 1985). An identical genetic basis for mimicry would perhaps be surprising given that the *Heliconius* subclades split from a common ancestor 10–15 million yrs ago and subsequently evolved their color patterns completely independently. Because species from the two clades cannot be interbred, it was not possible to address this question directly until recently. Today, comparative genetic maps anchored with homologous genes, and sequenced BAC contigs, permit the identification of the same chromosomes across species and co-localization of potentially homologous mimicry genes. Astonishingly, this comparative mapping work across *H. erato*, *H. melpomene*, and *H. cydno* has revealed that mimicry loci affecting similar mimetic patterns, or which influence similar wing pattern elements, routinely map to identical positions on homologous chromosomes (Joron et al., 2006b; Kronforst et al., 2006a). This is strongly suggestive of a conserved genetic basis for mimicry across *Heliconius*. Specific examples include the melanin patterning locus *Ac* of *H. cydno* and *H. melpomene*, which appears to be homologous to *Sd* in *H. erato* (Kronforst et al., 2006a). Similarly, a second melanin patterning locus, *Yb* in *H. cydno* and *H. melpomene*, appears to be homologous to *Cr* in *H. erato* (Baxter et al., 2010; Counterman et al., 2010; Kronforst et al., 2006a). The *Yb/Cr* genomic region has also evolved into a mimicry 'supergene', called the *P* locus, that controls wing patterning across the entire wing in *H. numata* (Joron et al., 2006b), a phenomenon associated with the existence of polymorphic inversions in that species (Joron et al., 2011). The emerging picture is one of a limited and conserved mimicry 'toolbox', shared across the genus, with the incredible diversity within and between species being generated by extensive allelic diversity at a small number of genes.

The best characterized example of convergent mimicry in *Heliconius* involves red coloration. Red wing patterning in *H. erato* is controlled by a single locus, *D* (Figure 3). For instance, allelic variation at *D* determines whether the wings of *H. erato* will have a broad red band across the forewings, red/orange patches at the base of the forewings, and/or red rays on the hindwings (Kapan et al., 2006). Similar phenotypes in *H. melpomene* appear to be the result of two tightly linked loci, *B* and *D* (Baxter et al., 2008). In *H. cydno*, red patterning takes a very different form, generally consisting of a brown oval shape on the ventral side of hindwings or small red spots and rays at the base of the wings. These traits are controlled by the linked loci *G* and *Br* (Chamberlain et al., 2011). Similar to other examples, comparative mapping experiments have shown that *D*, *B/D*, and *G* all map to the same genomic interval (Baxter et al., 2008; Kronforst et al., 2006a) and recent expression and association data point to a single gene, *optix*, as the red patterning locus itself (Reed et al., 2011). In fact, in situ experiments reveal that *optix* expression correlates perfectly with the position of all future red, orange or brown wing patterns in *Heliconius* (Reed et al., 2011).

The fact that the first *Heliconius* mimicry locus to be characterized at a molecular level is ancestrally involved in eye development is intriguing for at least two reasons. First, besides melanin, most of the pigments that color nymphalid butterfly wings, including those of *Heliconius*, are ommochrome pigments (Gilbert et al., 1988), which also function as filtering pigments in insect eyes. The emerging data for *optix* suggest that this eye-development pathway has been co-opted in the evolution of butterfly wing patterning. Secondly, there is another emerging link between wing coloration and mate preference in *Heliconius*. Behavioral experiments have shown that *Heliconius* butterflies not only exhibit wing pattern–based mate preference (Jiggins et al., 2001; Kronforst et al., 2006b), but that variation in mate preference is controlled by loci tightly linked to the mimicry genes (Chamberlain et al., 2009; Kronforst et al., 2006b; Merrill et al., 2011). The fact that wing color and color preference co-segregate in crosses and

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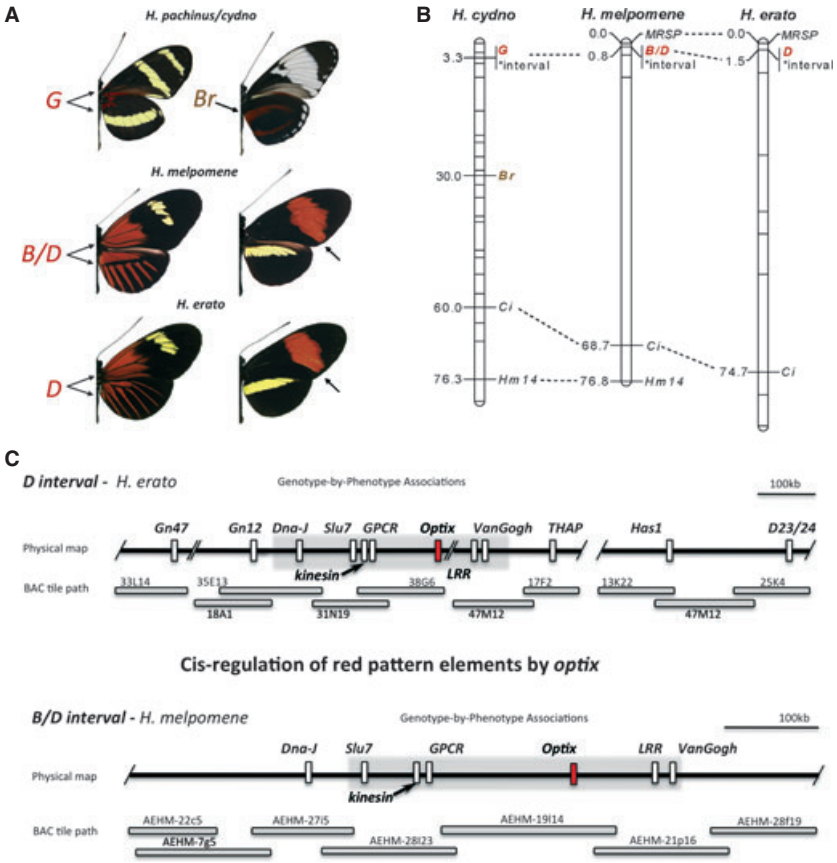

Figure 3. The genetic basis of mimicry in *Heliconius* butterflies. (A) Red wing patterning is controlled by large-effect, Mendelian loci in each *Heliconius* species, the *G* locus in *H. pachinus/cydno*, tightly linked *B/D* loci in *H. melpomene*, and the *D* locus in *H. erato*. (B) Comparative genetic maps reveal that these red patterning loci are located in similar positions on homologous chromosomes, suggestive of a conserved genetic basis for red patterning across species. (C) Further fine mapping and association mapping point to the same narrow interval in both *H. melpomene* and *H. erato*, and subsequent gene expression data identify the transcription factor *optix* as the gene controlling red, orange, and brown wing patterning across all *Heliconius* butterflies.

remain correlated in polymorphic populations may point to a functional link between wing patterning and behavior, one perhaps mediated by the shared use of ommochrome pigments in wing scales and ommatidia.

The *Heliconius* system is revealing that, at least for closely related species, phenotypic convergence because of wing pattern mimicry involves the same underlying genes. Beyond *Heliconius*, there is a huge amount of wing pattern diversity among butterflies yet much of this appears to be the product of variation in a conserved ground plan (Nijhout, 1986, 1991). Furthermore, crossing experiments in a variety of other butterflies have shown that drastic shifts in mimetic wing patterns are generally controlled by a switch-like genetic architecture (Nijhout, 1991). Together, these observations suggest that we may find a broadly conserved genetic basis for wing pattern divergence across butterflies. While comparative data outside *Heliconius* are still scarce, there are some preliminary observations that both do and do not support this hypothesis. For instance, supporting evidence comes from recent genetic mapping work in the butterfly *Bicyclus anynana* and the moth *Biston betularia*. Specifically, Beldade et al. (2009) mapped the genomic locations of four Mendelian wing patterning mutants in *Bicyclus* and found that three of these were located on chromosomes that are known to contain mimicry genes in *Heliconius*. Furthermore, recent work in *Biston betularia* has shown that the Mendelian locus that controls the famous shift to industrial melanism is located in the same location as the *Yb*, *Cr*, and *P* mimicry loci of *Heliconius* (van't Van't Hof et al., 2011). This same region, incidentally, also contains at least one of the *Bicyclus* wing pattern mutants, *Bigeye* (Beldade et al., 2009).

Evidence against a broadly conserved basis for wing patterning comes from *optix* itself. While this gene controls the distribution of red pigmentation across *Heliconius*, there does not appear to be a link between *optix* expression and red patterning in the nymphalid butterflies *Vanessa cardui* and *Agraulis vanilla* (Reed et al., 2011), which are closely related to *Heliconius*. Furthermore, switches in brown and orange patterning, as well as yellow and melanin, in *Heliconius numata* are all achieved chiefly via different gene arrangement allelomorphs in the supergene *P* (Joron et al., 2011), which is not linked to *optix*. Similarly, genetic mapping work in the polymorphic swallowtail butterfly *Papilio dardanus* has shown that its Mendelian mimicry supergene, which controls the entire wing pattern phenotype, maps to the genomic location of the gene *invected* (Clark et al., 2008). This region is not known to contain mimicry genes in other taxa such as *Heliconius*. Like other examples highlighted in this paper, the available data for butterflies

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suggest instances of both conserved and divergent genetic control of wing patterning. This heterogeneity is likely to be a product of the interplay of various forces including phylogeny, selection, and genetic or developmental constraints.

Pigment variation in lizards

Efforts to characterize the genetic basis of reptile color variation are still in their infancy, this system having not received as much attention as mammals and invertebrates like *Drosophila* and butterflies. However, studies of reptiles represent a tremendous opportunity to better understand genetic mechanisms underpinning ecologically important color traits. Color serves a number of ecological functions in reptiles. First, the dorsal color of reptiles can be important for camouflage. As with many other small, diurnal animals, reptiles are subject to intense visual predation. Therefore, many reptiles are cryptically colored to blend in with simple or complex backgrounds (e.g., white sand color-matching or horned-lizard stone mimicry, respectively; Rosenblum, 2006; Sherbrooke and Montanucci, 1988). Second, reptile coloration can serve as antipredator advertisements. Many reptiles exhibit bright aposematic warning coloration – some of these brightly colored reptiles are toxic while others are mimics of toxic forms (e.g., coral snakes and their mimics; Savage and Slowinski, 1992). Third, reptile coloration can play a role in intraspecific communication. Many reptiles use sexually dimorphic color patches during territorial or courtship interactions (Chan et al., 2009a; Cooper and Burns, 1987). Finally, reptile coloration is important for thermoregulation because color can influence heat acquisition for these ectotherms (Clusella Trullas et al., 2007; Forsman, 1995).

The ecological importance of reptile coloration has led to dramatic variation in coloration across individuals, populations, and species. Within populations, some reptiles have multiple color morphs (e.g., striped versus melanic polymorphism in garter snakes and throat color polymorphism in side-blotched lizards; King, 1988; Sinervo and Lively, 1996). Across populations, many reptiles exhibit different color morphs in different parts of their ranges because of natural selection, sexual selection, and/or genetic drift. For example, many reptiles are locally substrate matched, likely due to natural selection for predator avoidance (e.g., fence lizards with different color morphs on dark soil, white sand, and black lava substrates; Rosenblum et al., 2007). Other reptiles have locally differentiated signaling colors, possibly due to sexual selection (e.g., variation in male ornaments like *Anolis* dewlaps; Nicholson et al., 2007). Finally, some reptiles exhibit geographic variation in coloration that cannot be unambiguously linked to particular environmental pressures (e.g., legless lizards on islands/coasts are darker than their inland counterparts; Pearse and Pogson, 2000).

In addition to variation in color at the population and species levels, individuals of many reptile species can vary in color over time. First, many species can alter their coloration from moment to moment. Specifically, color can be rapidly changed in response to local conditions (e.g., temperature, stress, predators; Castrucci, 1997; Rosenblum, 2005; Stuart-Fox and Moussalli, 2009). Second, many reptiles change color seasonally – females of many species obtain bright coloration during the breeding season to communicate information about breeding status (Chan et al., 2009a; Hager, 2001). Third, many reptiles change color ontogenetically; for example, conspicuous tail coloration is observed in juveniles of several species, but not in adults (Hawlena et al., 2006). Although the physiological and adaptive significance of color variation has rarely been experimentally tested in reptiles, the dramatic color variation in this group is likely to have important fitness consequences.

Genetics of pigment variation in lizards

The mechanisms of production and variation of reptile coloration are well characterized at the structural level. Reptile skin contains a 'dermal chromatophore unit' with three primary cell types (Bagnara and Hadley, 1973). Xanthophores are pigment-containing cells that are primarily responsible for yellow/orange coloration. Iridophores do not contain pigment but are essential for blue/green structural coloration through the reflective properties of the cells. Melanophores contain melanin and determine the overall darkness of color patches. Melanin is contained in the deepest layer of the dermal chromatophore unit and can be dispersed or aggregated. The interaction of these three cell types determines the overall appearance of reptile color patches.

The genetic architecture of color traits is clearly expected to vary across traits and species. However, breeding studies suggest that at least some reptile color phenotypes are controlled by genes of large effect. For example, genetic crosses in the garter snake *Thamnophis sirtalis* have shown that the melanic (versus striped) phenotype is consistent with a simple Mendelian inheritance pattern (King, 2003). Similarly, breeding studies with the side-blotched lizard *Uta stansburiana* have indicated that there is likely a locus of major effect controlling the male throat color polymorphism (Sinervo and Svensson, 2002).

The identification of specific genes underlying reptile coloration can be informed by the detailed understanding of vertebrate pigmentation pathways developed from mammalian systems. Melanin-based traits have been a tractable focus for candidate-gene studies focused on the melanocortin-1 receptor gene (*Mc1r*). Regions of *Mc1r* are conserved enough across vertebrates to amplify small portions of the gene in many vertebrate taxa. Genome walking techniques have then been used to develop species-specific primers for a number of reptiles (Rosenblum et al., 2004). By comparison, less is

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known about the molecular mechanisms underlying red/yellow and blue/green color variation in reptiles. Red/yellow coloration is largely controlled by pterine and carotenoid pigments, and there are candidate genes in the pterine pathway that could be interrogated (Braasch et al., 2007; Salzburger et al., 2007). However, carotenoid pigments can be influenced by diet, stress, and maternal provisioning (Fitze et al., 2009; Weiss et al., 2011). Therefore, the interactions between genes and the environment may be more complex for red/yellow color patches. The genetics of blue/green coloration also may be challenging to unravel because reflective colors are determined by the interaction between melanin layers and the number, size, and spacing of reflecting platelets in the iridophore cell layer (Kuriyama et al., 2006; Morrison et al., 1996).

Examining the genetic basis of convergence in lizard pigmentation

One dramatic example of convergent evolution of reptile coloration is the pale lizards of White Sands, New Mexico (Figure 4). White Sands is a striking landscape of white gypsum dunes, which have formed recently, in the last 6000 yrs (Kocurek et al., 2007), and contrast starkly with the dark substrate of the surrounding Chihuahuan Desert. Several animal species exhibit pale forms on the dunes, presumably as an adaptation for crypsis. The lizards of White Sands are of particular interest because only three species have successfully colonized the gypsum dunes: the lesser earless lizard (*Holbrookia maculata*), the eastern fence lizard (*Sceloporus undulatus*), and the little striped whiptail (*Aspidoscelis inornata*). This reduced lizard assemblage is striking compared to more than a dozen lizard species in the nearby dark soil habitat. Blanched forms of the three White Sands lizards were first described more than 50 yrs ago and have since been recognized as subspecies or unique species (Dixon, 1967; Jones and Lovich, 2009; Lowe and Norris, 1956; Smith, 1943).

The White Sands lizards are a particularly compelling example of rapid convergent evolution. The three species are distantly related (Reeder and Wiens, 1996; Wiens et al., 2010), thus serving as independent evolutionary replicates. It is of interest to study independent lineages in precisely the same selective environment, in contrast to most studies of convergent evolution that compare closely related lineages that have colonized geographically separate but similar environments (Berner et al., 2009; Hoekstra et al., 2005; Nosil and Sandoval, 2008; Nosil et al., 2002; Steiner et al., 2009; Vines and Schluter, 2006). Further, because the White Sands formation is geologically recent and there are no geographic barriers separating White Sands from the surrounding dark soil habitat, understanding adaptive divergence between light and dark lizards is facilitated by comparatively little 'neutral' divergence for populations in different habitats (Rosenblum and Harmon, 2011).

A candidate-gene approach was used to understand whether *Mc1r* was associated with color variation in the White Sands lizards (Figure 4). Statistical associations between particular *Mc1r* mutations and color phenotype were identified in all three species (Rosenblum et al., 2004). Follow-up functional assays implicated the *Mc1r* mutations in blanched coloration for two of the three species (*S. undulatus* and *A. inornata,* Rosenblum et al.,

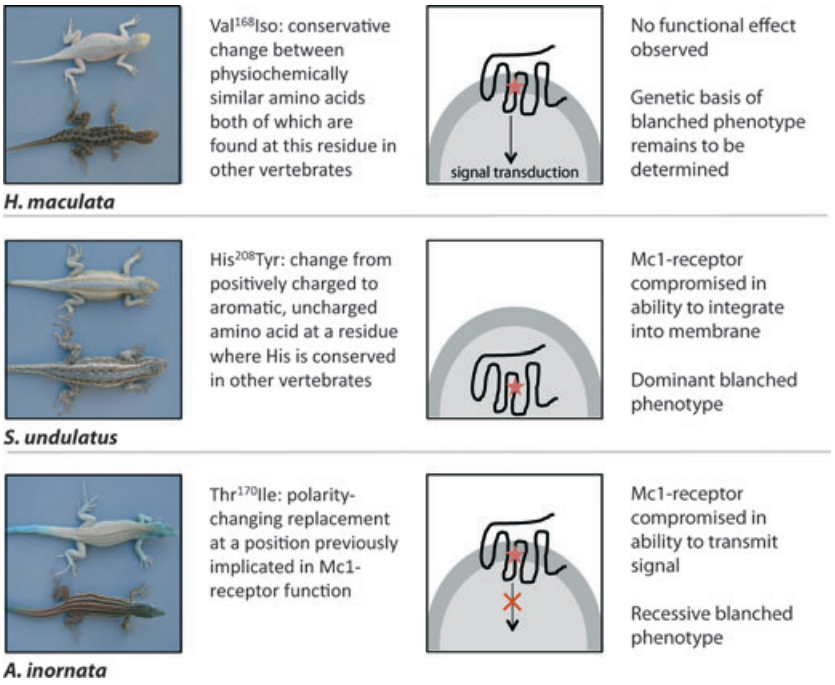

Figure 4. Genetic and functional basis of blanched color in three White Sands lizard species. Left panel: for each species, images of lizards from White Sands (top) and dark soil (bottom) habitats. Right panel: schematic of a melanocyte cell and a Mc1-receptor. Wildtype Mc1-receptors integrate into the melanocyte membrane and are effective signal transducers in the melanin production pathway. Mutations in *Mc1r* (schematized with red stars) have different functional effects in different species.

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2010). Specifically, cyclic AMP assays were used to determine the functional consequences of the focal mutations (His^{208}Tyr for *S. undulatus*, Thr^{170}Ile for *A. inornata* and Val^{168}Ile for *H. maculata*). The *H. maculata* mutation had no measurable effect on *Mc1r* signaling in this assay. However, for both *S. undulatus* and *A. inornata*, the 'blanched receptors' were compromised relative to the 'wild-type receptors'. An enzyme-linked immunosorbent assay (ELISA) also showed that cell-surface expression was particularly low for the *S. undulatus* blanched receptor. These results are consistent with a model in which blanched *S. undulatus* receptors do not integrate efficiently in the melanocyte membrane, whereas blanched *A. inornata* receptors integrate into the melanocyte membrane properly but are compromised in their signal transduction ability (Rosenblum et al., 2010).

Thus, studies of the genetics of blanched coloration in White Sands lizards reveal important similarities and differences across species. There are exciting similarities across species in the molecular mechanism for blanched coloration. Notably, mutations in the same gene underlie blanched phenotypes for *S. undulatus* and *A. inornata,* two distantly related lizards. In both species, structural mutations in the coding region of *Mc1r* 'break' a critical link in the melanin synthesis pathway. However, there are some important differences across species as well. First, blanched coloration in the third species *H. maculata* is controlled by a different and yet undiscovered mechanism. Second, there are differences in the functional consequences of the *Mc1r* mutations in *S. undulatus* and *A. inornata*. The *Mc1r* receptor is disrupted in two different ways in the two different species, a fact that has important ramification for the adaptive dynamics in this system. For example, the blanched allele is dominant in *S. undulatus* but recessive in *A. inornata*, an observation that is consistent with the functional mechanisms of disruption for the blanched alleles (Rosenblum et al., 2010). Allelic dominance is important for predicting the visibility of new adaptive alleles to natural selection (Orr, 2010) and thus can influence the distribution of adaptive alleles in nature. Finally, the differences between species are of particular interest because they demonstrate the important distinction between genetic and functional mechanisms of adaptation (Manceau et al., 2010).

Thus far, molecular studies of reptile coloration have primarily been association studies with *Mc1r* as a candidate gene. There are a number of important next steps for expanding research on the genetics of adaptation in reptiles. First, future studies should use a greater diversity of approaches – from genome-wide association studies to genetic mapping in controlled crosses. Second, future work should focus on genes and gene interactions beyond *Mc1r*. For example, it is important to understand whether additional loci interact with *Mc1r* to generate melanin-based phenotypes in lizards, and whether these interactions are similar to those described in other vertebrates. Third, it is important to ask whether convergent phenotypes have a similar molecular basis in a broader sample of reptiles. Melanism is an excellent focal phenotype because many reptile species have convergently evolved melanic dorsal coloration (e.g., associated with dark lava substrates and coastal or island regions; King, 1988; Pearse and Pogson, 2000; Rosenblum et al., 2004). However, melanin-based traits are only one aspect of reptile coloration. The enormous diversity in reptile color and pattern merits further investigation and could provide important advances in understanding the molecular basis of convergent evolution.

Emerging systems

In addition to the systems discussed above, there are many more systems in which the genetic basis of coloration is being studied. When examining an 'emerging' system for color variation, there are two major considerations: the degree of color and pattern variation and the ease and potential of doing genetics in the system. Some of the emerging systems have great genetic potential and molecular tools but have less striking color and pattern variation. Studying these systems is advantageous because it allows us to more quickly understand the actual genes responsible for pigmentation phenotypes. Other emerging systems have extreme color and pattern variation but few genetic tools. These systems are worthwhile because once the genetic and molecular tools are established, a greater understanding of more complex phenotypic variation can be achieved. Here, we first discuss the ideal characteristics of an emerging system for the study of color pattern convergence. Next, we discuss systems with developed genetic tools where the actual genes responsible for color variation have been examined. Finally, we discuss systems with marked color pattern diversity where the genetic and molecular tools are in the process of being developed.

There are several characteristics of any new system that will facilitate the genetic analysis of convergence. First, there must be color pattern variation within or among populations of the same species or between closely related species, and evidence for independent evolution of similar phenotypes (i.e., a phylogeny demonstrating independent phenotypic evolution). Second, species should be able to be reared in captivity and mated in a controlled way to generate experimental crosses. This is necessary for QTL analyses to dissect the genetic basis of complex traits and also for complementation analyses addressing whether the same or different loci are implicated in producing identical phenotypes in different populations. Third, species with short generation times, large clutches, and limited space requirements are most desirable to facilitate large

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sample sizes for captive breeding experiments. Fourth, there should be an assembled and annotated genome for the organism of interest or for one closely related to the target species with which it shares a large degree of synteny. This is required to positionally clone genes responsible for mapped traits, such as in QTL analyses. Fifth, techniques for in situ hybridization to examine the embryonic expression of different candidate genes involved in pigmentation are useful to examine the development and ontogeny of pigmentation. Sixth, it is helpful to have tools to perform gene knockdown or over-expression experiments to verify gene action. Finally, one should have experimental evidence for the evolutionary pressures and ecological role of pigmentation in nature, if specifically interested in adaptive evolution. Many systems may have limitations in one or more of these features, but the benefit of having multiple systems in which convergence in pigmentation is analyzed may outweigh the shortcomings of individual systems.

There are multiple fish systems that offer promise to build upon the excellent pigmentation work that has been carried out with zebrafish and relatives (Mills et al., 2007; Parichy, 2006, 2007). For instance, the three-spine stickleback, *Gasterosteus aculeatus* (Figure 5), meets all of the above criteria. Particular advantages include the presence of several freshwater populations with lighter pigmentation, a sequenced genome, BAC libraries, and methods for transgenesis and in situ hybridization (Chan et al., 2009b; Colosimo et al., 2005; Greenwood et al., 2011; Jones et al., 2012; Miller et al., 2007; Shapiro et al., 2004). Because of these tools, sticklebacks have proven to be an excellent system for the analyses of the genetic basis of convergence in many traits (Chan et al., 2009b; Colosimo et al., 2005; Cresko et al., 2004; Greenwood et al., 2011; Hohenlohe et al., 2010; Miller et al., 2007; Shapiro et al., 2004). There are also multiple pigmentation differences in marine versus freshwater populations. The genetic basis of darker pigmentation in a marine population compared to a freshwater population was investigated by QTL analysis, positional cloning, and allele-specific expression (Miller et al., 2007). The gene responsible was found to be *Kit ligand*. Furthermore, a patterning difference, vertical bars, present in freshwater populations, was also examined by QTL analysis although the genes responsible have not yet been determined (Greenwood et al., 2011).

Another emerging system with great promise for the genetic analysis of convergent evolution is the Mexican cave tetra, *Astyanax mexicanus* (Figure 5), a species in which cave-dwelling populations have been repeatedly derived from surface-dwelling ancestors (Dowling et al., 2002; Strecker et al., 2004; Wilkens, 1988). Advantages of this system include the development of embryonic techniques such as tissue transplants, in situ hybridization, morpholino knockdown, and overexpression by RNA injection (Yamamoto et al., 2004, 2009). Genetic tools include a BAC library, linkage map, and synteny with the zebrafish genome (Di Palma et al., 2007; Gross et al., 2008; Protas et al., 2006).

Cave populations have many color differences including albinism, reduced pigmentation, a yellowish color, and reduced iridiphores (iridescent chromatophores in fish) (Culver and Wilkens, 2000; Sadoglu, 1957; Sadoglu and Mckee, 1969). Therefore, this species is a good system in which to examine the genetic basis of color variation. So far, the genetic basis of albinism and reduced pigmentation has been examined. In two of the cave populations, different deletions in the same gene, *Oca2*, have been shown to cause the loss of function of the protein (Protas et al., 2006). In one other albino cave population, complementation tests suggest that an unidentified mutation in *Oca2*, possibly a regulatory mutation, is responsible for the albino phenotype. Two of these albino cave populations also have the 'brown' phenotype that causes reduced pigmentation (Sadoglu and Mckee, 1969). Each of these populations has a different mutation in the gene *Mc1r* (Gross et al., 2009). Furthermore, complementation tests implicate *Mc1r* in several additional populations that contain the 'brown' phenotype (Wilkens and Strecker, 2003). Therefore, in this particular species, convergent evolution of multiple pigmentation phenotypes has resulted from functionally similar variants in the same genes.

Figure 5. Emerging model systems for the study of adaptive pigmentation. Upper row: Three-spine stickleback (photo by Frank Chan); Happy face spiders (photos by Rosemary Gillespie and Geoff Oxford); Mexican cave fish (photo by Richard Borowsky). Lower row: *Anolis* lizards (photos by Jonathan Losos and Luke Mahler); Cichlid fish (*Pundamilia nyererei* and *P. pundamilia* photos by Ad Konings and Ole Seehausen, respectively). All photos used with permission of the owners.

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Other fish systems have truly amazing variation in pigmentation and pattern as well as molecular tools, for example cichlids (reviewed in Kocher, 2004). There are phylogenies for many taxa available, multiple species are readily bred in captivity, there is an effort underway to produce complete genomes from several taxa, and there is synteny with the *Takfugu rubripes* genome (Roberts et al., 2009; Streelman et al., 2003). In addition, there are many developmental and genetic tools available for cichlids (Roberts et al., 2009; Salzburger et al., 2007) and much is known about the ecological role of coloration in cichlids (Maan et al., 2010; Seehausen et al., 2008). These tools have allowed for the identification of the gene *Pax7* as responsible for the orange blotch color pattern (Roberts et al., 2009).

The techniques and tools discussed above are currently being developed in additional species with interesting color patterns. For example, lizards in the genus *Anolis* have evolved similar colorful phenotypes many times independently, both in terms of body color and pattern, but also an impressive diversity in dewlap, the extensible throat fan used in social displays, color, and pattern, including spots and stripes (Nicholson et al., 2007). This is an exciting system to study because there is a well-resolved phylogeny for most of the Caribbean species (Alfoldi et al., 2011); there is abundant variation in color and pattern within individuals and among populations and species; there is a recently published genome sequence (Alfoldi et al., 2011); and there is much known about the ecological role of color (Losos, 2009). Captive breeding of *Anolis* has been challenging, but breeding colonies have been established in several labs (Cox and Calsbeek, 2010). Given the complete genome sequence of *A. carolinensis*, genomic approaches to identify candidate loci associated with particular pigmentation phenotypes are likely to be fruitful. Controlled crosses for genetic dissection of complex traits will be time consuming given the relatively long generation time (several months) and small clutch size (Schneider, 2008), but efforts are underway to do just that. Finally, embryological and gene manipulation techniques for *Anolis* are being developed, and it is expected that tools for transgenic analysis and gene knockdown will be available in the near future. Despite the difficulties, the promise of the *Anolis* system lies in the high frequency of convergent pigment phenotypes (e.g., at least 20 independent origins of the yellow dewlap, and a similar number for red and orange) as well as the understanding of the ecological role of colorful pigmentation. Furthermore, there are multiple promising arthropod systems, in addition to *Drosophila* and butterflies, that show complex, convergent pigmentation phenotypes that may be amenable to genetic study (Figure 5). These include happy face spiders (Croucher et al., 2011; Gillespie and Oxford, 1998; Oxford and Gillespie, 2001) and ladybird beetles (Michie et al., 2010, 2011; Tan, 1946).

We have discussed but a few of the promising natural systems for the analysis of convergence in pigmentation and patterning, limiting ourselves to those with which we are most familiar. Even though any given system may not meet all of the desired criteria of a model system, substantial progress can still be made in understanding the genetic basis of convergent evolution in pigmentation and pattern. Modern genomic techniques permit the rapid assessment of genetic variation among phenotypes, the identification of differentially expressed genes among differently pigmented tissues within individuals or among individuals, populations and species. Furthermore, laboratories are increasingly capable of sequencing, assembling, and annotating new draft genomes. Controlled crosses are particularly useful to dissect the genetic basis of complex phenotypes, but genome scans of naturally varying populations can also provide substantial information (Schlotterer, 2003; Stinchcombe and Hoekstra, 2008; Storz, 2005). Although transgenics and gene manipulation tools are not currently available for most non-model species, these tools can theoretically be developed for most species. Therefore, we urge researchers to identify promising systems for investigation and to proceed boldly.

Conclusions

The great diversity of color patterns across animals offers a rich palette with which to explore the underlying genetic basis of morphological evolution. Furthermore, when paired with analyses of the fitness consequences of pigment variation (Mallet and Barton, 1989; Vignieri et al., 2010), such studies offer a rare opportunity to unlock the molecular basis of adaptation (Barrett and Hoekstra, 2011). By itself, convergent evolution of color pattern argues for adaptive evolution (Losos, 2011), although the selective advantage of specific traits is not always obvious. As we have highlighted here, a major question today is whether convergent pigmentation phenotypes arise by similar molecular mechanisms. Interpreting the answer to this question is not entirely straightforward, however. For a start, molecular convergence can occur at many levels – from specific mutations, to homologous genes or pathways, or even functionally similar or interacting pathways (Losos, 2011; Manceau et al., 2010). Even when it is possible to cleanly differentiate between shared or divergent mechanisms underlying convergent phenotypes, the biological significance of these alternatives remains a matter of debate.

Traditionally, evolutionary biologists have interpreted the molecular basis of convergent evolution in terms of constraint, with shared mechanisms indicative of development's role in limiting the means by which a system may respond to forces such as natural selection. However, it is actually a complex interaction among multiple evolutionary processes, including mutation, selection,

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drift, and constraint, which ultimately produce the end products we see today (Gompel and Prud'homme, 2009; Losos, 2011). In other words, the phenotypes and genotypes present in nature are not merely a product of what can and cannot occur, but also what has stood the test of time.

Our review has highlighted that there is substantial heterogeneity in the genetic basis of convergent pigmentation. For instance, a conserved set of genes is used to pattern Heliconius wings, and nymphalid butterflies appear to have evolved the gene network underlying eyespots only once. Similarly, the gene *Mc1r* has emerged as an important player in color pattern evolution in White Sands lizards, and this is also the case in many mammals and birds (Barsh, 1996; Hoekstra, 2006; Manceau et al., 2010; Mundy, 2005; Nadeau et al., 2006; Theron et al., 2001; Uy et al., 2009). In contrast, the genetic basis of color pattern variation in *Drosophila* is quite variable, sometimes involving the same genes but often times not. Given this heterogeneity among systems, an important next step will be to determine whether there is signal in this variation. As an example, if heterogeneity exists at the level of closely related species, this would be indicative of very little constraint. In contrast, if we generally see a shared genetic basis among closely related species, but divergence among more distantly related taxa, that form of heterogeneity would be consistent with substantial constraint. In truth, despite considerable interest and effort, we still have very little comparative data to address this question. No wonder, given the time and energy that goes into fully characterizing the genetic basis of color patterning in a single system. Future work in the field will need to move beyond complete characterization of a small number of case studies, scattered across the animal kingdom, to comprehensive investigation focused on taxa with strategic phylogenetic relationships. As an example, ongoing work in butterflies is stepping away from the few model species on the butterfly phylogeny to determine how the genetic basis of wing patterning evolves. Thankfully, advances in genomics offer new promise in our hunt for genetic mechanisms underlying adaptive pigmentation across all biological systems. Few other traits show such amazing diversity and repeated convergence across living organisms, making color patterning in natural populations a particularly promising system for continued investigation.

Acknowledgements

We thank the Radcliffe Institute for Advance Study at Harvard University, and Phyllis Strimling in particular, for hosting our Exploratory Seminar entitled 'The Genetics and Evolution of Animal Coloration', which served as a foundation for this paper. We also thank reviewers for their comments on the manuscript. Our research is funded by NSF (MRK, AK, AM, SPM, MP, EBR, CJS, HEH), NIH (MRK, GSB, HEH), BBSRC (JM), and the BEACON Center for Evolution in Action (EBR).

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