The Function of Pigmentation Genes in the Development and Evolution of Drosophila Mating Behavior

by

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Abstract

Phenotypic evolution within and between species involves correlated changes in traits that facilitate survival and reproduction. Mating behaviors, in particular, and their correlated anatomical structures enable animals to court and reproduce with mates using a mixture of visual, chemical, and mechanical cues. How genes and genomes evolve to generate correlated differences in these traits is unclear. In this thesis, I investigate how genes and genomes contribute to correlated differences in mating behavior and pigmentation in *Drosophila*. Using tissue-specific genetic manipulations, I illustrate how the *yellow* gene influences male mating success through its function in melanizing a secondary sexual character; using CRISPR/Cas9 genome editing, I demonstrate a role of the *ebony* and *tan* genes in cuticular hydrocarbon synthesis and natural variation; and using multiplexed shotgun genotyping, I map the genomic location of species differences in wing pigmentation and mating display, identifying new genes involved in pigmentation evolution and new evidence explaining how behavior and anatomy evolve together. Together, these data show 1) that behavioral development and evolution involves correlated changes in structures that animals use to interact with their environment, 2) that changes in these structures correlate with the behaviors that use them, 3) that changes in individual genes can generate these differences, and 4) that complex evolution of sex chromosomes can explain species correlated differences in sex-specific behavior and anatomy.
Chapter 1

Introduction

One cannot intelligently discuss behavior and structure separately. Behavior is what an animal does with its structure; structure is what an animal uses to behave

–Howard Evans, 1966

The problem

Animal species often interact with their environments using specialized structures that are adapted for specific activities. Correlations between behavior and anatomy exist as a consequence of both development and evolution: Males often grow bigger and more aggressive due to higher levels of testosterone, and females tend to select males with more stunning courtship rituals. How do correlations between behavior and anatomy originate and why might they persist (or not) during evolution? How does heritable variation shape these interactions? This thesis dissects the genetic and molecular basis of correlations between mating behavior and pigmentation in Drosophila to better understand how genes and genomes shape the development and coevolution of behavior and anatomy.

Mechanisms linking behavior with anatomy during development and evolution

In numerous cases, patterns of animal behavior correlate with the anatomical structures used during specific social and sexual activities (reviewed in West-Eberhard, 2003): Birds with crests display their crests (Mayr, 1963); lizards with dewlaps bob their
dewlaps (Jenssen, 1977); fishes with spots shake their spots (Endler, 1983). These patterns are observable on both macro- and microevolutionary time scales. Between species, animals show correlated gains or losses of behavior and structure that evolve repeatedly (reviewed in Brooks and McLennan, 1991). Gregariousness in caterpillars, for example, is phylogenetically correlated with aposematic coloration: more colorful species tend to be more social (Sillén-Tullberg, 1988). Within species, individuals show correlated changes in behavior and anatomy that often scale with growth or differentiate sexes. Changes in horn size in dung beetles, for example, are correlated with reproductive behavior: large horned males tend to guard females, hornless males tend to sneak, and hornless females tend to engage in brood care (Cook, 1990; Emlen, 1997; Moczek and Emlen, 2000). How do correlated differences in behavior and anatomy within species lead to correlated divergence between?

The ultimate source of biological variation is mutation. Correlated variation, whether segregating within or diverging between species, originates from mutational changes in gene regulation, gene function, and genome organization. Disentangling when, where, and how these mutations give rise to correlational change is the focus of developmental biology as much as it is evolution.

Pleiotropy

Some mutations are highly disruptive, causing changes in multiple traits throughout development. Mutations that alter multiple traits during development are said to be pleiotropic (Zhang and Wagner, 2013). Depending on where mutations arise in the genome, pleiotropy can manifest as a consequence of changes in gene regulation, protein function, or genome rearrangement. Mutations disrupting the function of transcription factors (TFs), for example, are often highly pleiotropic, because TFs regulate numerous genetic pathways (Wagner and Zhang, 2011). In butterflies, the optix gene encodes a homeobox containing TF (Seimiya and Gehring, 2000) that when disrupted alters multiple correlated components of wing scale structure and pigmentation (Zhang et al., 2017). Ectopic expression of the same gene in Drosophila induces ectopic eye
development (Seimiya and Gehring, 2000). The degree to which a mutation behaves pleiotropically, therefore, often depends on the pleiotropic nature of the affected gene.

How does pleiotropy link behavior with anatomy? Several TFs and cell-cell signaling molecules patterning the development of the nervous system in vertebrates and invertebrates also regulate the development of morphological structures (Lewis, 1998; Louvi and Artavanis-Tsakonas, 2006; Wittkopp and Beldade, 2009; Rideout et al., 2010; Robinett et al., 2010; Wagner and Zhang, 2011). The Ectodysplasin (Eda) gene, for example, encodes a tumor necrosis factor (TNF) transmembrane protein (Mikkola and Thesleff, 2003) that controls the development of armor plates, neuromasts, and schooling behavior in the three-spine stickleback (Gasterosteus aculeatus) (Colosimo et al., 2005; Mills et al., 2014; Greenwood et al., 2016). Pleiotropy at Eda is also associated with disease in humans that likely stems from its broad expression pattern in the skin, heart, nervous system, lung, liver, small intestine, and kidneys (Kere et al., 1996; Bayes et al., 1998; Montonen et al., 1998). But in sticklebacks, Eda expression specifically in neuromast cells is associated with both armor plate development (Mills et al., 2014) and schooling behavior (Greenwood et al., 2016), suggesting that developmental differences in TNF-mediated cell signaling within the same tissue underlies correlated variation in behavior and anatomy.

Pleiotropic mutations disrupting hormone synthesis and reception also contribute to correlated differences in behavior and anatomy during development. Hormones take many forms. In vertebrates, amines, peptides, proteins, and steroids can all behave as hormones (Nussey, 2001). One of the clearest examples illustrating how hormone signaling pairs behavior with anatomy involves the melanocortin system, including the five melanocortin receptors (MC1–5R) and the proopiomelanocortin (POMC) gene, which produces the peptide hormones adrenocorticotropin hormone (ACTH) and melanin-stimulating hormones (MSHs) (Ducrest et al., 2008). Knockout alleles of POMC in mice causes obesity and light coat color; knockout alleles of MC4R causes obesity and increased anxiety; and knockout alleles of MC5R causes higher hair lipid content and
decreased aggression (reviewed in Butler and Cone, 2002). Similar effects have been described in POMC-deficient humans (Kühnen et al., 2016). Pleiotropy in the melanocortin system is likely the consequence of multiple complimentary mechanisms: First, POMC post-transcriptional regulation is complex, leading to multiple peptide products with distinct functions (Ducrest et al., 2008); second, these peptide products are capable of binding different MCRs, each with their own unique expression profiles in the skin, brain, and endocrine glands (Butler and Cone, 2002); and third, POMC and MCR expression in the brain regulates feeding (Zhan et al., 2013) and stress response (Liu et al., 2013) each of which leads to changes in metabolism and growth. Mutations affecting POMC function, therefore, have the potential to echo throughout a cascade of hormone-mediated regulatory pathways controlling both behavioral and anatomical development.

Although mutations at Eda and MCRs are highly pleiotropic, causing disease malformations in humans and widespread phenotypic changes in model organisms, both Eda and MCRs have repeatedly contributed to anatomical evolution within and between species (reviewed in Martin and Orgogozo, 2013). In sticklebacks, natural variation at Eda has repeatedly caused armor plates to evolve in freshwater lake populations (Colosimo et al., 2005; Barrett et al., 2008). In bananaquits, bears, cows, chickens, dogs, foxes, humans, and lizards, natural variation at MC1R is strongly associated with changes in pigmentation (reviewed in Martin and Orgogozo, 2013). It remains unclear to what extent natural alleles at Eda and MC1R contribute to behavioral evolution. Changes in armor plate number and pigmentation intensity in natural populations, however, repeatedly covary with behavioral traits that take advantage of these anatomical differences (Ducrest et al. 2008, Greenwood et al., 2013; Greenwood et al., 2016). The challenge, then, is testing whether animals that carry different versions of the same pleiotropic gene show behavioral changes that correlate with anatomical evolution.

Linkage

Evolution also coordinates multiple traits through the action of multiple, co-inherited genes. Linkage disequilibrium occurs when physical associations between two or more
genetic variants, often on the same chromosome, cause the variants to be inherited together (Saltz et al., 2017). Like pleiotropy, linkage is a source of genetic correlation between behavior and anatomy.

Sex linkage is theorized to be one way evolution separates the development of sexually antagonistic traits between males and females (Rice, 1984). Sexually antagonistic traits involve phenotypes that are beneficial in one sex but deleterious in the other. The costly development of elaborate courtship displays and their correlated ornaments, for example, benefit males but not females in many animal species (Alcock and Rubenstein, 2019). Differences in dosage effects and sex determination cascades cause sex-linked genes to express sex-specific effects during development. Many sex-linked genes, then, are responsible for sexual dimorphism in both behavioral and anatomical development (Reinhold, 1998). In Drosophila and mice, Reinhold (1998) estimates as much as one-third of sexually dimorphic behavioral and anatomical differences are due to X effects. Between Drosophila melanogaster and D. simulans, divergence in gene expression is primarily explained by sex-biased gene expression, and an excess of female-biased genes are X-linked (Ranz et al., 2003). The repeated coevolution of behavior and anatomy within and between species, therefore, may be a consequence of species resolving sexual antagonism via mutations on the X chromosome. In 1978, Turner theorized this very scenario, suggesting that selection causes sex chromosomes to behave like sieves for mutations with sex-limited effects during the evolution of sexual dimorphism.

How do linked loci cause correlated differences within and between species? Early models inspired by the genetics of butterfly mimicry hypothesized that supergenes, multiple genes in tight linkage disequilibrium, were responsible for causing complex patterns of phenotypic evolution (Ford, 1964; Clark and Sheppard, 1972; Charlesworth and Charlesworth, 1975). In swallowtail butterflies (Papilio polytes), for example, both sexually dimorphic and female polymorphic wing pattern mimicry segregate as if controlled by a single locus (Clark and Sheppard, 1972). Surprisingly, genetic mapping localized these effects to numerous single nucleotide polymorphisms (SNPs) at a single gene, doublesex (dsx), rather than multiple tightly linked loci like Clark and Sheppard.
(1972) hypothesized (Kunte et al., 2014). \( dsx \) encodes a zinc-finger containing TF that controls sexual differentiation through alternative splicing in insects (Erdman and Burtis, 1993). An inversion mutation flanking the \( dsx \) locus in \( P. \) polytes reduced recombination at this region relative to the rest of the genome (Kunte et al., 2014). As has recently been shown in ruff (Lamichhaney et al., 2016), sparrows (Tuttle et al., 2016), and zebra finch (Kim et al., 2017), supergenes may exist as a consequence of inversions that facilitate the accumulation of mutations in tight linkage disequilibrium. In \( P. \) polytes, then, the \( dsx \) locus may also behave like a sieve, allowing the buildup of mutations that lead to correlated differences in wing patterning, possibly through complex alternative splicing mechanisms (Kunte et al., 2014).

Together these results suggest that genetic correlations controlling variation in both behavior and anatomy can be built through linkage and pleiotropy. In fact, it is not always clear how to separate both mechanisms, since multiple mutations in tight linkage can modify the function of a single pleiotropic gene, as in the case of \( dsx \). Similarly, the evolution of sexual dimorphism within species and correlated divergence between might often be part of the same problem. That is, genetic mechanisms that separate the expression of phenotypes between males and females can also generate differences between species, as in the case of sex chromosome evolution. But, there are still many unresolved issues: Most of the evidence explaining how genes and genomes evolve within and between species to generate phenotypic differences in behavior and anatomy are correlative. While birds, fish, and butterflies show some of the most elaborate diversity patterns in mating displays and ornamentation, it is extremely difficult to perform casual genetic experiments in these systems. As a consequence, in nearly all cases, the mechanistic relationship between individual gene mutations and their pleiotropic effects on mating success remains unclear. Understanding these mechanisms is a requirement to connect genotype to phenotype and genotype to environment during correlated evolution.
Solving the problem in *Drosophila*

In *Drosophila*, males engage in courtship rituals involving wing, leg, and body movements near females to initiate mating (Hall, 1994). These movements are correlated with rapidly evolving anatomical changes within and between species, including pigmentation patterning on the wings (Kopp and True, 2002), secondary sexual structures on the legs (Kopp, 2011), and lipid profiles on the body (Yew and Chung, 2015). Genetic tools are available to modify each of these phenotypes in live, freely behaving animals (Duffy, 2002). In this thesis, I take advantage of these tools to answers questions about the genetic basis of developmental and evolutionary change in correlated behavior and anatomy. Specifically, I perform a series of genetic experiments to study how correlations between mating behaviors and pigmentation come about. Each chapter illustrates the power of *Drosophila* to learn how genes and genomes function in the context of ecologically important traits.

**Thesis overview**

In chapter two, I investigate how a single mutation in the *yellow* gene influences male mating success in *Drosophila melanogaster*. Using tissue specific RNAi manipulations in combination with GAL4 and GAL80 tools, I show that *yellow* expression specifically in male-specific leg structures called sex combs is required for mating success. Loss of sex comb melanization in *yellow* mutants causes structural changes that reduce a male’s ability to grasp and mount females for copulation. These results highlight, unexpectedly, that anatomical changes themselves can modify animal behavior.

In chapter three, I show that the function of two pigmentation enzymes, Ebony and Tan, have reciprocal effects on cuticular hydrocarbon (CHC) synthesis. Specifically, loss of Ebony activity increases the abundance of long-chain CHCs, and loss of Tan activity increases the abundance of short-chain CHCs. These effects are partially explained by changes in dopamine signaling. Further, natural variation in *ebony* and *tan* expression
covaries with CHC profiles in the direction predicted by the mutants, suggesting that pigmentation and CHCs might evolve in coordination.

In chapter four, I perform QTL mapping between the sibling species *D. elegans* and *D. gunungcola* to study the genetic basis of divergence in wing pigmentation and mating display. Evolution on the X chromosome explains the majority of variation for both traits, due to the effects of co-localized QTLs. Fine-mapping revealed a ~400 kb region containing fifteen genes that behaves like a genetic switch controlling wing spot evolution. Through introgression mapping, I separated the effects of the wing spot locus from the effects of mating display, possibly as a consequence of epistasis. I also discovered new populations of *D. gunungcola* that perform mating displays similar to *D. elegans*, suggesting that behavioral divergence occurred more slowly than anatomical divergence between these species.

Finally, in the appendices, I include a series of supplemental figures accompanying each chapter and a review that synthesizes a database of genes and mutations contributing to pigmentation evolution in *Drosophila*. 
References


dysplasia is expressed in the developing epithelium, neuroectoderm, thymus, and bone. *Journal of Histochemistry & Cytochemistry, 46*(3), 281-289.


Chapter 2

The yellow Gene Influences Drosophila Male Mating Success Through Sex Comb Melanization

Abstract

Drosophila melanogaster males perform a series of courtship behaviors that, when successful, result in copulation with a female. For over a century, mutations in the yellow gene, named for its effects on pigmentation, have been known to reduce male mating success. Prior work has suggested that yellow influences mating behavior through effects on wing extension, song, and/or courtship vigor. Here, we rule out these explanations, as well as effects on the nervous system more generally, and find instead that the effects of yellow on male mating success are mediated by its effects on pigmentation of male-specific leg structures called sex combs. Loss of yellow expression in these modified bristles reduces their melanization, which changes their structure and causes difficulty grasping females prior to copulation. These data illustrate why the mechanical properties of anatomy, and not just neural circuitry, must be considered to fully understand the development and evolution of behavior.

1 This chapter is in review at eLife as: Massey, J. H., Chung, D., Siwanowicz, I., Stern, D. L., Wittkopp, P. J. The yellow gene influences Drosophila male mating success through sex comb melanization
Introduction

“The form of any behavior depends to a degree on the form of the morphology performing it” -Mary Jane West-Eberhard, 2003

Over 100 years ago in Thomas Hunt Morgan’s fly room, Alfred Sturtevant described what is often regarded as the first example of a single gene mutation affecting behavior (Sturtevant, 1915; reviewed in Drapeau et al., 2003; Cobb, 2007; Greenspan 2008): he noted that yellow mutant males, named for their loss of black pigment that gives their body a more yellow appearance (Figure 2-1A), mated successfully with wild-type females much less often than wild-type males. In 1956, in what is often regarded as the first ethological study (reviewed in Cobb, 2007; Greenspan 2008), Margaret Bastock compared courtship of yellow mutant and wild-type males and concluded that despite all courtship actions being present, loss of yellow function likely reduces courtship vigor or drive, leading to copulation inhibition (Bastock 1956). Despite more recent data consistent with this hypothesis (Drapeau et al. 2003), the precise mechanism by which the yellow gene affects male mating success in *D. melanogaster* has remained a mystery. Consequently, Bastock’s statement about yellow from her 1956 paper is equally true today: “It seemed worthwhile therefore to examine more closely one example of a gene mutation affecting behavior and to ask two questions, (1) how does it bring about its effect? [and], (2) what part might it play in evolution?”

The *D. melanogaster* yellow gene encodes a protein hypothesized to act either structurally (Geyer et al., 1986) or enzymatically (Wittkopp et al., 2002) in the synthesis of dopamine melanin, and a Yellow homolog has been shown to bind dopamine and other biogenic amines in the sand fly *Lutzomyia longipalpis* (Xu et al., 2011). The interaction between Yellow and dopamine might explain the protein’s effects on male mating success because dopamine acts as a modulator of male courtship drive in *D. melanogaster* (Zhang et al., 2016). These effects of dopamine are mediated by neurons expressing the gene fruitless (*fru*) (Zhang et al., 2016), which is a master regulator of sexually dimorphic behavior in *D. melanogaster* that can affect every component of courtship and copulation (reviewed in Villella and Hall, 2008). *fru* has also been shown
to regulate expression of yellow in the central nervous system (CNS) of male D. melanogaster larvae (Drapeau et al., 2003). These observations suggest that the pleiotropic effects of yellow on male mating success might result from effects of yellow in the adult CNS, particularly in fru-expressing neurons. Consistent with this hypothesis, functional links between the pigment synthesis pathway and behavior mediated by the nervous system have previously been reported for other pigmentation genes (Hotta and Benzer, 1969; Heisenberg, 1971; Borycz et al., 2002; Richardt et al., 2002; True et al., 2005; Suh and Jackson, 2007).

Results and Discussion

fruitless-expressing cells do not mediate the effect of yellow on male mating success

D. melanogaster males perform multiple behaviors, including tapping, chasing, singing, and genital licking, before attempting to copulate with females by curling their abdomen and grasping the female (Figure 2-1B, Movie 1). In one-hour trials, we found that virgin males homozygous for a null allele of the yellow gene (y1) successfully mated with wild-type virgin females only 3% of the time, whereas wild-type males mated with wild-type virgin females 93% of the time (Figure 2-1C). Videos of mating trials indicated that the difference in mating success between wild-type and yellow males did not come from differences in courtship activity (Figure 2-1D-H) (compare Movies 1 and 2), but rather from differences in the ability of yellow and wild-type males to initiate copulation (compare Movies 3 and 4).

To determine whether yellow activity in fru-expressing cells is responsible for this difference in mating success, we used the UAS-GAL4 system (Brand and Perrimon, 1993) to drive expression of yellow-RNAi (Dietzl et al., 2007) with fruGAL4 (Stockinger et al., 2005), knocking down native yellow expression in these cells. We also used fruGAL4 to drive yellow expression in y1 mutants. In both cases, we found no significant effect on male mating success (Figure 2-2A,B), showing that expression of yellow in fru-expressing cells is neither necessary nor sufficient for yellow’s effect on male mating success.
Doublesex-expressing cells require yellow for normal male mating success

To continue searching for cells responsible for yellow’s effects on mating, we examined a 209 bp sequence 5’ of the yellow gene called the “mating-success regulatory sequence” (MRS) because deletion mapping indicated it was required for male mating success (Drapeau et al. 2006). We hypothesized that the MRS might contain an enhancer driving yellow expression and found that ChIP-seq data indicates the Doublesex (Dsx) transcription factor binds to this region in vivo (Clough et al., 2014). Like fru, dsx expression is required to specify sex-specific behaviors in D. melanogaster (Rideout et al., 2010; Robinett et al., 2010; reviewed in Villella and Hall, 2008; Yamamoto and Koganezawa, 2013), suggesting that yellow expression regulated by Dsx through the MRS enhancer might be responsible for its effects on male mating behavior. We found that reducing yellow expression in dsx-expressing cells with either of two different dsx\textsuperscript{GAL4} drivers (Robinett et al., 2010; Rideout et al., 2010) strongly reduced male mating success (Figure 2-2C, Supplementary Figure S2-1A), whereas restoring yellow activity in cells expressing dsx\textsuperscript{GAL4} in y\textsuperscript{l} mutants significantly increased male mating success compared with y\textsuperscript{l} controls (Figure 2-2D, Supplementary Figure S2-1B). Video recordings of male flies with reduced yellow expression in dsx-expressing cells showed the same mating defect observed in y\textsuperscript{l} mutants: males seem to perform all courtship actions normally, but repeatedly failed to copulate (Movie 5). We therefore conclude that yellow expression is required in dsx-expressing cells for normal male mating behavior.

To determine whether the MRS sequence might be the enhancer mediating yellow expression in dsx-expressing cells that affect male mating success, we manipulated yellow expression with GAL4 driven by a 2.7kb DNA region located 5’ of yellow that includes the wing, body, and putative MRS enhancers (Gilbert et al., 2006, Supplementary Figure S2-2A). Altering yellow expression with this GAL4 driver modified pigmentation as expected but did not affect male mating success (Supplemental Figure S2-2B-D), possibly because this GAL4 line did not show any detectable expression in the adult CNS (Supplementary Figure S2-2E). To test more directly whether the MRS was necessary for male mating success, we deleted 152 bp of the 209 bp MRS sequence using CRISPR/Cas9 gene editing (Bassett et al., 2013) (Supplemental
Figure S2-2F,G). We found that this deletion had no significant effect on male mating success (Supplemental Figure S2-2H), contradicting the previous deletion mapping data (Drapeau et al., 2006). We conclude therefore that yellow expression in dsx-expressing cells affecting mating behavior must be mediated by other cis-regulatory sequences associated with the yellow gene.

dsx-expressing cells outside the CNS require yellow for normal male mating success

Although dsx is expressed broadly throughout the fly (Robinett et al., 2010; Rideout et al., 2010), we hypothesized that its expression in the nervous system would be responsible for yellow’s effects on mating because yellow has been reported to be expressed in the adult brain (Hinaux et al., 2018) and behavioral effects of other pigmentation genes are mediated by neurons (Hotta and Benzer, 1969; Heisenberg, 1971; Borycz et al., 2002; True et al., 2005). However, we found that suppressing yellow expression in the larval CNS, dopaminergic neurons, or serotonergic neurons (Supplementary Figure S2-3), or in all neurons (Figure 2-2E) or all glia (Figure 2-2F), had no significant effect on male mating success. Specifically reducing yellow expression in either all dsx-expressing neurons (Figure 2-2G) or all dsx-expressing glutamatergic neurons that are required for genital coupling (Pavlou et al., 2016) (Figure 2-2H) also had no significant effect on male mating success. In addition, when we examined yellow expression in adult brains, we were only able to observe non-specific signal at the anterior of the adult brain in females (Figure 2-2J,K). Given this lack of evidence that yellow is required in neuronal cells for normal male mating behavior, we limited dsx\textsuperscript{GAL4} activation of yellow expression in y\textsuperscript{l} mutants to non-neuronal cells and found that these flies exhibited a substantial increase in male mating success compared with y\textsuperscript{l} mutant males (Figure 2-2I), showing that yellow expression in non-neuronal dsx-expressing cells is required for normal male mating behavior.

To identify which non-neuronal dsx-expressing cells require yellow expression for normal male mating success, we screened ten dsx-enhancer GAL4 lines that each contains a different ~3 kb region of dsx noncoding sequence (Figure 2-2L; Pfeiffer et al., 2008). Two of these lines, 42D04-GAL4 and 40F03-GAL4, significantly decreased male mating
success when driving yellow-RNAi (Figure 2-2M). These two GAL4 drivers contain overlapping sequences from intron 2 of dsx (Figure 2-2L), suggesting that their similar effects result from reduction of yellow expression in the same cells. Line 42D04-GAL4 had stronger effects than 40F03-GAL4 (Figure 2-2N), so we performed all further analyses with this line. Males with yellow reduced by 42D04-GAL4 performed courtship behavior in a pattern similar to y¹ mutant males: males performed all precopulatory courtship behaviors normally, but repeatedly failed to copulate, even after hours of attempts (Movie 6). These data indicate that some or all cells in which 42D04-GAL4 drives expression require yellow expression for normal male mating behavior.

Sex combs require yellow expression for normal male mating success

42D04-GAL4 drives expression in a sexually dimorphic pattern in multiple neurons of the adult male (Figure 2-3A,B) and female CNS (Supplemental Figure S2-4A,B), consistent with previously described dsxGAL4 expression in the posterior cluster, the abdominal cluster, and, in males, in the prothoracic TN1 neurons (Robinett et al., 2010). 42D04-GAL4 also drives expression in male and female larval CNS and genital discs, with expression in the genital tissues persisting into the adult stage only in females (Supplemental Figure S2-4C-G). Finally, we observed 42D04-GAL4 expression at the base of the sex combs (also observed by Robinett et al. 2010), which are modified bristles used during mating (Cook, 1975; Ng and Kopp 2008; Hurtado-Gonzales et al., 2015) that are present only on the first tarsal segment of adult male forelegs (Figure 2-3C-F). Yellow protein is expressed in sex combs (Hinaux et al., 2018, Figure 2-3G,H), where it is presumably required for synthesis of black dopamine melanin in the sex comb “teeth”. This expression of yellow in sex comb cells is driven by enhancer sequences in the yellow intron (Supplementary Figure S2-5), potentially explaining why manipulating yellow expression using GAL4 driven by sequences 5’ of the yellow gene failed to affect mating. Driving expression of yellow-RNAi with 42D04-GAL4 eliminated expression of an mCherry tagged version of the native Yellow protein in sex combs and strongly reduced black melanin in the sex combs (Figure 2-3I-L) but not the abdomen (Supplemental Figure S2-4J).
To test the impact of yellow expression in sex combs on male mating behavior, we used 42D04-GAL4 to drive yellow-RNAi, but inhibited the function of 42D04-GAL4 in the CNS with nysb-GAL80 (courtesy of Julie Simpson). These flies showed no GAL4 activity in the CNS (Figure 2-3M,N), but lost black melanin in the sex combs (Figure 2-3O) and had significantly reduced male mating success (Figure 2-3P). High-speed videos (1000 frames per second) revealed that yellow mutant (y1) males fail repeatedly to grasp the female abdomen with their sex combs when attempting to mount and copulate (Movie 7), whereas wild-type males more readily grasp the female with their melanized sex combs and initiate copulation efficiently (Movie 8). These observations suggest that yellow expression in sex combs affects their melanization, which in turn affects their function.

Sex comb melanization is required for efficient grasping, mounting and copulation

To test whether sex comb melanization (as opposed to some other unknown effect of losing yellow expression in sex combs) is critical for male sexual behavior, we suppressed expression of Laccase2 (Arakane et al., 2005; Riedel et al., 2011) in sex combs using 42D04-GAL4 and Laccase2-RNAi (Dietzl et al., 2007). Laccase2 is required to oxidize dopamine into dopamine quinones and thus acts upstream of Yellow in the melanin synthesis pathway (Figure 2-4A; Riedel et al., 2011). Males with Laccase2 suppressed in sex combs lacked both black and brown dopamine melanin, making these sex combs appear translucent (Figure 2-4B). These males displayed strongly reduced mating success compared with wild-type males (Figure 2-4C) and behavioral defects similar to those observed for y1 mutants (Movies 9,10), including inefficient grasping of the female for mounting and copulation. We noticed, however, that flies with Laccase2-RNAi driven by 42D04-GAL4 also showed a loss of melanin in the aedeagus (Supplementary Figure S2-6A), which is the main part of the male genitalia used for copulation, despite no visible expression of 42D04-GAL4 in the adult male genitalia (Supplementary Figure S2-4G) nor changes in aedeagus pigmentation in y1 mutants (Supplementary Figure 2-6A). We therefore used subsets of the 42D04 enhancer (Supplementary Figure S2-6B) to drive expression of Laccase2-RNAi, separating the effects of expression in the sex combs from expression in the genitalia (Supplementary
Male mating success was reduced when \textit{Laccase2} suppression reduced melanization in the sex combs, but not the genitalia (Supplementary Figure S2-6D-G).

How can sex comb melanization affect sex comb function? In insects, melanization impacts not only the color of the adult cuticle but also its mechanical stiffness (Xu et al., 1997; Kerwin et al., 1999; Vincent and Wegst, 2004; Andersen, 2005; Arakane et al., 2005; Suderman et al., 2006; Riedel et al., 2011; Noh et al., 2016). For example, expressing \textit{Laccase2-RNAi} in \textit{D. melanogaster} wings softens the cuticle to such a degree that the wings collapse (Riedel et al., 2011). Butterflies lacking dopamine melanin due to loss of \textit{yellow} or another gene required for melanin synthesis, \textit{Dopa decarboxylase}, show changes in the fine structure of their wing scales (Matsuoka and Monteiro, 2018), and we also observed structural changes in \textit{D. melanogaster} sex comb teeth lacking \textit{yellow} or \textit{Laccase2} expression using scanning electron microscopy (SEM), with a crack appearing in one of the \textit{Laccase2-RNAi} comb teeth (Figure 2-4D). We conclude that these structural changes in sex combs are responsible for inhibiting the \textit{yellow} mutant male’s ability to grasp a female for mounting and copulation (Movie 10). Interestingly, Wilson et al. (1976) also proposed “that there may be a structural basis for the behavioural effects of the [yellow] mutant” based on their observations of behavior in \textit{yellow} mutant males.

Data from other Drosophila species are also consistent with this structural hypothesis. Specifically, \textit{yellow} mutants in \textit{D. subobscura}, \textit{D. pseudoobscura}, and \textit{D. gaucha}, all of which have sex combs, show reduced male mating success (Rendel, 1944; Tan, 1946; Frias and Lamborot, 1970; Pruzan-Hotchkiss et al., 1992) whereas \textit{yellow} mutants in \textit{Drosophila willistoni}, a species that lacks sex combs (Kopp, 2011; Atallah et al., 2014), do not (Da Silva et al., 2005). Sex comb morphology is highly diverse among species that have sex combs (Kopp, 2011), but these structures generally seem to be melanized (Supplementary Figure S2-7; Tanaka et al., 2009) and used to grasp females (Movies 11-15). Our high-speed video recordings of mating in \textit{D. anannasae}, \textit{D. bipectinata}, \textit{D. kikkawai}, \textit{D. malerkotiana}, and \textit{D. takahashi} show that differences in sex comb morphology (Supplementary Figure S2-7) correspond with differences in how (where on
the female and with which part of the male leg) the male grasps the female prior to copulation (Movies 11-15).

It remains unclear how *D. willistoni* males (and males of other species without sex combs) are able to efficiently grasp females prior to copulation (Movie 16). Differences in females might be part of the answer, however, as *D. melanogaster* y1 mutant males are able to mate with y1 mutant females at rates similar to wild-type males (Bastock 1956, Dow 1976, Heisler 1984, Liu et al., 2019; Supplementary Figure S2-8A). That said, removing all melanin from *D. melanogaster* sex combs by knocking down Laccase-2 reduced mating efficiency with y1 females, suggesting that the brown melanin remaining in y1 sex-combs (Figure 2-4B) played a role in the mating success of y1 males with y1 females (Supplementary Figure S2-8B).

Taken together, our data show that melanization of a secondary sexual structure affects mating in *D. melanogaster*. Specifically, we find that the reduced mating success of *D. melanogaster* yellow mutant males, which was perceived as a behavioral defect for decades, is caused by changes in the morphology of the structures used during mating. These observations underscore that behavior cannot be understood by studying the nervous system alone; anatomy and behavior function and evolve as an interconnected system.

**Materials and Methods**

**Fly stocks and maintenance**

The following lines were used for this work: y1 [which was backcrossed into a wild-type (*Canton-S*) line for 6 generations before starting our experiments; the y1 allele contains an A to C transversion in the ATG initiation and is considered a null allele (Geyer *et al.*, 1990)]; *Canton-S* as wild-type (courtesy of Scott Pletcher); *UAS-yellow-RNAi* obtained from the Vienna Drosophila Resource Centre (VDRC) (Dietzl *et al.*, 2007, KK106068); y1; UAS-y (BDSC 3043); *elav-GAL4* (BDSC 49226); *nsyb-GAL4* (BDSC 39171); *repo-GAL4* (BDSC 7415); *dsx* 

\( ^{GAL4} \) (Robinett *et al.*, 2010) (courtesy of Bruce Baker); *dsx* 

\( ^{GAL4} \)
(Rideout et al., 2010) (courtesy of Stephen Goodwin); fru\textsuperscript{GAL4} (Stockinger et al., 2005) (courtesy of Barry Dickson); the following Janelia enhancer trap GAL4 lines (Pfeiffer et al., 2008): 40A05-GAL4 (BDSC 48138), 41D01-GAL4 (BDSC 50123), 42D02-GAL4 (BDSC 41250), 41F06-GAL4 (BDSC 47584), 41A01-GAL4 (BDSC 39425), 42D04-GAL4 (BDSC 47588), 40F03-GAL4 (BDSC 47355), 39E06-GAL4 (BDSC 50051), 42C06-GAL4 (BDSC 50150), 40F04 (BDSC 50094); y\textsuperscript{mCherry} (courtesy of Nicolas Gompel); nsyb-GAL80 (courtesy of Julie Simpson); UAS-Laccase2-RNAi obtained from the VDRC (Dietzl et al., 2007, KK101687); dsx\textsuperscript{GAL4-DBD} (Pavlou et al., 2016) (courtesy of Stephen Goodwin); BDSC 6993; BDSC 49365; BDSC 6927; BDSC 45175; BDSC 3740; BDSC 5820; BDSC 8848 (courtesy of Shinya Yamamoto); BDSC 7010 (courtesy of Shinya Yamamoto); TPH-GAL4 (courtesy of Shinya Yamamoto); wing-body-GAL4 (BDSC 44373); D. melanogaster yellow 5' up EGFP reporter (Kalay and Wittkopp, 2010) (courtesy of Gizem Kalay); D. melanogaster yellow intron EGFP reporter (Kalay and Wittkopp, 2010) (courtesy of Gizem Kalay); vGlut\textsuperscript{vP16-AD} (Gao et al., 2008) (courtesy of Stephen Goodwin); BDSC 6993; BDSC 49365; BDSC 6927; BDSC 45175; BDSC 3740; BDSC 5820; BDSC 8848 (courtesy of Shinya Yamamoto); BDSC 7010 (courtesy of Shinya Yamamoto); TPH-GAL4 (courtesy of Shinya Yamamoto); wing-body-GAL4 (BDSC 44373); D. melanogaster yellow 5' up EGFP reporter (Kalay and Wittkopp, 2010) (courtesy of Gizem Kalay); D. melanogaster yellow intron EGFP reporter (Kalay and Wittkopp, 2010) (courtesy of Gizem Kalay); vasa-Cas9 (BDSC 51324); UAS-cytGFP (courtesy of Janelia Fly Core); pJFRC12-10XUAS-IVS-myrc::GFP (courtesy of Janelia Fly Core). All flies were grown at 23°C with a 12 h light-dark cycle with lights on at 8AM and off at 8PM on standard corn-meal fly medium.

**Behavior**

**Mating assays**

Virgin males and females were separated upon eclosion and aged for 4-7 d before each experiment. Experiments were carried out at 23°C on a 12 h light dark cycle with lights on at 8 AM and off at 8 PM on standard corn-meal fly medium. Males were isolated in glass vials, and females were group housed in standard plastic fly vials at densities of 20-30 flies. All mating assays were performed at 23°C between 8-11AM or 6-9PM. For each assay replicate, a single virgin male and female fly were gently aspirated into a 35 mm diameter Petri dish (Genesee Scientific, catalog #32-103) placed on top of a 17 inch LED light pad (HUION L4S) and immediately monitored for 60 min for courtship and copulation activity. All genotypes tested initiated courtship (including tapping, chasing,
wing extension, genital licking, and attempted copulation) towards the female. Any genotype that copulated within the 60 min window was noted. Except for the experiment described in Figure 2-8, all female targets in mating assays were wild-type (Canton-S). Percent mated in 60 min was then calculated as the number of replicates that mated divided by the total number of replicates and multiplied by 100.

Courtship analysis

For courtship analysis, 60 min videos were recorded using Canon VIXIA HF R500 camcorders mounted to Manfrotto (MKCOMPACTACN-BK) aluminum tripods. To calculate courtship indices in Figure 2-1 between wild-type and y1 males, the amount of time males spent engaged in courtship: tapping, chasing, wing extension, genital licking, or attempted copulation was quantified for the first 10 min of the assay and divided by the total 10 min period. We chose to quantify courtship activity within the first 10 min of the assay, because wild-type (Canton-S) males will often begin copulating after this window, while y1 males will continue to court throughout the entire 60 min period. Wing extension bouts were quantified by noting every unilateral wing extension bout for each genotype within the first 10 min of the assay.

Song analysis

Courtship song was recorded as described previously (Arthur et al., 2013). All genotypes were recorded simultaneously. Song data was segmented (Arthur et al., 2013) and analyzed (http://www.github.com/dstern/BatchSongAnalysis) without human intervention. P-values for one-way ANOVAs were estimated with 10,000 permutations (http://www.mathworks.com/matlabcentral/fileexchange/44307-randanova1).

High-speed video capture

For high-speed video capture of attempted mounting and copulation events, virgin males and females were isolated upon eclosion and aged for 4-7 d before each assay. Using a Fascam Photron SA4 (courtesy of Gwyneth Card) mounted with a 105 mm AF Micro Nikkor Nikon lens (courtesy of Gwyneth Card), we recorded individual pairs of males and females that were gently aspirated into a single well of a 96 well cell culture plate
(Corning 05-539-200) partially filled with 2% agarose and covered with a glass coverslip. We recorded mounting and copulation attempts at 1000 frames per second (fps) and played back at 30 fps. Most wild-type males attempted mounting 3-5 times before copulating, whereas y1, yellow-RNAi, and Laccasse2-RNAi males repeatedly attempted mounting without engaging in copulation, mirroring the videos we captured on the Canon VIXIA HF R500 at 30 fps.

**Imaging sex combs and genitalia**

Sex comb images highlighting different melanization states (Figure 2-3I, J, O; Figure 2-4B) were taken using a Zeiss Axio Cam ERc 5s mounted on a Zeiss Axio Observer A1 Inverted Microscope. Front legs were cut and placed sex comb side down on a microscope slide (Fisher brand 12-550-123) and imaged through a 40x objective. Images were processed using AxioVision LE software. Abdomens and genitalia images highlighting different melanization states of the aedeagus and female genital bristles were captured using a Canon EOS Rebel T6 camera mounted with a Canon MP-E 65 mm macro lens. Genitalia images were processed in Adobe Photoshop (version 19.1.5) (Adobe Systems Inc., San Jose, CA).

Focus Ion Beam Scanning Electron Microscope (FIB-SEM) images (Figure 2-4D) were taken by placing individual, dissected legs on carbon tape adhered to a SEM pin stud mount with sex combs facing up. The samples were then coated with a 20-nm Au layer using a Gatan 682 Precision Etching and Coating System, and imaged by SEM in a Zeiss Sigma system. The samples were imaged using a 3-nA electron beam with 1.5 kV landing energy at 2.5MHz.

**Immunohistochemistry and confocal imaging**

**Central Nervous System**

Dissections, immunohistochemistry, and imaging of fly central nervous systems were done as previously described (Aso et al., 2014). In brief, brains and VNCs were dissected.
in Schneider’s insect medium and fixed in 2% paraformaldehyde (diluted in the same medium) at room temperature for 55 min. Tissues were washed in PBT (0.5% Triton X-100 in phosphate buffered saline) and blocked using 5% normal goat serum before incubation with antibodies. Tissues expressing GFP were stained with rabbit anti-GFP (ThermoFisher Scientific A-11122, 1:1000) and mouse anti-BRP hybridoma supernatant (nc82, Developmental Studies Hybridoma Bank, Univ. Iowa, 1:30), followed by Alexa Fluor® 488-conjugated goat anti-rabbit and Alexa Fluor® 568-conjugated goat anti-mouse antibodies (ThermoFisher Scientific A-11034 and A-11031), respectively. Tissues expressing mCherry-tagged Yellow protein (ymCherry) were stained with rabbit anti-dsRed (Clontech 632496, 1:1000) and rat anti-DN-Cadherin (DN-Ex #8, Developmental Studies Hybridoma Bank, Univ. Iowa, 1:100) as neuropil marker, followed by CyTM3-conjugated goat anti-rabbit and CyTM5-conjugated goat anti-rat antibodies (Jackson ImmunoResearch 111-165-144 and 112-175-167), respectively. After staining and post-fixation in 4% paraformaldehyde, tissues were mounted on poly-L-lysine-coated cover slips, cleared, and embedded in DPX as described. Image z-stacks were collected at 1 µm intervals using an LSM710 confocal microscope (Zeiss, Germany) fitted with a Plan-Apochromat 20x/0.8 M27 objective. Images were processed in Fiji (http://fiji.sc/) and Adobe Photoshop (version 19.1.5) (Adobe Systems Inc., San Jose, CA).

**Sex combs and genitalia**
Adult flies were 2-7 d old and pupae were 96 h old after pupal formation (APF) for the EGFP reporter experiment summarized in Supplementary Figure S2-5. Flies were anesthetized on ice, submerged in 70% ethanol, rinsed twice in phosphate buffered saline with 0.1 % Triton X-100 (PBS-T), and fixed in 2% formaldehyde in PBS-T. Forelegs and genitalia/abdomen tips were removed with fine scissors and mounted in Tris-buffered (pH 8.0) 80% glycerol. Serial optical sections were obtained at 1.5 µm or 0.5 µm intervals on a Zeiss 880 confocal microscope with a LD-LCI 25x/0.8 NA objective (genitalia) or a Plan-Apochromat 40x/1.3 NA objective (appendages/tarsal sex combs). The native fluorescence of GFP, mCherry and autofluorescence of cuticle were imaged using 488, 594 and 633 lasers, respectively. Images were processed in Fiji (http://fiji.sc/),

Statistics

Statistical tests were performed in R for Mac version 3.3.3 (R Core Team 2018) using Fisher’s exact tests to test for statistically significant effects of 2 x 2 contingency tables, Chi-square tests to test for statistically significant effects of contingency tables greater than 2 x 2 with Bonferroni corrections for multiple comparisons, and two-tailed Student’s t-tests to test for statistically significant effects of pairwise comparisons of continuous data with normally distributed error terms. For song analysis, one-way ANOVAs were performed in MATLAB version R2017a (The MathWorks, Inc.).

Generation of the mating regulatory sequence (MRS) deletion line

Using the 209 bp region mapped in Drapeau et al. (2006) between -300 and -91 bp upstream of yellow’s transcription start site, we designed two single guide RNA (gRNA) target sites at -291 bp and -140 bp that maximized the MRS deletion region, given constraints of identifying NGG PAM sites required for CRISPR/Cas9 gene editing (Supplementary Figure S2-1A,B). We in-vitro transcribed these gRNAs using a MEGAscript T7 Transcription Kit (Invitrogen) following the PCR-based protocol from Bassett et al. (2013). Two 1 kb homology arms were PCR amplified from the yellow locus immediately upstream and downstream of the gRNA target sites using the forward and reverse primers with NcoI and BglII tails, respectively, for the Left Arm (5’- TTACCATGGGGGATCAAGTTGAACCAC-3’, 5’- GGAGATCTGGCCTTCATCGACATTTA-3’) and the forward and reverse primers with Bsu36I and MluI tails, respectively, for the Right Arm (5’- TACATCCCTAAGGCGCTTGATTACCCGAAACT-3’, 5’- TATACGCGTGCTGCCATGCTATGGCTTTC-3’) and cloned into pH-DsRed-attp (Gratz et al., 2014; Addgene Plasmid # 51019) in two steps, digesting first with NcoI and BglII (Left Arm) to transform the Left Arm and second with Bsu36I and MluI (Right Arm) to
transform the Right Arm, flanking the 3xP3::DsRed, attP, and LoxP sites. Homology arms were ligated into pHD-DsRed-attP using T4 DNA Ligase (ThermoFisher Scientific), and products were transformed into One Shot TOP10 (Invitrogen) DH5 alpha competent cells. Purified donor plasmid was then co-injected at 500 ng/uL with the two gRNAs at 100 ng/uL total concentration into a *vasa-Cas9* (BDSC 51324) line. Flies were then screened for DsRed expression in the eyes, and Sanger sequenced verified for a 3xP3::DsRed replacement of the MRS region (Supplementary Figure S2-2F). We confirmed that we deleted 152 bp of the 209 bp region based on Sanger sequencing the CRISPR/Cas9 cut sites (Supplementary Figure S2-2F). Next, we crossed y<sup>AMRS+3xP3::DsRed</sup> with a Cre-expressing fly line (courtesy of Bing Ye, University of Michigan) to excise 3xP3::DsRed and screened for flies that lost DsRed expression in the eyes. Finally, we PCR-gel verified that DsRed was indeed removed in creation of the y<sup>AMRS</sup> line using the forward and reverse primers, respectively (5’-
CAGTCGCCGATAAGATGAACACTG-3’, 5’-
CAAGGTGATCAGGGTCACAAGGATC-3’) (Supplementary Figure S2-2G).

**Generation of the 42D04-GAL4 enhancer sub-fragment pBPGUw lines**

Enhancer sub-fragments (2 kb, 2 kb, 1.3 kb, 1.3 kb, and 1.3 kb for 42D04_A,B,C,D,E-GAL4, respectively) were synthesized as IDT gene blocks (sequences copied below) based off of the 42D04 *D. melanogaster dsx* enhancer sequence (FBsf0000164494) (Supplementary Figure S2-7). The gene blocks were designed with 5’ and 3’ Gibson tails to facilitate Gibson assembly (Gibson *et al.*, 2009) into the GAL4 plasmid pBPGUw (Pfeiffer *et al.*, 2008; Addgene Plasmid #17575) after digestion with FseI and AatII. Products were transformed into Mix and Go! DH5 alpha competent cells (Zymo). Clones were selected by ampicillin resistance on Amp-LB plates (60mg/mL). Purified plasmids were injected at 500 ng/uL into the phiC31 integrase-expressing 86Fb landing site line *BDSC 24749* (courtesy of Rainbow Transgenics) for phiC31 attP-attB integration and screened for using a mini-white marker.
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References


Figure 2-1 The *Drosophila melanogaster* *yellow* gene is required for male mating success

(A) Photographs comparing wild-type and yellow (y1) body pigmentation (Nicolas Gompel). (B) Snapshots from videos illustrating *D. melanogaster* courtship behaviors. (C) y1 males (yellow) showed significantly lower mating success levels compared to wild-type males (black) in non-competitive, one-hour trials. Sample sizes are shown at the top of each barplot. (D-H) y1 males showed similar levels of courtship activity and song compared to wild-type males. (D) Courtship index: the proportion of time a male engages in courtship activity divided by the total observation period. (E) Wing extension bouts: the number of unilateral wing extensions during the observation period. (F) Pulses per minute. (G) Sine per minute. (H) Inter pulse interval. (D-H) Show individual points that represent single fly replicates. Circles represent means and lines SD. Significance was measured using Fisher's exact test in (C), Student's t-tests (two-tailed) in (D-E), and one-way ANOVA in (F-H). ****P<0.0001. n.s., not significant.
Figure 2-2 yellow expression in non-neuronal doublesex-expressing cells, but not fruitless-expressing cells, is necessary and sufficient for male mating success

(A,B) Neither expressing yellow-RNAi nor yellow-cDNA in fruit-expressing cells using fruGAL4 (Stockinger et al., 2005) affected male copulation. (C) Expressing yellow-RNAi in dsx-expressing cells using dxsGAL4 (Robinett et al., 2010) significantly inhibited male mating success. (D) Expressing yellow in dsx-expressing cells using dxsGAL4 in a y1 mutant background was sufficient to restore male mating success. (E,F) Expressing yellow-RNAi using pan-neuronal (elav-GAL4 and nvyb-GAL4) and pan-glia (repo-GAL4) drivers did not affect male mating success. (G) Restricting yellow-RNAi expression to dxs-expressing neurons using the split-GAL4 technique, combining dxsGAL4-DBD (Pavlou et al., 2016) with elavVP16-AD (Luan et al., 2006), did not affect male mating success. (H) Restricting yellow-RNAi expression to dxs-expressing glutamatergic neurons using the split-GAL4 technique, combining dxsGAL4-DBD (Pavlou et al., 2016) with vGluTVP16-AD (Gao et al., 2008) did not affect male mating success. (I) Expressing yellow in dxs-expressing cells restricted outside the CNS using dxsGAL4 and nvyb-GAL80 (courtesy of Julie Simpson) in a y1 mutant background significantly increased male mating success. (J,K) Brain and ventral nerve cord of adult male and female y1mCherry flies stained with anti-N-Cadherin (N-cad) antibody labeling neuropil (white) and anti-DsRed antibody labeling Yellow::mCherry (red). We observed sparse, inconsistent signal outside the CNS at the top of the brain in males (white arrow), and especially females (white arrow), but we were unable to confirm a previous report that y1mCherry is expressed in the adult brain (Hinaux et al., 2018). (L) Diagram of the male exon structure of the dsx locus highlighting 10 genomic fragments between 1.7 and 4 kb used to clone Janelia enhancer trap GAL4 drivers (Pfeiffer et al., 2008). Black boxes indicate coding exons. White boxes indicate 5' and 3' UTRs, and the arrow in exon 2 denotes the transcription start site. (M) Expressing yellow-RNAi using each Janelia dsx-GAL4 driver identified 42D04-GAL4 and 40F03-GAL4 as affecting male mating success when compared with the yellow-RNAi control. (N) A replicate experiment comparing 42D04-GAL4 and 40F03-GAL4 effects on male mating success with both GAL4 and UAS parental controls confirmed the significant effect of 42D04-GAL4 but not 40F03-GAL4. We attribute differences in the 40F03-GAL4 effect between (M) and (N) to between experiment variability in the levels of male mating success; each common genotype tested in (M), for example, mated at higher levels in (N), but 42D04-GAL4 consistently showed a significant effect relative to controls. Sample sizes are shown at the top of each barplot. Significance was measured using Chi-square tests with Bonferroni corrections for multiple comparisons. *P<0.05, ***P<0.001. n.s., not significant.
Figure 2-3: Yellow expression in non-neuronal 42D04-GAL4 expressing cells is necessary for sex comb melanization and male mating success.

(A, B) Brain and ventral nerve cord of adult male fly stained with anti-GFP (green) antibody for myrGFP expressed using 42D04-GAL4 and counterstained with anti-nC82 (magenta) for neuropil. (C) Wild-type (wt) D. melanogaster adult male fly highlighting the location of sex combs (Nicolas Gompel). (D) Close up of a wild-type (wt) sex comb on the first tarsal segment (ts1) of the front leg (courtesy of Nicolas Gompel). (E) Bright field illumination of a male front leg expressing cytGFP (green) in sex-comb cells using 42D04-GAL4. (F) Confocal image of the sex comb cells expressing cytGFP (green) with 42D04-GAL4 and leg cuticle autofluorescence (blue). (G) Confocal image of a ymCherry male leg highlighting native ymCherry sex comb expression (red). (H) Zoomed in confocal image shown in (G) with leg cuticle autofluorescence (blue) and native ymCherry sex comb expression (red). (I) Wild-type (wt) sex comb. (J) Loss of black melanin in sex combs in males expressing yellow-RNAi using 42D04-GAL4. (K) Co-localization of ymCherry (red) at the base of the sex comb cells expressing cytGFP (green) with 42D04-GAL4. (L) Loss of ymCherry (red) at the base of the sex comb cells expressing cytGFP (green) and yellow-RNAi using 42D04-GAL4. (M, N) Brain and ventral nerve cord of adult male expressing nsyb-GAL80 to block GAL4 activity in the CNS, stained with anti-GFP (green) antibody for myrGFP expressed using 42D04-GAL4, and counterstained with anti-nC82 (magenta) for neuropil. (O) Loss of black melanin in sex combs in nsyb-GAL80 males expressing yellow-RNAi using 42D04-GAL4. (P) Expressing yellow-RNAi using 42D04-GAL4 in males expressing nsyb-GAL80 significantly inhibited male mating success. Scale bars in (I), (J), and (O) measure 12.5 µm. Sample sizes are shown at the top of each barplot. Significance was measured using Chi-square tests with Bonferroni corrections for multiple comparisons. *P<0.05, ***P<0.001.
Figure 2-4 Sex comb melanization is specifically required for male mating success

(A) Simplified version of the insect melanin synthesis pathway. (B) Light microscopy images of sex combs from wild-type (wt), y1, and 42D04-GAL4; UAS-Laccase2-RNAi males. Expressing Laccase2-RNAi in sex combs completely blocked melanin synthesis. (C) Expressing Laccase2-RNAi using 42D04-GAL4 in males significantly inhibited male mating success. (D) Scanning Electron Microscopy (SEM) of sex combs from wild-type (wt), y1, and Laccase2-RNAi males (expressed using 42D04-GAL4). Compared to wild-type, sex comb teeth in y1 mutants appeared thinner and smoother, whereas Laccase2-RNAi sex comb teeth appeared even smoother than y1 mutants, and one comb tooth had a visible crack in the cuticle (white rectangle, enlarged on the right). Scale bars in (B) measure 12.5 µm. Sample sizes are shown at the top of each barplot. Significance in was measured using Chi-square tests with Bonferroni corrections for multiple comparisons. ****P<0.0001.
Abstract

Pleiotropic genes are genes that affect more than one trait. For example, many genes required for pigmentation in the fruit fly *Drosophila melanogaster* also affect traits such as circadian rhythms, vision, and mating behavior. Here, we present evidence that two pigmentation genes, *ebony* and *tan*, which encode enzymes catalyzing reciprocal reactions in the melanin biosynthesis pathway, also affect cuticular hydrocarbon (CHC) composition in *D. melanogaster* females. More specifically, we report that *ebony* loss-of-function mutants have a CHC profile that is biased toward long (>25C) chain CHCs, whereas *tan* loss-of-function mutants have a CHC profile that is biased toward short (<25C) chain CHCs. Moreover, pharmacological inhibition of dopamine synthesis, a key step in the melanin synthesis pathway, reversed the changes in CHC composition seen in *ebony* mutants, making the CHC profiles similar to those seen in *tan* mutants. These observations suggest that genetic variation affecting *ebony* and/or *tan* activity might cause correlated changes in pigmentation and CHC composition in natural populations. We tested this possibility using the *Drosophila* Genetic Reference Panel (DGRP) and found that CHC composition covaried with pigmentation as well as levels of *ebony* and

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tan expression in newly eclosed adults in a manner consistent with the ebony and tan mutant phenotypes. These data suggest that the pleiotropic effects of ebony and tan might contribute to covariation of pigmentation and CHC profiles in Drosophila.

**Introduction**

When organisms adapt to novel environments, genetic changes often cause multiple traits to evolve. In some cases, organisms invading similar environments undergo similar shifts for suites of traits. In the threespine stickleback, for example, marine populations independently invading freshwater lake habitats have repeatedly evolved similar changes in defensive armor, behavior, and body shape (Walker and Bell, 2000; Schluter *et al*., 2004; Wark *et al*. 2011). Such correlated evolution might result from (i) selection favoring a particular suite of traits (i.e. selection targeting multiple unlinked loci), (ii) selection favoring a trait that is genetically linked to genes affecting other traits, or (iii) selection favoring a trait that varies due to genetic variation at a pleiotropic gene affecting multiple traits. In the case of the threespine stickleback, genetic variation linked to a single major gene, *Eda*, has been found to explain correlated differences in these traits among populations (Albert *et al*., 2008; Greenwood *et al*., 2016), suggesting that pleiotropy has played a role. Studies in various other plant and animal species also support the hypothesis that pleiotropy contributes to the coevolution of correlated traits (e.g., McKay *et al*., 2003; McLean *et al*., 2011; Duveau and Felix 2012; Nagy *et al*. 2018).

In insects, genes determining body color are often pleiotropic. For example, in *Drosophila*, the yellow gene is required for the synthesis of black melanin and also affects mating behavior (Bastock, 1956; Drapeau *et al*., 2003; Drapeau *et al*., 2006). The genes pale and Dopa-decarboxylase, which encode enzymes that synthesize tyrosine-derived precursors for pigmentation, are also pleiotropic, affecting both body color and immunity (reviewed in Wittkopp and Beldade, 2009; Takahashi, 2013). In addition, prior work suggests that pigmentation genes might also affect cuticular hydrocarbon (CHC) profiles, which can affect desiccation (Gibbs, 1997; Gibbs, 1998; Foley and Telonis-Scott, 2011) and mate choice (reviewed in Yew and Chung, 2015). Specifically, a
receptor for the tanning hormone *bursicon* and levels of the biogenic amine dopamine, which both affect cuticle pigmentation in *Drosophila melanogaster*, have been shown to influence CHC composition (Marican et al., 2004; Wicker-Thomas and Hamann, 2008; Flaven-Pouchon et al. 2016).

Here, we test whether the *ebony* and *tan* genes of *D. melanogaster*, which are required for the synthesis of dark melanins and yellow sclerotins from dopamine, respectively, also affect CHC composition. The *ebony* gene encodes a protein that converts dopamine into N-β-alanyl dopamine (NBAD), and the *tan* gene encodes a protein that catalyzes the reverse reaction, converting NBAD back into dopamine (Figure 3-1A). We report that loss-of-function mutations in both *ebony* and *tan* altered CHC length composition relative to wild-type flies in opposing directions. These opposing effects on CHC length composition are consistent with *ebony* and *tan*'s opposing biochemical functions in dopamine metabolism (Figure 3-1A). Indeed, pharmacological inhibition of dopamine synthesis in *ebony* mutants caused a *tan*-like CHC length profile. To examine the possibility that variation in *ebony* and/or *tan* activity might cause correlated changes in pigmentation and CHC composition in a natural population, we used lines from the *Drosophila* Genetic Reference Panel (DGRP) to test for covariation between pigmentation and CHC composition. We found that CHC length composition covaried not only with pigmentation but also with levels of *ebony* and *tan* expression in a manner consistent with the mutant analyses. In the discussion, we compare our data to studies of clinal variation in CHC composition and pigmentation to determine whether the pleiotropic effects we see might have contributed to correlated evolution of these traits.

**Materials and Methods**

*Fly stocks and maintenance*

The following lines were used: P excision line *tan*\textsuperscript{20A} (True et al., 2005) (courtesy of John True, Stony Brook University); the *UAS-ebony-RNAi* effector line was obtained from the Vienna Drosophila Resource Centre (Dietzl et al., 2007, KK106278); *dsx\textsuperscript{GAL4}* (Rideout et al., 2010) (courtesy of Stephen Goodwin, Oxford University); *OK72-GAL4* (Ferveur et al., 1997) (courtesy of Scott Pletcher, University of Michigan); *pannier-GAL4* (Calleja et
al. 2000) was obtained from the Bloomington Drosophila Stock Center (BDSC 3039); *vasa-Cas9* (Gratz et al., 2014, BDSC 51324) (courtesy of Rainbow Transgenics Inc.). All flies were grown at 23°C with a 12 h light-dark cycle on standard corn-meal fly medium.

**DGRP stocks**

The following inbred *D. melanogaster* lines from the DGRP (Ayroles et al. 2009; Mackay et al. 2012; Huang et al. 2014) were used in this study: RAL-208, RAL-303, RAL-324, RAL-335, RAL-357, RAL-358, RAL-360, RAL-365, RAL-380, RAL-399, RAL-517, RAL-555, RAL-705, RAL-707, RAL-732, RAL-774, RAL-786, RAL-799, RAL-820, RAL-852, RAL-714, RAL-437, RAL-861 and RAL-892. These lines consist of the set of 20 lines used in Miyagi et al. (2015) and additional 3 dark lines (RAL-714, RAL-437, and RAL-861), which were added to avoid line specific effects from a limited number of dark lines. All flies were grown at 25°C with a 12 h light-dark cycle on standard corn-meal fly medium.

**Generation of ebony CRISPR lines**

New loss-of-function ebony mutants were constructed by synthesizing two single guide RNAs (gRNA), using a MEGAscript T7 Transcription Kit (Invitrogen), following the PCR-based protocol from Bassett et al. (2014), that target ebony’s first coding exon and co-injecting these at a total concentration of 100 ng/µL into embryos of a *D. melanogaster* vasa-Cas9 line (Gratz et al., 2014; BDSC 51324) (Supplementary Figure S3-1). These gRNAs were previously found to generate a high level of heritable germline transformants (Ren et al., 2014; Supplementary Figure S3-1). We screened for germline transformants based on body pigmentation and confirmed via Sanger sequencing three unique ebony loss-of-function alleles, *ebony*\textsuperscript{CRISPR(1,2)} containing a 55 bp deletion, and *ebony*\textsuperscript{CRISPR(3)} and *ebony*\textsuperscript{CRISPR(4)}, each containing an in-frame 3 bp deletion (Supplementary Figure S3-1). Each deletion caused flies to develop dark body pigmentation, indicating loss of Ebony activity (Figure 3-1B, Supplementary Figure S3-2A).

**CHC extraction and measurements**
For Figures 3-1 and 3-2 and Supplementary Figures S3-2–S3-5, CHCs were extracted and analyzed as described below (CHC names and formulas are summarized in Supplementary Table S3-1). For the analyses using the DGRP (Figures 3-3 and 3-4, Supplementary Figure S3-6), all CHC data for females were obtained from Dembeck et al. (2015b); however, in the case of GC/MS peaks composed of more than two combined CHC components that differed in CHC chain length, the non-branched CHC chain length was used. Also, CHCs that were not detected in all strains were removed from the analyses.

**Extraction**

For each experiment, five replicate CHC samples of virgin female flies were prepared for each genotype or pharmacological treatment group. All ebony and tan mutant CHC extractions were performed on 3–4 d old virgin females. We restricted our analysis to virgin females, because previous evidence studies suggested that a link between dopamine and CHC composition occurs in females but not males (Marican et al., 2004; Wicker-Thomas and Hamann, 2008). For pharmacological experiments, 1–2 d old virgin females were treated for 4 d prior to CHC extraction. For GAL4/UAS experiments (Brand and Perrimon, 1993), virgin females were tested at 10–12 d. For each sample, 5 flies were placed in a single glass vial (Wheaton 224740 E–C Clear Glass Sample Vials) on ice. 120 µL of hexane (Sigma Aldrich, St Louis, MO, USA) spiked with 10 µg/mL of hexacosane (Sigma Aldrich) was added to each vial and sealed with a cap. Vials were incubated at room temperature for 20 mins. 100 µL of the cuticular extract was removed, transferred into a clean vial (Wheaton 0.25 mL with low volume insert), and stored at -20°C.

**GC/MS analysis**

Gas chromatography mass spectrometry (GC/MS) analysis was performed on a 7820A GC system equipped with a 5975 Mass Selective Detector (Agilent Technologies, Inc., Santa Clara, CA, USA) and a HP-5ms column ((5%-Phenyl)-methylpolysiloxane, 30 m length, 250 µm ID, 0.25 µm film thickness; Agilent Technologies, Inc.). Electron ionization (EI) energy was set at 70 eV. One microliter of the sample was injected in
splitless mode and analyzed with helium flow at 1 mL/min. The following parameters were used: column was set at 40°C for 3 min, increased to 200°C at a rate of 35°C/min, then increased to 280°C at a rate of 20°C/min for 15 min. The MS was set to detect from m/z 33 to 500. Chromatograms and spectra were analyzed using MSD ChemStation (Agilent Technologies, Inc.). CHCs were identified on the basis of retention time and EI fragmentation pattern. The relative abundance for each CHC signal was calculated by normalizing the area under each CHC peak to the area of the hexacosane signal. To eliminate multicollinearity among sample peak amounts, a log-contrast transformation was applied to the resulting proportional values, using nC27 as the denominator (Yew et al., 2011; Blow and Allen, 1998):

$$\text{logcontrast CHC}_n = \log 10 \left( \frac{\text{proportion (CHC}_n\text{)}}{\text{proportion (C27 alkane)}} \right)$$

To determine the relative change in CHC length between two genotypes, experimental groups, or groups of DGRP strains, the difference in relative intensity of individual CHC intensities of each group was calculated:

$$\text{Difference} = \text{logcontrast CHC}_a - \text{logcontrast CHC}_b$$

These values were then plotted against CHC chain length.

**Ultraviolet laser desorption ionization mass spectrometry (UV-LDI MS)**

For intact fly analysis, individual animals were attached to a glass cover slip using adhesive pads (G304, Plano, Wetzlar, Germany). The cover slips were mounted on a custom-milled sample holder containing a rectangular, 1.8 mm deep well. Sample height was adjusted by choosing a stack of 0.2 mm-thick adhesive pads (G3347, Plano). Mass spectra were generated using a prototype orthogonal-extracting mass spectrometer (oTOF-MS) as described previously (Yew et al. 2011). The oTOF-MS was equipped with a modified oMALDI2 ion source (AB Sciex, Concord, Canada) and an N$_2$ laser ($\lambda = 337$ nm) operated at a pulse repetition rate of 30 Hz. N$_2$ was used as buffer gas at p = 2 mbar. This elevated pressure is critical to achieve an efficient collisional cooling environment for generation of weakly-bound [M + K]$^+$ ions that constituted the major molecular ion
species. Before starting the actual measurements, external mass calibration was achieved with red phosphorus, resulting in a mass accuracy of approximately 25 ppm. Approximately 900 laser shots were placed at one position to achieve a mass spectrum (30 s @30 Hz). All spectra were acquired in positive ion mode and processed using MS Analyst software (Analyst QS 2.0, AB Sciex, Concord, Canada).

**Pharmacology Experiments**

For pharmacological treatments, standard corn-meal fly medium was liquefied and cooled to ca. 60°C before the addition of each respective drug or solvent control. Ten 1–2 d old virgin females were placed in the vials for 4 d. To inhibit tyrosine hydroxylase activity, we prepared a 36 mM alpha methyl tyrosine (L-AMPT) (Sigma Aldrich) diet. The pH of the solution was adjusted with concentrated HCl until the drug dissolved. A solvent control diet solution was prepared using identical procedures. For the dopamine treatments, 1 mM and 10 mM L-dopa precursor (Methyl L-DOPA hydrochloride) (Sigma Aldrich) were dissolved in water before adding to liquefied fly media.

**RNA extraction**

Female virgin flies were collected within 1 h of eclosion, and the heads were removed in Rnalater (Ambion) to separate the effect from transcripts in non-epidermal head tissues. The remaining head-less body samples were stored in Rnalater at -80°C until use. Three body samples from each line were placed in a 2 mL microtube with 400 µL TRIzol Reagent (Thermo Fisher Scientific, Tokyo, Japan) and an equivalent volume of 1.2 mm zirconia silica beads (Bio Medical Science). After shaking the tube at 3,200 rpm for 2 min using a Beads Crusher µT-12 (TAITEC, Koshigaya, Japan), 160 µl chloroform was added and mixed thoroughly. Total RNA in the aqueous phase was subsequently purified using silica-gel (Wakocil 5SIL, Wako, Osaka, Japan) based on the method of Boom et al. (1990) and was quantified using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific).

**Quantitative real-time PCR (qRT-PCR)**
First strand cDNA was synthesized from 1 µg total RNA by using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Kusatsu, Japan). qRT-PCR was performed in a 25 µl reaction volume with SYBR Premix Ex Taq II Tli RNaseH Plus (Takara Bio) on a Thermal Cycler Dice TP800 (Takara Bio). Primer pairs used for RT-qPCR were ebony: 5′−CTTAGTGTGAAACGGCCACAG−3′ and 5′−GCAGCGAACCCATCTTGAA−3′; tan: 5′−GTTGAGGGGCTTCGATAAGA−3′ and 5′−GTCCATCCGAAAGATCCTG−3′; Act57B: 5′−CGTGTCTCCTCTCTGTTGCAGA−3′ and 5′−ACCGCGAGCGATTAAACGT−3′; Rp49: 5′−TCGGATCGATATGCTAGCT−3′ and 5′−TCGGATCGGATACCGATTG−3′. Act57B and Rp49 were used as internal control. Two replicate PCR reactions were performed for each cDNA sample and three biological replicates were obtained for each line.

Grouping DGRP lines based on pigmentation scores and ebony/tan expression levels

The DGRP lines (N = 155) with both pigmentation scores in Dembeck et al. (2015a) and CHC profiles in Dembeck et al. (2015b) were grouped into dark, intermediate, and light pigmentation lines using the pigmentation scores of the abdominal tergites from Dembeck et al. (2015a). The scores ranged from 0 for no dark pigmentation to 4 for 100% dark pigmentation in increments of 0.5, and were averaged across 10 individuals per line. Pigmentation grouping was done based on the score delimitations that split the lines most evenly into three groups. For the 5th tergite (A5), lines were categorized into following groups: dark (1.5 < score, N = 53), intermediate (1 < score ≤ 1.5, N = 56), and light (score ≤ 1, N = 49). For the 6th tergite (A6), lines were categorized into following groups: dark (3 < score, N = 51), intermediate (2 < score ≤ 3, N = 55), light (score ≤ 2, N = 49).

The 23 DGRP lines with varying ebony and tan expression levels were grouped into low, intermediate, and high expression lines using the qRT-PCR data. Since the normalized quantities are continuous values, grouping was done based on standard deviations (SD). For the ebony expression, lines were categorized into following groups: low (expression
< mean - 0.5SD, N = 6), intermediate (mean - 0.5SD ≤ expression ≤ mean + 0.5SD, N = 9), and high (mean + 0.5SD < expression, N = 8). For the tan expression, lines were categorized into following groups: low (expression < mean - 0.5SD, N = 10), intermediate (mean - 0.5SD ≤ expression ≤ mean + 0.5SD, N = 7), and high (mean + 0.5SD < expression, N = 6).

Statistics
All statistical tests were performed in R for Mac version 3.3.3 (R Core Team 2018) using one-way ANOVAs to test for statistically significant effects between more than two groups and post-hoc Tukey HSD tests for multiple pairwise comparisons. We used Spearman’s rank correlation coefficient \( \rho \) to test for the significance of the association. All pairwise tests were two-tailed, and the level of significance was set as \( \alpha = 0.05 \).

Results

Loss-of-function mutations in ebony and tan have reciprocal effects on CHC length profiles
To determine whether the ebony gene affects cuticular hydrocarbons (CHCs), we created three new ebony mutant alleles via CRISPR/Cas9 gene editing. One allele, \( \text{ebony}^{\text{CRISPR(1,2)}} \), contained a 55 bp deletion that caused a frame-shift in ebony’s coding sequence (Supplementary Figure S3-1C). Flies homozygous for this \( \text{ebony}^{\text{CRISPR(1,2)}} \) allele showed dark body pigmentation similar to that described previously for loss-of-function ebony mutants (Bridges and Morgan, 1923) (Figure 3-1B). We measured CHC profiles in 3–4 d old \( \text{ebony}^{\text{CRISPR(1,2)}} \) virgin females using gas chromatography (GC/MS) and found that \( \text{ebony}^{\text{CRISPR(1,2)}} \) flies showed lower levels of total alkanes relative to 3–4 d old virgin females from the strain the guide RNAs were injected into (i.e., un-injected \( \text{vasa-Cas9} \)) (Figure 3-1C, One-way ANOVA: \( F_{9,40} = 4494, P < 2.0 \times 10^{-16} \); post-hoc Tukey HSD was significant for alkanes: \( P < 1.0 \times 10^{-5} \)).

We then tested whether \( \text{ebony}^{\text{CRISPR(1,2)}} \) females had different proportions of individual CHCs. We calculated the average difference in individual log-contrast transformed CHC
relative intensities (see Materials and Methods) between $ebony^{CRISPR(1,2)}$ flies and un-injected $vasa-Cas9$ control flies and plotted these values against CHC chain length (varying from 21 carbons (C) to 29C) (Figure 3-1D, Supplementary Table S3-1). We found that $ebony^{CRISPR(1,2)}$ flies tended to show lower levels of short chain CHCs (<25C) and higher levels of long chain CHCs (>25C), suggesting that disrupting the function of ebony causes a CHC lengthening effect (Figure 3-1D, Spearman's $\rho = 0.83$, P < 1.0 x 10^-5).

The two other ebony alleles generated using CRISPR/Cas9 gene editing ($ebony^{CRISPR(3)}$ and $ebony^{CRISPR(4)}$) each had a single 3 bp in-frame deletion in the first coding exon (Supplementary Figure S3-1D,E), suggesting that they might have less severe effects on Ebony activity than the $ebony^{CRISPR(1,2)}$ allele containing a 55 bp deletion causing a frame-shift. Consistent with this prediction, these ebony mutants also showed darker body pigmentation than wild-type flies (Supplementary Figure S3-2A), but did not show any bias toward longer CHCs (Supplementary Figure S3-2B,C, $ebony^{CRISPR(3)}$: Spearman's $\rho = 0.22$, P = 0.34; $ebony^{CRISPR(4)}$: Spearman's $\rho = 0.07$, P = 0.78).

To better understand the effects of reduced ebony expression on CHCs, we knocked down ebony expression in specific cell types using $ebony$-RNAi (Dietzl et al., 2007). First, we drove expression of $ebony$-RNAi with the $dsx^{GAL4}$ driver (Rideout et al., 2010), which causes RNAi expression in the cuticle, fat body, CNS, and oenocytes among other tissues. We observed darker pigmentation in $dsx^{GAL4} > UAS$-$ebony$-RNAi flies than control flies (data not shown), suggesting that the $ebony$-RNAi effectively targeted and knocked down ebony expression. These $dsx^{GAL4} > UAS$-$ebony$-RNAi flies also showed a pattern of CHC lengthening similar to the $ebony^{CRISPR(1,2)}$ mutants when compared to $dsx^{GAL4} / +$ control flies but not when compared to $UAS$-$ebony$-RNAi / + control flies. This result might be due to leaky $UAS$-$ebony$-RNAi expression in the latter control flies that makes their profiles more similar to those of $dsx^{GAL4} > UAS$-$ebony$-RNAi flies (Supplementary Figure S3-3A, B, relative to $dsx^{GAL4} / +$ control: Spearman’s $\rho = 0.58$, P < 0.007; relative to $UAS$-$ebony$-RNAi / + control: Spearman’s $\rho = 0.19$, P = 0.42).
We hypothesized that the effect on CHCs might be due to reducing ebony expression specifically in oenocytes because these cells synthesize many CHC precursor compounds (Wigglesworth, 1970). Therefore, we drove expression of *ebony-RNAi* using the *OK72-GAL4* driver that is also expressed in oenocytes (Ferveur et al., 1997). These flies showed no significant difference in CHC length profiles (Supplementary Figure S3-3C, Spearman’s ρ = -0.01, P = 0.96), suggesting that ebony expression in non-oenocyte tissues expressing doublesex affects the overall length proportion of CHCs.

Next, we asked whether loss-of-function mutations in the tan gene also affect CHC composition. Specifically, we examined CHC composition in 3–4 d old virgin females carrying a *tan*20A null allele, which contains an imprecise P-element excision that results in a 953 bp deletion that includes the presumptive promoter region (True et al., 2005). Because tan encodes a protein that catalyzes the reverse of the reaction catalyzed by Ebony (Figure 3-1A), we predicted that tan mutants might show the opposite effects on CHC composition. Similar to the *ebony*CRISPR(1,2) mutants, *tan*20A females showed differences in the overall abundance of alkanes, but also total CHCs, monoenes, and methyl branched CHCs (Figure 3-1E, One-way ANOVA: F9,40 = 3586, P < 2.0 x 10^-16; post-hoc Tukey HSD was significant for total summed CHCs: P < 0.01, total summed alkanes: P < 0.001, total summed monoenes: P < 0.001, and total summed methyl branched: P < 0.001). More importantly, *tan*20A (*w^1118 tan*20A) females tended to show higher levels of short chain CHCs relative to long chain CHCs when compared to *w^1118 Canton-S* (CS) control flies, as predicted (Figure 3-1F, Spearman’s ρ = -0.62, P = 0.0043). Together, these results suggest that *ebony* and *tan* have reciprocal effects on both pigmentation synthesis (reviewed in True, 2003 and True et al., 2005) and CHC length profiles. We note that this conclusion contradicts Wicker-Thomas and Hamann (2008)’s report that CHC profiles were similar in *ebony* or *tan* loss-of-function mutants and wild-type flies; however, the ebony and tan alleles used in this prior work might not have been nulls.

*Pharmacological inhibition of tyrosine hydroxylase activity reverses the CHC lengthening effect in *ebony*CRISPR(1,2) flies*
We hypothesized that ebony and tan might have reciprocal effects on CHC length profiles because of their effects on dopamine metabolism. For example, because ebony encodes a protein that converts dopamine into NBAD (Figure 3-1A), we hypothesized that loss-of-function ebony mutants might accumulate dopamine (as reported in Hodgetts and Konopka, 1973) and that this dopamine might be shunted into other pathways, possibly affecting CHC lengthening. To explore this hypothesis, we fed 1–2 d old adult female ebony\textsuperscript{CRISPR(1,2)} flies a tyrosine hydroxylase inhibitor, alpha methyl tyrosine (L-AMPT), for four days to determine whether inhibiting dopamine synthesis would reverse the CHC lengthening pattern we observed in ebony\textsuperscript{CRISPR(1,2)} flies. Relative to ebony\textsuperscript{CRISPR(1,2)} solvent-fed control flies, ebony\textsuperscript{CRISPR(1,2)} flies fed 36 mM L-AMPT did indeed reverse the CHC lengthening pattern we observed in ebony\textsuperscript{CRISPR(1,2)} flies, resulting in a shortening of CHCs similar to that observed in tan\textsuperscript{20A} flies (Figure 3-2A, Spearman’s $\rho = -0.48$, $P = 0.03$). Feeding 1–2 d old adult flies L-AMPT did not, however, affect body pigmentation (data not shown), consistent with body pigmentation being determined prior to and soon after eclosion (Hovemann et al., 1998). We also fed ebony\textsuperscript{CRISPR(4)} flies a 36 mM dose of L-AMPT to see if we could induce CHC shortening in an ebony mutant with unchanged CHC length composition. Similar to ebony\textsuperscript{CRISPR(1,2)} fed flies, we detected a significant negative correlation when comparing ebony\textsuperscript{CRISPR(4)} fed flies to an ebony\textsuperscript{CRISPR(4)} solvent-fed control (Supplementary Figure S3-4, Spearman’s $\rho = -0.57$, $P = 0.009$).

We next hypothesized that tan\textsuperscript{20A} flies might have lower levels of circulating dopamine, because tan encodes a protein that converts NBAD back into dopamine (Figure 3-1A). To determine whether elevating dopamine levels in tan mutants would affect CHCs, we fed tan\textsuperscript{20A} females a dopamine precursor, methyl L-DOPA hydrochloride (L-DOPA precursor), to see if elevating dopamine levels could reverse the CHC shortening pattern we observed in tan\textsuperscript{20A} flies; however, neither the 1 mM nor 10 mM L-DOPA precursor treatments seemed to affect CHC length profiles when compared to tan\textsuperscript{20A} solvent-fed control flies (Figure 3-2B, C, Spearman’s $\rho = 0.17$, $P = 0.50$; Spearman’s $\rho = 0.01$, $P = 0.97$, respectively). We also fed tan\textsuperscript{20A} flies a higher 100 mM dose of the L-DOPA precursor, but all of these flies died before CHC extraction; these flies also showed
darker cuticle pigmentation consistent with elevated dopamine. Finally, we fed 1 mM and 10 mM doses of L-DOPA precursor to wild-type (w^{1118} CS) females to see if we could induce CHC lengthening in a wild-type genetic background; instead, we observed a slight CHC shortening effect for the 1 mM dose and no effect for the 10 mM dose (Supplementary Figure S3-5, Spearman’s $\rho = -0.52$, $P = 0.02$; Spearman’s $\rho = -0.36$, $P = 0.12$, respectively). Together, these results indicate that inhibiting tyrosine hydroxylase activity in ebony mutants causes a CHC shortening effect like that observed in tan^{20A} flies; however, increasing dopamine levels through feeding does not cause a CHC lengthening effect.

**UV-LDI MS data suggests that ebony’s effects on pigmentation and CHC length profiles are not linked at the level of the cuticle**

Pigmentation synthesis in insect cuticles involves the secretion of biogenic amines (such as dopamine) by epidermal cells into the developing cuticle where they are oxidized into quinones that can form melanins or sclerotins that crosslink proteins (Figure 3-1A; reviewed in True, 2003 and Riedel et al., 2011). To determine whether ebony’s effects on CHC length profiles depend on their function in pigmentation and sclerotization of the fly cuticle, we measured the relative abundance of individual CHCs in virgin females with different levels of pigmentation across the body. We crossed pannier-GAL4 (Calleja et al. 2000) females with males from the UAS-ebony-RNAi effector line to generate flies with a dark, heavily melanized stripe down the dorsal midline (Figure 3-3A). We then used UV laser desorption/ionization mass spectrometry (UV-LDI MS) to take repeated measurements of CHCs along the thorax of females, targeting inside and outside the dark stripe (Figure 3-3A). Although we observed an upward trend in abundance from short to long CHCs, we did not detect a significant CHC lengthening effect like that observed between ebony^{CRISPR(1,2)} flies and un-injected vasa-Cas9 females (Figure 3-3B, Spearman’s $\rho = 0.58$, $P = 0.13$). Within the black cuticle, most CHCs detected by UV-LDI MS showed a decrease in abundance relative to brown cuticle (Figure 3-3B). This result suggests that ebony does not affect CHC length profiles through the pigmentation/sclerotization synthesis pathway, at least at the level of CHC/pigment deposition in the cuticle.
Abdominal pigmentation covaries with CHC length profiles in the Drosophila Genetic Reference Panel (DGRP)

The effects of *ebony* and *tan* mutants on CHC profiles described above suggest that variation in these genes might contribute to variation in both pigmentation and CHC profiles. Recently, Dembeck et al. (2015a,b) analyzed the genetic architecture of abdominal pigmentation and CHC composition in female *D. melanogaster* lines from the Drosophila Genetic Reference Panel (DGRP): Dembeck et al. (2015a) quantified abdominal pigmentation intensity in the 5th and 6th abdominal tergites (A5 and A6), and Dembeck et al. (2015b) investigated CHC profiles from the majority of the panel, but the relationship between the two traits was not examined. Using data from the 155 DGRP lines for which both pigmentation scores and CHC profiles were published, we tested the hypothesis that natural variation in pigmentation covaries with natural variation in CHC length profiles. In order to investigate CHC composition in a way that was comparable to the experiments described above, we divided the 155 DGRP lines into dark (N = 53), intermediate (N = 56), and light (N = 46) pigmentation groups using the 5th abdominal tergite (A5) pigmentation scores (0–4) from Dembeck et al (2015a). Next, we tested whether females from dark, intermediate, or light pigmentation groups showed differences in their abundance of CHCs with different chain lengths relative to the 155 DGRP line average. We found that the group with the darkest A5 pigmentation showed lower levels of short chain CHCs and higher levels of long chain CHCs relative to the 155 line average (Figure 3-4A, Spearman’s ρ = 0.44, P < 0.01); the group with intermediate A5 pigmentation showed no relationship with CHC chain length (Figure 3-4B, Spearman’s ρ = 0.002, P = 0.98); and the group with lightest A5 pigmentation showed the opposite pattern as the dark group (Figure 3-4C, Spearman’s ρ = -0.57, P = 1.0 x 10⁻³). We also compared CHC profiles in dark (N = 51), intermediate (N = 55), and light (N = 49) groups based on pigmentation of the 6th abdominal tergite (A6), and found that, unexpectedly, the dark group did not show a significant CHC lengthening effect (Supplementary Figure S3-6A, Spearman’s ρ = 0.19, P = 0.25), and the intermediate group showed a CHC lengthening effect (Supplementary Figure S3-6B, Spearman’s ρ = 0.44, P < 0.01). However, the light group showed a significant CHC shortening effect as
expected (Supplementary Figure S3-6C, Spearman’s \( \rho = -0.68 \), \( P < 1.0 \times 10^{-5} \)). These data suggest that darkly pigmented DGRP females show a pattern of CHC lengthening similar to the darkly pigmented loss-of-function \textit{ebony}^{CRISPR(1,2)} flies, and lightly pigmented DGRP females show a pattern of CHC shortening similar to lightly pigmented loss-of-function \textit{tan}^{20A} flies.

\textit{ebony} and \textit{tan} expression covaries with CHC length profiles in the DGRP

The DGRP genome-wide association (GWAS) study from Dembeck et al. (2015a) revealed that top variants associated with pigmentation are in \textit{ebony}, \textit{tan}, and \textit{bab1}, consistent with variation in ebony expression level observed in the DGRP lines (Miyagi et al. 2015) and associations between pigmentation and these genes in studies of other \textit{D. melanogaster} populations (Rebeiz et al. 2009a,b; Telonis-Scott et al. 2011; Takahashi and Takano-Shimizu 2011; Bastide et al. 2013; Endler et al. 2016; 2018). We therefore hypothesized that the differences in CHC length profiles seen in darkly and lightly pigmented DGRP females might be a consequence of expression variation at \textit{ebony} and/or \textit{tan}.

Using qRT-PCR, we quantified ebony and tan expression within 1 h after eclosion, which is when pigments determining adult body color are actively produced, in a sample of 23 DGRP lines that showed variable pigmentation. We then tested whether variation in \textit{ebony} and \textit{tan} expression covaried with CHC length profiles by categorizing the 23 DGRP lines into groups of low, intermediate, and high \textit{ebony} or \textit{tan} expression levels based on the qRT-PCR results, examining the average difference in individual CHC abundances between each expression group relative to the 23 line average, and plotting these values against CHC chain length (Figure 3-5).

Consistent with our hypothesis, the DGRP lines with low ebony expression showed lower levels of short chain CHCs, lines with high \textit{ebony} expression showed higher levels of short chain CHCs, and lines with intermediate expression showed no change in CHC profiles (Figure 3-5A–C, Spearman’s \( \rho = 0.67 \), \( P < 1.0 \times 10^{-6} \), Spearman’s \( \rho = -0.61 \), \( P < 1.0 \times 10^{-5} \), Spearman’s \( \rho = -0.10 \), \( P = 0.50 \), respectively). Reciprocally, the DGRP lines
with low or intermediate tan expression showed a slight increase in short chain CHCs, and lines with high tan expression showed a significant decrease in short chain CHCs (Figure 3-5D,F, Spearman’s ρ = -0.29, P = 0.05, Spearman’s ρ = -0.32, P = 0.03, Spearman’s ρ = 0.50, P < 0.001, respectively). Taken together, our results suggest that differences in ebony and tan gene expression have pleiotropic effects on both pigmentation and CHC length profiles that might cause these traits to covary in natural D. melanogaster populations.

**Discussion**

Pigmentation genes are often pleiotropic, with effects on vision, circadian rhythms, immunity, and mating behavior (reviewed in Wittkopp and Beldade, 2009; Takahashi, 2013). Here, we show that ebony and tan also affect CHC production, with the two genes altering CHC length profiles in opposing directions: ebony^{CRISPR(1,2)} mutants had significantly higher levels of long chain CHCs, and tan^{20} mutants had significantly higher levels of short chain CHCs. Our results suggest 1) that ebony and tan have a previously undescribed role in CHC synthesis and/or deposition and 2) that pleiotropy of both genes might influence the covariation of pigmentation and CHC composition.

*Considering the pleiotropic effects of ebony and tan through changes in dopamine metabolism*

Previous work has shown that changes in dopamine metabolism influence CHC composition in Drosophila melanogaster. Specifically, females homozygous for loss-of-function Dopa-decarboxylase (Ddc) temperature-sensitive alleles showed changes in CHC composition that could be reversed with dopamine feeding (Marican et al., 2004; Wicker-Thomas and Hamann, 2008). Additionally, inhibiting dopamine synthesis by feeding wild-type females the tyrosine hydroxylase inhibitor L-AMPT altered CHC composition in a similar direction as the loss-of-function alleles (Marican et al., 2004; Wicker-Thomas and Hamann, 2008). We found that feeding with L-AMPT affects CHC length composition, causing ebony^{CRISPR(1,2)} and ebony^{CRISPR(3)} mutants to have a more tan^{20}-like CHC length profile (Figure 3-2A and Supplementary Figure S3-4). This result suggests that ebony and tan may affect CHC length composition through dopamine...
metabolism, but feeding tan\textsuperscript{20} and wild-type females dopamine did not lead to CHC lengthening (Figure 3-2B,C and Supplementary Figure S3-5). Why did L-AMPT feeding affect CHC length composition while dopamine feeding did not? One possible reason is that L-AMPT is a potent inhibitor of tyrosine hydroxylase activity (Spector et al., 1965), which processes tyrosine that flies ingest, whereas dopamine feeding might not cause significant changes in dopamine abundance in tissues relevant to CHC synthesis.

Another gene suggesting a possible link between CHC composition and dopamine is the \textit{D. melanogaster} apterous gene. Loss of apterous gene function causes an increase in the proportion of long chain CHCs (Wicker and Jallon, 1995), and \textit{apterous} mutants also show high levels of dopamine (Grutenko et al., 2003; Grutenko et al., 2005; Grutenko et al., 2012). These mutants also show low levels of juvenile hormone (JH) (Altaratz et al., 1991), and treating decapitated females with methoprene to increase JH synthesis caused a decrease in long chain CHCs (Wicker and Jallon, 1995). The CHC lengthening and increased dopamine levels seen in \textit{apterous} mutants resemble \textit{ebony} mutants, but it is unknown whether \textit{ebony} mutants show altered JH profiles. Further evidence supporting a role of JH and other ecdysteroids in determining CHC chain length comes from houseflies (Blomquist et al., 1987). In \textit{D. melanogaster}, ecdysteroid signaling was found to be required not only for CHC synthesis but also survival of the oenocyte cells that synthesize CHCs (Chiang et al., 2016). An interesting future direction would be to test whether changes in dopamine metabolism in \textit{ebony} or \textit{tan} mutants influence CHC length composition through JH signaling. More broadly, a thorough genetic analysis focused on tissue-specific manipulation of dopamine is needed to deepen our understanding about its role in CHC synthesis.

\textit{CHC lengthening in \textit{ebony} mutants does not seem to depend on changes at the level of the cuticle}

Data from our tyrosine hydroxylase inhibition experiments supported the hypothesis that elevated dopamine levels in \textit{ebony} mutants (as reported in Hodgetts and Konopka, 1973) affect CHC lengthening; however, it remains unclear which cells require \textit{ebony} expression (and possibly dopamine metabolism) to influence CHC synthesis. We
hypothesized that *ebony*-dependent changes of the fly cuticle itself might affect CHC deposition during fly development or CHC extraction in the laboratory, and found that all but one detected CHC showed an overall decrease in abundance in dark cuticle relative to light cuticle. We note that these differences might be due to changes in the physical properties of dark versus light cuticle as they interact with the UV-LDI instrument. We also note that *ebony*<sup>CRISPR(3)</sup> and *ebony*<sup>CRISPR(4)</sup> mutants had darkly pigmented cuticle like *ebony*<sup>CRISPR(1,2)</sup> mutants but CHC length profiles similar to wild-type flies, suggesting that *ebony* and *tan*’s effects on CHC length composition can be separated from their role in pigmentation synthesis. For example, *ebony* expression in glia is necessary for normal circadian rhythms in *D. melanogaster* but not pigmentation (Suh and Jackson, 2007). It is also possible that *ebony* actually affects CHC composition through changes in pigmentation precursors within epidermal cells underneath the cuticle, which might not have been detected by our UV-LDI MS analysis in the thorax. We tested whether knocking down *ebony* in oenocytes in the abdomen affected CHC length composition and found that it did not, thus the specific cells required for *ebony* and *tan*’s effects on CHC synthesis remain unknown.

*Patterns of CHC composition and pigmentation along clines in natural populations*

Identifying the pleiotropic effects of *ebony* and *tan* on pigmentation and CHCs is important because it suggests that these genes might contribute to the covariation of both traits in natural populations. For example, selection for *ebony* or *tan*-dependent pigmentation variation might also cause variation in CHC length composition without selection acting directly on this trait. Alternatively, selection for long chain CHCs with higher melting temperatures (Gibbs and Pomonis, 1995; Gibbs, 1998) in drier climates might cause a correlated increase in pigmentation intensity. Indeed, we found that variation in abdominal pigmentation covaries with both *ebony* and *tan* gene expression as well as CHC length profiles in directions predicted by *ebony* and *tan* mutants among the DGRP lines, which were derived from flies isolated from a single, natural population (Ayroles et al. 2009; Mackay et al. 2012; Huang et al. 2014). However, this finding does not necessarily imply variation in both traits is caused by the same gene(s) nor that these traits will always co-evolve; for example, individuals with dark pigmentation may
coincidentally possess alleles that are in linkage disequilibrium that cause a CHC lengthening phenotype. Comparing the phenotypic frequency of pigmentation and CHC length composition phenotypes within and between the same populations that are undergoing adaptation to common environments will help answer this question. In Africa, for example, *D. melanogaster* populations repeatedly show a strong positive correlation between elevation and dark pigmentation, suggesting that environments at high altitudes might select for darkly pigmented flies (or some other trait that correlates with pigmentation) (Pool and Aquadro, 2007; Bastide et al., 2014). It will be interesting to know whether these populations also show an increase in abundance of long chain CHCs.

Both pigmentation and CHC length profiles vary along altitudinal and latitudinal clines in natural *Drosophila* populations, suggesting that ecological factors such as humidity or temperature play a role in shaping variation in at least one of these traits. At higher altitudes or latitudes, populations often showed darker pigmentation profiles in Europe, India, and Australia (Heed and Krishnamurthy, 1959; David et al., 1985; Capy et al., 1988; Das, 1995; Munjal et al., 1997; Parkash and Munjal, 1999; Pool and Aquadro, 2007; Telonis-Scott et al., 2011; Parkash et al., 2008a; Parkash et al., 2008b; Matute and Harris, 2013). In Africa, however, latitude and pigmentation intensity showed a negative correlation, so this relationship is not universal (Bastide et al., 2014). For CHCs, Rajpurohit et al. (2017) reported that *D. melanogaster* populations at higher latitudes showed more short chain CHCs, whereas populations at lower latitudes showed more long chain CHCs in the United States. Frentiu and Chenoweth (2010) similarly found that populations at high latitudes along a cline in Australia showed more short chain CHCs and fewer long chain CHCs. These patterns do not match predictions based on the pleiotropy we observed: flies at higher latitudes tend to have darker pigmentation and higher levels of short chain CHCs whereas *ebony*^{CRISPR(1,2)} mutants, for example, have darker pigmentation and lower levels of short chain CHCs. To the best of our knowledge, pigmentation (nor *ebony* or *tan* expression) and CHC length composition have not been simultaneously measured in flies from the same cline, making it difficult to discern whether pigmentation and CHC composition covary in the wild in ways predicted by the
mutant data. For example, Frentiu and Chenoweth (2010) measured CHCs from populations along the east coast of Australia, but they did not include populations from higher latitude coastal regions with darker pigmentation and lower ebony expression in newly eclosed adults (Telonis-Scott et al. 2011). Comparing variation in both traits within and between populations along latitudinal and/or altitudinal clines will make it clearer if and to what extent pigmentation and CHC composition covary and whether variation in these features is accompanied by changes in ebony and tan expression.

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References


A

Tyrosine → Tyrosine hydroxylase → DOPA → DOPA decarboxylase → Dopamine → Ebony → Nβ-alanyl dopamine (NBAD) → PO

Dopamine melanin (black) → Yellow → PO

Dopamine melanin (brown)

NBIAD sclerotin (yellow-tan)

B

WT ebonyCRISPR(1,2) tan20A

C

![Graph C](image)

D

Spearman's rho = 0.83

\[ P < 1.0 \times 10^{-5} \]

Differences between ebonyCRISPR(1,2) and un-injected vasa-Cas9 control (%)

E

![Graph E](image)

F

Spearman's rho = -0.62

\[ P = 0.0043 \]

Differences between tan20A and w1118 Canton-S control (%)

Σ Total CHCs

Σ Alkanes

Σ Monoenes

Σ Dienes

Σ Methyl Branched
Figure 3-1 *ebony* and *tan* affect pigmentation and CHC composition in female *Drosophila melanogaster*.

(A) Insect sclerotization and pigmentation synthesis pathway. Ebony converts dopamine into N-β-alanyl dopamine (NBAD) which is oxidized into yellow-colored NBAD sclerotin. Tan catalyzes the reverse reaction, converting NBAD back into dopamine that can be oxidized into black and brown melanins. (B) Photographs highlighting the effects of *ebony*<sup>CRISPR(1,2)</sup> (darker) and *tan*<sup>20A</sup> (lighter) on body pigmentation compared to the un-injected *vasa-Cas9* control line (*WT*). (C) Summary of *ebony*<sup>CRISPR(1,2)</sup> effects on total summed CHC classes relative to un-injected *vasa-Cas9* control females. (D) Difference in log-contrast of relative CHC intensity between *ebony*<sup>CRISPR(1,2)</sup> and un-injected *vasa-Cas9* control flies. (E) Summary of *tan*<sup>20A</sup> effects on total summed CHC classes relative to *w<sup>1118 Canton-S* control females. For (D) and (E), each triangle represents a single replicate of CHCs extracted from five pooled individuals (N = 5 replicates per genotype). (F) Difference in log-contrast of relative CHC intensity between *tan*<sup>20A</sup> and *w<sup>1118 Canton-S* control flies. Results of Tukey HSD post-hoc tests following One-way ANOVA are shown: * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 3.2 Effects of pharmacological treatments on CHC lengthening in ebony\textsuperscript{CRISPR(1,2)} and tan\textsuperscript{20A} mutants

(A) Difference in log-contrast of relative CHC intensity between ebony\textsuperscript{CRISPR(1,2)} females fed 36mM alpha methyl tyrosine (L-AMPT) and ebony\textsuperscript{CRISPR(1,2)} females fed a solvent control. (B) Difference in log-contrast relative of CHC intensity between tan\textsuperscript{20A} females fed 1 mM methyl L-DOPA hydrochloride (L-DOPA precursor) and tan\textsuperscript{20A} females fed a solvent control. (C) Difference in log-contrast of relative CHC intensity between tan\textsuperscript{20A} females fed 10 mM L-DOPA precursor and tan\textsuperscript{20A} females fed a solvent control.
Figure 3-3 UV laser desorption/ionization mass spectrometry (UV-LDI MS) did not detect differences in short versus long CHCs between lightly and darkly pigmented cuticle.

Female *pannier-GAL4* flies were crossed to *UAS-ebony-RNAi* males to generate flies with a dark, heavily melanized stripe down the dorsal midline. (A) The UV-LDI MS lasers were targeted to light brown or dark black cuticle within the same fly (*N* = 3 biological replicates). (B) Difference in relative CHC intensity between black and brown cuticle.
Figure 3-4 Abdominal pigmentation co-varies with CHC length profiles in the *Drosophila* Genetic Reference Panel (DGRP)

Pigmentation scores and CHC data were obtained from Dembeck et al. (2015a,b). (A) Difference in log-contrast of relative CHC intensity between DGRP females with darkly-pigmented 5th abdominal tergites (A5) (1.5 < score, N = 53) and the 155 line average. (B) Difference in log-contrast of relative CHC intensity between DGRP females with intermediately-pigmented A5 (1 < score ≤ 1.5, N = 56) and the 155 line average. (C) Difference in log-contrast of relative CHC intensity between DGRP females with lightly-pigmented A5 (score ≤ 1, N = 49) and the 155 line average.
Figure 3-5 Variation in *ebony* and expression co-varies with CHC length profiles in the DGRP

CHC data was obtained from Dembeck et al. (2015b), and *ebony* and *tan* expression was quantified via qRT-PCR for 23 DGRP lines. (A) Difference in log-contrast of relative CHC intensity between DGRP females with low *ebony* expression and the 23 line average. (B) Difference in log-contrast of relative CHC intensity between DGRP females with intermediate *ebony* expression and the 23 line average. (C) Difference in log-contrast of relative CHC intensity between DGRP females with high *ebony* expression and the 23 line average. (D) Difference in log-contrast of relative CHC intensity between DGRP females with low *tan* expression and the 23 line average. (E) Difference in log-contrast of relative CHC intensity between DGRP females with intermediate *tan* expression and the 23 line average. (F) Difference in log-contrast of relative CHC intensity between DGRP females with high *tan* expression and the 23 line average.
Chapter 4

Genetic Dissection of Correlated Divergence in Wing Pigmentation and Mating Display

Abstract

Species differences in sexual traits involve correlated changes in morphology and behavior. The evolution of mating displays in particular often highlight diverse pigmentation patterns that distinguish males from females and closely related species. How the genome evolves to cause these correlated changes is not well understood. We investigated the genetic basis of correlated divergence in wing pigmentation and mating display between the sibling species *D. elegans* and *D. gunungcola*. Divergence in both traits map to a co-localized region on the X chromosome. Within this region, we mapped a single, ~440 kb locus that behaves like a genetic switch controlling wing spot divergence. Divergence in mating display also involved loci on the autosomes. Introgression mapping on the X chromosome and field observations suggest that wing spot and mating display divergence can be separated, possibly as a consequence of epistatic interactions between the X and autosomes.
Introduction

Animals often use colorful morphological structures to communicate with prospective mates during courtship. Most famously, male peacocks display their elaborately decorated plumage during courtship rituals to lure females (Petrie et al., 1991; Petrie and Halliday, 1994; but see Takahashi et al., 2008). In vertebrates and invertebrates, pigmented bodies or wings often evolve together with specific components of courtship behavior that animals use to display their colorful anatomy (Loxton, 1979; Endler, 1991; Sinervo et al., 2000; White et al., 2015). These correlated differences evolve both within and between populations, frequently distinguishing males from females or closely related species (Gray and McKinnon, 2007; McKinnon and Pierotti, 2010). Little is known about how pigmented body parts and courtship behaviors evolve together at the level of the genome. In just a few cases, linkage mapping and genome-wide association studies (GWAS) have shown that loci affecting pigmentation patterning tend to co-localize with loci affecting variation in mating behaviors (Lindholm and Breden, 2002; Kronforst et al., 2006; Thomas et al., 2008; Kupper et al., 2016; Lamichhaney et al., 2016; Merrill et al., 2019; reviewed in McKinnon and Pierotti, 2010). That is, physical linkage of genes or mutations on the same chromosome underlie phenotypic correlations between mating behavior and pigmentation. Interestingly, these loci also tend to explain much of the variation observed for both traits. A key challenge is determining how frequently these patterns of genomic architecture underlie correlated evolution and whether the same pleiotropic or separate linked loci are involved.

Disentangling whether pleiotropic or physically linked loci underlie patterns of correlated evolution between pigmentation and mating behavior is important for understanding how genomes create, maintain, and shape adaptive differences between sexes and species. If two beneficial traits are genetically correlated due to separate, physically linked loci, simulations predict that natural or sexual selection (e.g., through predation or female choice) must actively work to minimize recombination to maintain linkage (Charlesworth and Charlesworth, 1976); it has been hypothesized that one solution to this problem might involve the evolution of chromosomal inversions that suppress recombination between two or more linked loci (Kirkpatrick and Barton, 2006). Alternatively, mutations
at a single pleiotropic gene could cause correlated components of pigmentation and mating behavior to evolve simultaneously. The likelihood that mutation or recombination disrupt genetic correlations, however, will likely depend on locus-specific rates of recombination, mutation, and the distance between physically linked loci (Paaby and Rockmann, 2013). Distinguishing between these genetic modes of phenotypic evolution will require, in part, mapping species correlated differences at higher resolution in an attempt to recombine tightly linked loci.

In the Oriental *Drosophila melanogaster* species group, male-specific wing spots are phylogenetically correlated with mating displays (Kopp and True, 2002). Males that possess wing spots tend to perform elaborate wing display dances during courtship, turning their dorsal wing surfaces toward the female and waving them up and down; males without spots lack display behavior (Kopp and True, 2002). Correlated gains or losses of both traits have evolved repeatedly (Kopp and True, 2002). In two closely related species from this group, *D. elegans* (Bock and Wheeler, 1972) males possess wing spots and perform wing displays (Figure 4-1A), while males in its sibling species, *D. gunungcola* (Sultana et al., 1999), have lost both (Figure 4-1A) (Kopp and True, 2002; Prud’homme et al., 2006; Yeh et al., 2006). Previously, Yeh et al., (2006) and Yeh and True (2014) took advantage of the fact *D. elegans* and *D. gunungcola* form fertile F1 hybrid female offspring in the lab to study the genetic basis of wing spot and wing display divergence. Through linkage mapping, they discovered that evolution of linked loci on the X chromosome contributed to divergence in both traits (Yeh and True, 2014). It remains unclear, however, whether the same or different loci on the X chromosome underlie correlated differences in wing spot and wing display between these species.

To further dissect the genetic basis of wing spots and wing display divergence between *D. elegans* and *D. gunungcola*, we generated several hundred backcross recombinant male progeny segregating for both traits. We assembled chromosome-length scaffolds of *D. elegans* and applied Multiplexed Shotgun Genotyping (MSG) (Andolfatto et al., 2011) to estimate recombination crossover positions across the genome; we also generated quantitative measures of both wing spots and wing display behavior to estimate the effect
size of loci contributing to divergence. Finally, we generated advanced, recombinant introgressions on the X chromosome in an attempt to separate quantitative trait loci (QTL) underlying wing spots and wing display behavior.

Materials and Methods

Fly stocks

Species stocks were kept on a 12 h light-dark cycle at 23ºC on University of Michigan R food diet (http://lab-express.com/flyfoodsupplies.htm#rfood), containing molasses as a sugar source. The D. elegans HK (Hong Kong) and D. gunungcola SK (Sukarami) lines used in this study were a gift from John True (Stony Brook University). Fisherbrand filter paper (cat# 09-790-2A) was added to the food when 3rd instar L3 larvae developed to facilitate pupation.

Generating hybrid progeny

Virgin male and females of D. elegans HK and D. gunungcola SK were isolated upon eclosion and stored in groups of ten for one week on University of Michigan M food (http://lab-express.com/flyfoodsupplies.htm#rfood), standard cornmeal diet with 20% higher agar content. Virgin males from D. elegans HK were crossed to virgin females from D. gunungcola SK, and virgin males from D. gunungcola SK were crossed to virgin females from D. elegans HK in groups of ten males and ten females to generate fertile F1 female and sterile F1 male hybrids. These crosses generally took 3-4 weeks to produce hybrid progeny. The switch from R food to M food for interspecific crosses was necessary, because R food tended to accumulate condensation and bacterial growth much faster than M food when few flies occupied a vial. Since crossing D. elegans HK and D. gunungcola SK to generate F1 hybrids tends to take several more weeks than within species crosses, the switch to M food diet allowed for maximum breeding time and the development of dozens of hybrid progeny. Once hybrid females eclosed from both interspecific cross directions, they were pooled into the same vial and aged for ten days. We did not keep track of F1 hybrid female maternity, because previous work (Yeh and True, 2014) found no effect of F1 hybrid female maternity on trait means in F2 backcross
populations. Multiple high density groups of ~60 hybrid females were then backcrossed to ~60 virgin male *D. elegans HK* flies in individual vials on M food diet to create the *D. elegans HK* backcross recombinant population (724 individuals). To create the *D. gunungcola SK* backcross recombinant population (241 individuals), groups of ~60 hybrid females were backcrossed to ~60 virgin male *D. gunungcola SK* flies in individual vials on M food diet; this backcross was significantly less successful at producing recombinant progeny than the *D. elegans HK* backcross direction.

**Behavioral assays**

Virgin *D. elegans HK* females aged at least 10 days were isolated upon eclosion and stored in groups of 30-40 for courtship assays. F1 hybrid and recombinant backcross males were isolated individually in M food vials using CO2 upon eclosion for at least 5 days before each courtship assay. For each assay, a single individual male was gently aspirated into a custom designed 70 mm diameter bowl arena adapted from methods described in Simon and Dickinson (2010). Next, a single virgin *D. elegans HK* female was aspirated into the chamber and videotaped for 20 min immediately after, using a Canon VIXIA HF R500. Videos were recorded between 0900 and 1600 at 23°C. *D. elegans HK* virgin females were used in all courtship assays in case any *D. elegans HK* female cues were necessary to elicit male wing display behavior. After each assay, both the male and female were aspirated back into an M food vial for up to 5 days after which each male was frozen in individual 1.5 mL Eppendorf tubes for wing spot quantification (see Quantification of wing spots), genomic DNA (gDNA) extraction, and sequencing (see Library preparation and sequencing). All courtship videos (~900 total, available upon request) were transferred to external hard drives for wing display quantification (see Quantification of wing display behavior).

**Quantification of wing display behavior**

F1 hybrid and recombinant males from both backcross directions performed variable wing display behaviors during courtship (Figure 4-2D, as described previously in Yeh et al., 2006; Yeh and True, 2014). To generate quantitative measurements of wing display variation between individuals, each courtship video was played using QuickTime
(version 10.4) (Apple Inc., Cupertino, CA) software in a MacOS environment and digital screenshots were manually taken for each wing display bout, defined as bilateral wing extensions performed near the female (Supplemental Figure S4-1). Next, for each individual fly, wing display screenshots were compared to each other to identify the maximum wing display bout per fly, defined by comparing the distance between the tips of each wing relative to the center of the fly. These maximum wing display screenshots were then imported into ImageJ software (version 1.50i) (Wayne Rasband, National Institutes of Health, USA; http://rsbweb.nih.gov/ij/) to manually measure the “Maximum wing display angle” for F1 hybrid and recombinant males. In ImageJ, each screenshot image was inverted using the “Find Edges” function to enhance the contrast between the arena background and the edges of the fly wings (Supplemental Figure S4-1). Next, the “Polygon Selections” tool was used to fit an ellipse around the fly body using the “Fit Ellipse” function (Supplemental Figure S4-1). A Macros function (Supplementary File S4-2) was then used to generate major and minor axes inside the ellipse to identify the center of the fly body (Supplemental Figure S4-1). Finally, the “Angle Tool” was used to measure the “Maximum wing display angle” centering the vertex at the intersection of the major and minor axes and extended from wing tip to wing tip (Supplemental Figure S4-1). “Maximum wing display angle” varied between ~50º and ~220º between backcross recombinant individuals (Figure 4-2D).

Quantification of wing spots
Since wing spots fully form ~24 h after eclosion in D. elegans HK, all parental male D. elegans HK, D. gunungcola SK, F1 hybrids, and backcross recombinants were aged at least 7 days before being frozen at -20°C in 1.5 mL Eppendorf tubes. Next, using a 20 Gauge stainless steel syringe tip (Techcon) (cat# TE720100PK) the right wing of each fly was cut away from the thorax and placed on a glass microscope slide (Fisherbrand) (cat# 12-550-15) to image using either a Leica MZFLIII stereoscope equipped with a Leica DC480 microscope camera or a Canon EOS Rebel T6 camera equipped with a Canon MP-E 65 mm macro lens. Each camera was calibrated using an OMAX 0.1 mm slide micrometer to define pixel density in ImageJ software. JPEG images of wings were imported into ImageJ to measure wing spot size relative to total wing area (wing spot size
/ total wing area). Total wing area (wing length x wing width) was approximated using length and width proxies (Figure 4-2A) following methods described in Yeh and True (2014). Using the “Polygon Selections” tool, the margins of black pigmentation defining each “Wing spot size” (Figure 4-2A) was traced and the polygon area quantified in mm² using the “Measure” function. “Wing spot size” varied between 0.15 mm² and 0 mm² (spotless) between recombinant individuals.

Library preparation and sequencing
We estimated chromosome ancestry “genotypes” for 724 D. elegans HK backcross progeny and 241 D. gunungcola SK backcross progeny with a single Multiplexed Shotgun Genotyping (MSG) (Andolfatto et al., 2011) library using 965 barcoded adaptors following methods described in Cande et al., (2012). In brief, to extract gDNA from all male backcross individuals, single flies were placed into individual wells of 96-well (Corning) (cat# 3879) plates containing a single steel grinding bead (Qiagen) (cat# 69989). Eleven plates in total were prepared for 965 individual gDNA extractions. gDNA was isolated and purified using the solid tissue extraction procedure from a Zymo Quick-DNA 96 Kit (cat# D3012) and a paint shaker to homogenize tissue. gDNA was tagmented using a hyperactive version of Tn5 transposase charged with annealed adaptor oligos following the methods described in (Picelli et al., 2014). Unique barcoded adaptor sequences were ligated to each sample of tagmented gDNA with 14 cycles of PCR using NEB OneTaq 2x Master Mix (cat# M0482S), and all samples were pooled into a single multiplexed sequencing library. Agencourt AMPure XP beads (Beckman Coulter) (cat# A63881) were used to size select ~150-800 bp fragments and eluted in 35 uL of molecular grade water (Corning) (cat# MT46000CI). The library was quantified by qPCR and sequenced in a single lane of Illumina HiSeq by the Janelia Quantitative Genomics Team.

In addition to generating the backcross sequencing library, both D. elegans HK and D. gunungcola SK parental species were sequenced at 20x coverage using an Illumina MiSeq Reagent Kit (v.3, 600 cycle PE) to facilitate genome assembly. In brief, gDNA was extracted using a Zymo Quick-DNA Microprep Kit (cat# D4074) from 10 pooled
females for each species and quantified on a Qubit 2.0 (Invitrogen). These samples were sent to the University of Michigan DNA Sequencing Core to prepare 300 bp PE libraries, which were quantified by qPCR and sequenced in a single lane of Illumina MiSeq.

**Genome assembly**

In brief, Illumina reads from all 965 F2 backcross recombinants were used to perform MSG on the Baylor College of Medicine *D. elegans* genome assembly (https://www.ncbi.nlm.nih.gov/bioproject/62315). Using custom script in R and Python (https://github.com/masseyj/elegans), the recombination fraction between the Baylor and MSG contigs was calculated and plotted to manually tabulate joins and splits between newly assembled contigs. These new contigs were then used to assemble chromosome length scaffolds in *D. elegans*.

**Marker generation with Multiplexed Shotgun Genotyping**

Following methods described previously (Andolfatto *et al.*, 2011; Cande *et al.*, 2012), we used the MSG software pipeline (https://github.com/JaneliaSciComp/msg/tree/master/instructions) to perform data parsing and chromosome ancestry estimation to generate markers for QTL analysis. In brief, using data from the Illumina backcross sequencing library (see File S4-1 for the number of reads per individual), we mapped reads to the assembled *D. elegans HK* and *D. gunungcola SK* parental genomes to estimate chromosome ancestry for each backcross individual. We generated 3,425 and 3,121 markers for the *D. elegans HK* and *D. gunungcola SK* backcrosses, respectively (Supplementary Files S4-3, S4-4), for QTL analysis.

**QTL analysis**

QTL analysis was performed using R/qtl (Broman *et al.*, 2003) in R for Mac version 3.3.3 (R Core Team 2018) in a MacOS environment. Ancestry data for both backcross directions were imported into R/qtl using a custom script (https://github.com/dstern/read_cross_msg), which directly imports the conditional probability estimates by the Hidden Markov Model (HMM) of MSG (Andolfatto *et al.*, 2011).
2011) into R/qtl. We performed genome scans with a single QTL model using the “scanone” function of R/qtl and Haley-Knott regression (Haley and Knott, 1992) for “Wing spot size” and “Maximum wing display angle”. Significance of QTL peaks at $\alpha = 0.01$ was determined by performing 1000 permutations of the data. Effect sizes for each QTL peak were individually estimated by comparing the mean “Wing spot size” or “Maximum wing display angle” between individuals that inherited either D. elegans HK or D. gunungcola SK alleles at each QTL peak position. Since we detected multiple QTL peaks on separate chromosomes for “Maximum wing display angle”, we tested for the presence of epistatic interactions using two methods: First, we performed a two-way ANOVA comparing the effect of each QTL peak in multiple QTL peak genetic backgrounds and found no evidence of an interaction (Figure 4-2F). Second, we performed genome-wide pairwise tests using the “scantwo” function of R/qtl and Haley-Knott regression to test for non-additive interactions across all markers and found no significant LOD scores for any marker pairs (Supplementary Figure S4-2, Supplementary Table S4-1; Supplementary Table S4-2).

Note, for several QTL peaks, we noticed that a significant proportion of the chromosome reached the LOD significance threshold even though the QTL peak intervals were much smaller. For example, the 95% Bayes credible interval (Broman and Sen, 2009) for the D. elegans backcross wing spot QTL on the X chromosome is only 435,676 bp even though the majority of the chromosome (~25 Mbp) climbs above the LOD threshold at 3.56. We attribute this pattern, in part, to low recombination frequencies in both backcross directions. On average, we detected only two crossover events genome-wide per fly (Supplementary Figure S4-3).

**Annotating the wing spot QTL interval**

To annotate genes within the ~440 Kbp fine-mapped wing spot locus (Figure 4-3B, we performed nucleotide BLAST (BLASTn) (Johnson et al., 2008) searches against the D. melanogaster genome (taxid: 7227) using ~10 Kbp windows of assembled D. elegans chromosome regions spanning the QTL interval. Using the “GBrowse” tool on Flybase (Thurmond et al., 2019), we mapped regions of microsynteny to identify the orientation
of each gene and exported the respective *D. melanogaster* coding region (CDS) FASTA sequence to align with the *D. elegans* X chromosome.

**Generating X chromosome advanced recombinant introgressions**

To isolate the QTL effects for “Wing spot size” and “Maximum wing display angle” localized to the X chromosome according to the *D. elegans HK* backcross experiment (Figure 4-2), F1 hybrid females were generated using the procedures described above. F1 hybrid females were then backcrossed towards *D. elegans HK*, and F2 backcross males lacking wing spots were isolated to measure “Maximum wing display angles” during courtship as described above. This procedure was repeated for seven generations to generate F3-F9 backcross individuals: F2 backcross females were backcrossed towards *D. elegans HK*, and F3 backcross males lacking wing spots were isolated to measure “Maximum wing display angles” (and so on to F9; Figure 4-4A). At each generation, an attempt was made to create stable introgression lines of advanced recombinant males lacking wing spots, but all failed to produce offspring, suggesting that *D. gunungcola SK* genomic regions on the X chromosome might also be linked to sterility factors. After seven generations of backcrossing, gDNA from all backcross males lacking wing spots was extracted and sequenced for MSG as described above. Backcross males lacking wing spots from F4-F9 were homozygous for *D. elegans HK* genomic regions across all autosomes but varied for the amount of *D. gunungcola SK* genome regions on the X chromosome (Supplementary Figure S4-4). Due to low levels of recombination (Supplementary Figure S4-3), however, the vast majority of introgressed individuals contained large 5-15 Mbp linked blocks of *D. gunungcola SK* X chromosome (Figure 4-4B).

**Video capture of *D. elegans* and *D. gunungcola* in Indonesia**

*D. elegans* is distributed across Southeast Asia, including Japan, Malaysia, Hong Kong, the Philippines, Taiwan, and Indonesia, often occupying Ipomoea or Brugmansia flowers. *D. gunungcola* is distributed throughout Indonesia in regions that partly overlap with *D. elegans* (Hirai and Kimura, 1997; Sultana *et al*., 1999; Ishii *et al*., 2002; Suwito *et al*., 2002). Using Canon VIXIA HF R500 camcorders mounted to Manfrotto (cat#
MKCOMPACTACN-BK) aluminum tripods, we captured videos of *D. elegans* and *D. gunungcola* courting conspecific females on *Ipomoea indica* and *Brugmansia candida* flowers at National Central University (Taoyuan City, Taiwan), Bumiaji (Indonesia), and Coban Rondo (Indonesia).

**Statistics**

Statistical tests were performed in R for Mac version 3.3.3 (R Core Team 2018) using Student’s t-test (two-tailed) to test for statistically significant effects of pairwise comparisons of continuous data with normally distributed error terms. For tests comparing more than two groups, one-way ANOVAs were performed with post-hoc Tukey HSD for pairwise comparisons adjusted for multiple comparisons. See “QTL analysis” methods for statistical tests used during QTL mapping.

**Results and Discussion**

*Evolution of loci on the X chromosome contributed to divergence in wing spots and wing display behavior*

*D. elegans* males perform elaborate wing display dances in front of females during courtship, highlighting the presence of darkly pigmented wing spots (Figure 4-1A). Its sibling species, *D. gunungcola*, lost wing spots (Prud’homme *et al*., 2006) and lack the ability to perform wing displays (Figure 4-1A,B). Although these species diverged 2-2.8 Myr (Prud’homme *et al*., 2006), they are still capable of reproducing to form viable F1 hybrids in the lab (Yeh *et al*., 2006; Yeh and True, 2014). To compare the effects of evolution on the X chromosome to divergence in wing spots and wing display behavior, we quantified variation in wing spot size and wing display behavior between reciprocal F1 hybrid males, inheriting their X chromosome from either *D. elegans* or *D. gunungcola* mothers and autosomes from both species’ parents. As previously reported (Yeh *et al*., 2006; Yeh and True, 2014), F1 hybrid males inheriting the X chromosome from *D. elegans* mothers (F1E) possessed wing spots (although smaller than *D. elegans*), while F1 hybrid males inheriting the X chromosome from *D. gunungcola* mothers (F1G) did not (Figure 4-1B,C). We also confirmed reports (Yeh *et al*., 2006; Yeh and True, 2014) of
differences in wing display behavior between F1E and F1G hybrids. While both F1 hybrids performed wing displays during courtship, F1G hybrid males performed much more variable wing displays (Figure 4-1B). Specifically, F1G hybrids failed to open their wings as widely as F1E hybrids during display performance (Figure 4-1B). We quantified variation in wing display behavior between F1 hybrids by measuring the maximum bilateral wing display angles (Figure 4-1B) during courtship (see Methods), finding that F1E hybrids performed wing displays similar to *D. elegans* males, while F1G males showed, on average, lower display angles (Figure 4-1C). Together these data confirm previous reports (Yeh et al., 2006; Yeh and True, 2014) that evolution on the X chromosome contributed significantly to divergence in wing spot size and wing display behavior between *D. elegans* and *D. gunungcola*.

*Evolution at a single locus explains the majority of wing spot divergence, but evolution at multiple, additive loci contributed to wing display divergence*

To identify the location of loci contributing to divergence in wing spot size and wing display behavior on the X chromosome and autosomes, we quantified variation in both traits (Figure 4-2A,D) in hundreds of F2 backcross recombinant male flies and estimated genome-wide chromosome ancestry using MSG (Andolfatto et al., 2011) for quantitative trait locus (QTL) mapping (Broman and Sen, 2009). Scanning for single, additive QTL in the *D. elegans* backcross population, we identified highly significant QTL peaks on the X chromosome for both wing spot size and wing display behavior as predicted based on our comparisons between reciprocal F1 hybrids (Figure 4-2B,E; Table 4-1). Scanning for single, additive QTL in the *D. gunungcola* backcross population similarly revealed a highly significant QTL peak on the X chromosome for wing spot size but only a marginally significant QTL peak for wing display behavior (Figure 4-2B,E; Table 4-1). In both backcross directions, we identified a QTL peak on Muller Element B for wing display behavior and a peak on Muller Element E for the *D. gunungcola* backcross (Figure 4-2E; Table 4-1).

The X-linked QTL peak for wing spot size in both backcross directions explains almost all of the difference in wing spot size between *D. elegans* and *D. gunungcola* (Figure 4-
QTL peaks for wing display behavior showed smaller effects (Figure 4-2F). Since we detected QTL peaks on separate chromosomes for wing display behavior in both backcross directions, we estimated the effect size of each to test for any possible epistatic interactions. In the *D. elegans* backcross, the effects from X chromosome and Muller Element B QTL peaks were approximately additive; that is, for the *D. elegans* backcross, we did not detect a significant interaction when comparing the effect of the X-linked QTL peak in either homozygous or heterozygous genetic backgrounds at the Muller Element B QTL peak (Figure 4-2F). Similarly, we did not detect significant interactions between QTL peaks at Muller Element B and E for the *D. gunungcola* backcross (Figure 4-2F). We also performed a two-dimensional genome scan (see Methods) in both backcross directions to test for genome-wide evidence of epistatic loci contributing to wing display divergence and found no significant interactions (Supplementary Figure S4-2; Supplementary Table S4-1; Supplementary Table S4-2). Thus, the wing display QTLs behave approximately additively in both backcrosses, while divergence in wing spot size is primarily controlled by a single major locus.

Some QTL peaks for wing display behavior were present in only one backcross direction (Figure 4-2E). This is likely a consequence of performing an F2 backcross rather than an F2 intercross to map QTL. For example, the peak on Muller Element E in the *D. gunungcola* backcross is likely caused by *D. gunungcola* recessive alleles that are detectable in only the *D. gunungcola* backcross. The absence of a large effect X chromosome QTL peak in the *D. gunungcola* backcross is less clear (Figure 4-2E). Although we did not detect evidence of epistatic loci contributing to wing display divergence in our two-dimensional genome scan (Supplementary Figure S4-2; Supplementary Table S4-1; Supplementary Table S4-2), it is possible that the presence of many small-effect *D. gunungcola* alleles in the *D. gunungcola* backcross masked the effect of the X chromosome. Our current sample size (N = 147) is likely too small to measure these effects. It is clear, however, that evolution at loci on multiple chromosomes contributed to wing display divergence between *D. elegans* and *D. gunungcola* (Figure 4-2E; Table 4-1).
The wing spot locus maps to a narrow 440 kb region containing omb

The interval estimates (Table 4-1) for the QTL peaks explaining wing spot size variation in both backcross directions mapped to a narrow region on the X chromosome. We aligned X chromosomes from recombinants with crossover positions immediately flanking the wing spot QTL peak to more closely compare the effect of this region on wing spot size (Figure 4-3A). Strikingly, the ~440 kbp wing spot locus seems to act like a switch, turning on or off the wing spot between recombinants varying in D. elegans and D. gunungcola alleles at this region (Figure 4-3A). To identify potential candidate genes contributing to this switch effect, we annotated the loci within this region (see Methods) and discovered it contains omb (Figure 4-3B), a T-box-containing transcription factor (Pflugfelder et al., 1992a; Pflugfelder et al., 1992b) previously implicated in pigmentation development (Thompson, 1959; Kopp and Duncan, 1997), pigmentation evolution (Brisson et al., 2004), and distal wing patterning (Grim and Pflugfelder, 1996). In D. melanogaster, gain- and loss-of-function omb alleles cause expansion and contraction of abdominal pigmentation bands, respectively (Kopp and Duncan, 1997). In D. polymorpha, variation in abdominal pigmentation patterning is strongly associated with polymorphisms at the omb locus (Brisson et al., 2004). omb, therefore, is the strongest candidate gene for wing spot divergence; however, we cannot presently rule out the other 14 genes mapped within this region.

Surprisingly, wing spot divergence did not map to yellow, a pigmentation candidate gene that was previously thought to contribute to wing spot divergence between D. elegans and D. gunungcola (Prud’homme et al., 2006; reviewed in Massey and Wittkopp, 2016). Like omb, yellow has been implicated in pigmentation development (Geyer et al., 1986; Geyer et al., 1987; Wittkopp et al., 2002a; Wittkopp et al., 2002b) and evolution (Gompel et al., 2005; Prud’homme et al., 2006). Yellow protein expression prefigures wing spot patterning in multiple spotted Drosophila species (Wittkopp et al., 2002b; Gompel et al., 2005; Prud’homme et al., 2006). Using wing GFP reporter constructs in D. melanogaster, Prud’homme et al. (2006) mapped variation in wing GFP patterning to a few divergent nucleotides at the D. elegans and D. gunungcola yellow wing enhancer region, suggesting that D. gunungcola lost wing spots, at least in part, due to cis-
regulatory evolution at yellow. In D. elegans, yellow maps to 11.41 Mbp on the X chromosome, ~650 Kbp downstream of the fine-mapped wing spot region (Figure 4-3A). In two instances, recombinants that inherited the entire D. gunungcola yellow locus in physical linkage with the D. elegans wing spot region still possessed dark wing spots (Figure 4-3A). To test whether divergence in wing spot size alone, rather than wing spot presence, maps closer to yellow, we removed spotless recombinants from our analyses and found that the position of the QTL peak did not change for the D. elegans backcross (Supplementary Figure S4-4; Supplementary Table S4-3). For the D. gunungcola backcross, however, we discovered new QTL peaks on Muller Element C and E that explained variation in wing spot size, independent of wing spot presence (Supplementary Figure S4-4; Supplementary Table S4-3; Masset et al., in prep). These results suggest that recessive D. gunungcola alleles linked to Muller Element C and E contributed to wing spot size evolution (Massey et al., In prep). cis-regulatory evolution at yellow, however, likely did not play as significant a role in wing spot divergence between D. elegans and D. gunungcola as previously anticipated (Prud’homme et al., 2006).

**Spotless advanced recombinants perform D. elegans-like wing display behavior**

In the D. elegans backcross, QTL peaks explaining both wing spot size and wing display behavior co-localize on the X chromosome (Figure 4-2B,E). To disentangle whether this genetic correlation is a consequence of physical linkage between loci affecting each trait independently or a single, pleiotropic gene, we introgressed regions of the D. gunungcola X chromosome into a D. elegans genetic background through repeated backcrossing (Figure 4-4A, see Methods). At backcross generations F4-F9, we quantified maximum wing display angles for spotless recombinants and estimated genome-wide chromosome ancestry using MSG (Andolfatto et al., 2011). By the third backcross, recombinants were only segregating for D. elegans and D. gunungcola alleles on the X chromosome and were homozygous for D. elegans autosomes (Supplementary Figure S4-5). Although we repeated backcrossing for eight generations, we recovered very few uniquely recombined X chromosomes. Instead, likely due to low levels of recombination (Supplementary Figure S4-3), the vast majority of spotless recombinants inherited similar 5-15 Mbp X chromosome haplotypes from D. gunungcola (Figure 4-4B). Still, one of these haplotypes
contained an estimated crossover position in between the *D. elegans* backcross wing display and wing spot QTL peaks, inheriting the *D. elegans* wing display QTL peak in physical linkage with the *D. gunungcola* QTL peak (Figure 4-4B). Unexpectedly, these, and all of the advanced backcross recombinants, performed wing displays indistinguishable from *D. elegans* wing display behavior (Figure 4-4B). That is, spotless recombinants performed maximum wing display angles as well as *D. elegans* whether or not they inherited *D. gunungcola* loci linked near the wing display QTL peak (Figure 4-4B). These results suggest that in the process of introgressing the wing spot QTL peak from *D. gunungcola* into an *D. elegans* genetic background, the effects of the X-linked wing display QTL were lost.

Why does introgressing the *D. gunungcola* wing spot locus into a *D. elegans* genetic background fail to cause correlated changes in wing display behavior? One possibility is that *D. gunungcola* loci linked to the Muller Element B QTL peak must be present in the same genetic background as *D. gunungcola* X-linked loci to detect effects on wing display behavior. In the *D. elegans* backcross, however, wing display QTL peaks localized to these chromosomes behaved approximately additively with each other, suggesting that each locus should affect wing display variation independent of the other (Figure 4-2F). Another possibility is that small effect *D. gunungcola* autosomal loci that we failed to detect in the F2 backcross experiment potentiate the X chromosome effect on wing display divergence. Alternatively, we might have failed to capture the true X-linked wing display locus in the process of introgression. Regardless, spotless advanced recombinants that inherited the X-linked *D. gunungcola* wing spot locus (and several Mbp of linked loci) performed maximum wing display angles indistinguishable from *D. elegans* (Figure 4-4B), suggesting that through either undetected epistatic interactions or physical linkage these correlated traits are separable.

*D. gunungcola* lacking wing spots perform wing displays in the wild

When *D. elegans* and *D. gunungcola* diverged 2-2.8 Myr (Prud’homme et al., 2006), did wing spots or wing display behavior evolve first, or did they evolve simultaneously? That is, were wing spots and wing displays ever separated in nature? Since wing display
behavior likely diverged due to multiple loci (Figure 4-2E; Table 4-1), and wing spots due to a single major locus (Figure 4-2B; Table 4-1), we hypothesized that the evolution of wing display behavior likely took longer than the evolution of wing spots. Currently, only one *D. gunungcola* line exists in the laboratory (Sultana *et al*., 1999), and it does not perform any type of wing display during courtship (Figure 4-1A; Kopp and True, 2002; Yeh *et al*., 2006; Yeh and True, 2014), making it impossible to test this hypothesis with presently available stocks. To determine whether *D. gunungcola* populations in the wild are still segregating for wing spot or wing display variation, we isolated new lines from Indonesia and recorded videos of their courtship behavior on flowers (see Methods). *D. gunungcola* isolated at all field sites lacked wing spots (unpublished observation); however, we also observed for the first time *D. gunungcola* males performing wing displays towards conspecific females (Figure 4-5). Males approached females, initiating bilateral wing extensions during courtship, however, unlike *D. elegans* wing displays in the wild (Figure 4-5), *D. gunungcola* males did not turn their dorsal wing surfaces towards the female (Figure 4-5) and instead held their wings out flat similar to F1G courting males (Figure 4-1B). These are the first, to our knowledge, observations of *D. gunungcola* performing wing display behavior. These results suggest that loci affecting wing display variation are still segregating in natural *D. gunungcola* populations, while the genetic loss of wing spots appears to be fixed. Within these *D. gunungcola* populations, therefore, wing spot divergence preceded the loss of wing display behavior.

**Conclusions**

In the laboratory, *D. elegans* and *D. gunungcola* show divergent wing spot and wing display behavior (Figure 4-1A). QTL mapping identified a single major locus, including the *omb* gene, on the X chromosome that acts like a genetic switch controlling wing spot loss in *D. gunungcola* (Figure 4-3). Divergence in wing display behavior involved a major locus on the X chromosome but also multiple loci on the autosomes that behave approximately additively with each other (Figure 4-2E,F). Although the X-linked QTL regions for wing spots and wing display behavior co-localized, introgressing the wing spot locus from *D. gunungcola* into *D. elegans* did not cause a correlated change in wing display behavior (Figure 4-4). Different genetic mechanisms, therefore, likely caused
divergence in wing spots and wing display behavior between *D. elegans* and *D. gunungcola*: A single genetic switch mechanism controls wing spot loss in *D. gunungcola*, however, multiple loci (both on the X and autosomes) contributed to wing display divergence, and the X-linked *D. gunungcola* wing spot locus on its own is insufficient to cause wing display differences (Figure 4-4).

The precise mechanism underlying the genetic correlation linking wing spot and wing display divergence to the X chromosome remains unclear. Isolating the effects of the wing spot locus independent of the wing display region through introgression (Figure 4-4) suggests that physical linkage between X-linked genes is responsible for the genetic co-localization pattern. During introgression, for example, the causal wing display locus might have been lost due to recombination. This would suggest, contrary to the *D. elegans* backcross results (Table 4-1), that the causal wing display locus maps to a region downstream of 15 Mb, since the *D. gunungcola* introgression regions spanned ~0-15 Mb (Figure 4-4). Alternatively, divergence in wing spot and wing display might involve the same pleiotropic gene or tightly linked loci; while the *D. gunungcola* wing spot locus is sufficient to turn off the wing spot in *D. elegans*, the wing display locus may interact epistatically with *D. gunungcola* autosomal wing display loci that together reduce wing display angles. This would require that both loci are present in the same genetic background to affect wing display variation. Presently, our data do not support this hypothesis, given all wing display loci behaved approximately additively in the backcross experiments (Figure 4-2F; Supplementary Figure S2; Supplementary Table S-1; Supplementary Table S-2). Nevertheless, our current estimates of epistasis are likely significantly underpowered. Future studies aiming to fine-map the causal wing display locus, therefore, will likely only succeed if *D. gunungcola* autosomal loci (possibly linked to Muller Element B) are present in the same genetic background as the mapping population.

Wing spot and wing display divergence were previously described as being perfectly phylogenetically correlated in the Oriental *Drosophila melanogaster* species group (Kopp and True, 2002). Males in species that possess wing spots perform wing displays in front
of females during courtship, and males in species that lack wing spots do not. We mapped the genomic architecture of interspecific divergence for both traits between *D. elegans* and *D. gunungcola* to test whether or not these traits are genetically and phenotypically separable. We learned that both traits can be separated, possibly due to epistatic interactions between the X and autosomes. A complimentary approach to solving this problem is to ask whether both traits have ever been separated in natural populations. Surprisingly, we discovered that *D. gunungcola* populations in the wild are still segregating for components of wing display behavior yet appear to be fixed for the loss of wing spots (Figure 4-5). These observations suggest that *D. gunungcola* completely lost wing spots before they completely lost wing displays. Interestingly, however, wild *D. gunungcola* males do not perform wing displays as well as *D. elegans* (Figure 4-5). Males appear to lack the ability to turn their dorsal wing surfaces toward the female during wing displays, and wing waving behavior is much slower, resembling F1G hybrid courtship (Figure 4-1B). The components of wing display behavior that have diverged in natural *D. gunungcola* populations, therefore, are the actions that appear to make wing spots clearly visible during courtship. In future work, mapping how well males turn their dorsal wing surfaces during wing display within and between multiple *D. gunungcola* and *D. elegans* populations will likely help disentangle the genetic mechanisms underlying correlated divergence patterns between these species.

**Acknowledgements**

We thank members of the Wittkopp and Stern labs for helpful discussions. For fly strains, we thank John True. We also thank Shu-Dan Yeh for helpful discussions, advice about rearing *D. elegans* and *D. gunungcola*, for laying the groundwork to study both species in the wild, and for hosting J.H.M. in Taiwan and Indonesia. Funding: University of Michigan, Department of Ecology and Evolutionary Biology, Peter Olaus Okkelberg Research Award, National Institutes of Health (NIH) training grant T32GM007544, and Howard Hughes Medical Institute Janelia Graduate Research Fellowship to J.H.M.; NIH R01 GM089736 and 1R35GM118073 to PJW.
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gunungcola. G3: Genes, Genomes, Genetics, 4(11), 2079-2093.
A. *Drosophila elegans HK* vs *Drosophila gunungcola SK*

B. "F1E" hybrid ♀ *D. ele* x ♂ *D. gun* vs "F1G" hybrid ♀ *D. ele* x ♂ *D. gun*

C. Comparison of wing spot size (mm²) for *D. elegans* (♀), *D. gunungcola* (♂), and their F1 hybrid (♀, ♂).

D. Comparison of max wing display angle (degrees) for *D. elegans* (♀), *D. gunungcola* (♂), and their F1 hybrid (♀, ♂).
Figure 4-1 Wing pigmentation and wing display behavior in *D. elegans*, *D. gunungcola*, and F1 hybrids

(A) Males in *D. elegans* (left) possess wing spots and perform bilateral wing display behaviors in front of females during courtship (VIDEO). Males in *D. gunungcola* (right) lost wing spots (Prud’homme and Gompel et al., 2006) and do not perform wing displays (VIDEO). (B) F1 hybrid males inheriting their X chromosome from *D. elegans* mothers (F1E, left) possess wing spots and perform wing display behavior like *D. elegans* (VIDEO). F1 hybrid males inheriting their X chromosome from *D. gunungcola* mothers (F1G, right) are spotless and perform wing displays with low bilateral wing angles (VIDEO). (C) Quantification of wing spot size (see Methods) in male *D. elegans* and F1E. Wing spots are slightly larger in *D. elegans* than F1E (Student’s t-test; t = -2.8057; df = 11.43; p = 0.017; two-tailed). (D) Quantification of maximum bilateral wing display angles during courtship (see Methods) in male *D. elegans* and F1 hybrids. F1G hybrids showed lower maximum wing display angles than *D. elegans* and F1E hybrids (One-way ANOVA: F<sub>2,71</sub> = 20.92; p < 7.18 x 10<sup>-8</sup>; post-hoc Tukey HSD was significant between *D. elegans* and F1G: p < 2.0 x 10<sup>-7</sup> and between F1E and F1G: p < 7.1 x 10<sup>-5</sup>). Gray triangles represent individual replicates. * P < 0.05, *** P < 0.001.
Figure 4-2 QTL analysis and effect plots for wing pigmentation and wing display behavior in *D. elegans* and *D. gunungcola* backcross males

(A) Wing spots vary in size and shape in *D. elegans* and *D. gunungcola* backcross recombinants. Wing spots were traced (pink) and quantified relative to proxies for total wing area (length x width) using ImageJ (VERSION) software (see Methods). (B) Wing pigmentation QTL map for the *D. elegans* (red) and *D. gunungcola* (blue) backcross. LOD (logarithm of the odds) is indicated on the y-axis. The x-axis represents the physical map of Muller Elements X, B, C, D, E, and F based on the *D. elegans* assembled genome. Individual SNP markers are indicated with black tick marks along the x-axis. Horizontal red and blue lines mark p = 0.01 for the *D. elegans* and *D. gunungcola* backcross, respectively. (C) Effect plots for the X chromosome QTL peak from the *D. elegans* backcross (left) and *D. gunungcola* backcross (right). (D) Maximum wing display angles varied in *D. elegans* and *D. gunungcola* backcross recombinants. Maximum wing display angles were quantified by measuring the angle between each wing tip using ImageJ (VERSION) software (see Methods). (E) Maximum wing display QTL map for the *D. elegans* (red) and *D. gunungcola* (blue) backcross. LOD is indicated on the y-axis. The x-axis represents the physical map of Muller Elements X, B, C, D, E, and F based on the *D. elegans* assembled genome. Individual SNP markers are indicated with black tick marks along the x-axis. Horizontal red and blue lines mark p = 0.01 for the *D. elegans* and *D. gunungcola* backcross, respectively. (F) Effect plots for the X chromosome and Muller Element B QTL peaks from the *D. elegans* backcross (left) and for the Muller Element B and E QTL peaks from the *D. gunungcola* backcross (right). No epistatic interaction was detected comparing the combined effects of each QTL peak on maximum wing display angle (see Methods) (Two-way ANOVA: $F_{1,402} = 0.146; p = 0.70$ for the *D. elegans* backcross; Two-way ANOVA: $F_{1,141} = 0.875; p = 0.35$ for the *D. gunungcola* backcross). Gray triangles represent individual replicates.
Figure 4-3 Fine-mapping the wing spot locus

(A) *D. elegans* and *D. gunungcola* backcross recombinants containing X chromosome breakpoints immediately flanking the wing spot QTL peak were aligned to compare the effects of each on wing pigmentation. Regions in red represent *D. elegans* linked loci, and regions in blue represent *D. gunungcola* linked loci. Recombinants possessing *D. elegans* loci to the left of ~10.32 Mbp (left panel) are spotless, while recombinants possessing *D. elegans* loci to the right of ~10.74 Mbp (left panel) possess dark wing spots. Similarly, recombinants possessing *D. gunungcola* loci to the right of ~10.95 Mbp (right panel) are spotless. The effect of the fine-mapped wing spot locus between ~10.32 and ~10.74 Mbp seems to act like a switch, turning on or off wing pigmentation. (B) Two recombinants define the wing spot locus to a ~440 Kbp region containing 15 candidate genes. *omb* is the strongest wing pigmentation candidate gene given evidence from prior work (see Results and Discussion).
A) Diagram showing genetic and phenotypic traits across different hybrid backcross generations.

B) Chromosomal map showing wing display and spotless males across different hybrid generations. The graph indicates that there is no significant difference (ns) in the maximum wing display angle between the genotypes and phenotype.
(A) Schematic illustrating the crossing procedure used to generate advanced recombinant introgressions (see Methods). Briefly, hybrid females (D. elegans genome in red, D. gunungcola genome in blue) were repeatedly backcrossed towards D. elegans males to generate F2-F9 advanced recombinant males. At backcross generations F4-F9, males lacking wing spots were placed in courtship assays with virgin D. elegans females (see Methods) to quantify maximum wing display angles and then genotyped via MSG (Andolfatto et al., 2011). By the third backcross generation, advanced recombinants were only segregating for different species alleles on the X chromosome and were homozygous D. elegans on the autosomes (FIGURE). (B, Top panel) Due to low levels of recombination (FIGURE), replicates of only three unique introgression haplotypes were recovered. None of the haplotypes possessed dark wing spots (although a light wing spot “shadow” is visible, perhaps due to phenol oxidase expression in the wing spot region) as a consequence of inheriting D. gunungcola loci (blue) linked to the wing spot QTL peak. (B, Bottom panel) Quantification of max wing display angles from the haplotypes in the top panel. Max wing display angles for each haplotype were not different than D. elegans males (One-way ANOVA: F3,57 = 0.451; p = 0.72). Gray triangles represent individual replicates. n.s., not significant.
Both male *D. elegans* (left) and male *D. gunungcola* (middle and right) perform a type of wing display behavior towards females on *Ipomoea indica* (left, middle) and *Brugmansia candida* (right) flowers in Taiwan (*D. elegans*) and Indonesia (*D. gunungcola*). *D. gunungcola* males completely lack wing spots, and their wing displays are slower and have lower angles (wings were extended out flat at ~180°) than *D. elegans*. 
<table>
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<sup>a</sup> LOD drop 1.5 support interval

Table 0-1 QTLs detected for wing spot size and maximum wing display angle divergence
Chapter 5

Discussion and Future Directions

As facts accumulated it became evident that each gene produces not a single effect, but in some cases a multitude of effects on the characters of the individual.

-Thomas Hunt Morgan, 1935

Several results summarized in this thesis were unexpected. First, dissecting the effects of the yellow gene on male mating success revealed a previously unrecognized function for melanin in Drosophila reproductive behavior; second, quantifying the effects of ebony and tan knockout alleles on cuticular hydrocarbon (CHC) composition demonstrated a new function for pigmentation genes in lipid synthesis; and third, performing unbiased, high-resolution quantitative trait locus (QTL) mapping in interspecific hybrids identified new genes involved in pigmentation evolution and new mechanisms associated with behavioral evolution. All together, this thesis provides novel, causal evidence describing how gene pleiotropy manifests through the action of pigmentation enzymes to coordinate behavior and anatomy and how the genome evolves to generate species differences in these traits. Conclusions from each chapter compel multiple, future directions.

What a single mutation teaches us about behavior and evolution

For decades, pigmentation mutants in plants and animals have served developmental and evolutionary biologists as clear examples of how genotypes connect to phenotypes (Bateson, 1903; Bridges and Morgan, 1923; Wright, 1987; Jackson et al., 1994; Wittkopp et al., 2002; Wittkopp et al., 2003; Lin and Fisher, 2007; Hubbard et al., 2010). What has
remained a puzzle is how mutations affecting pigmentation act pleiotropically to disrupt behavior patterns (Takahashi, 2013). In chapter two, I set out to solve one of these mysteries by applying sophisticated genetic tools in *Drosophila melanogaster* to a classic problem: How and why do *yellow* mutant males mate less successfully than wild-type flies? My results for this chapter are clear and unexpected: *yellow* flies mate poorly because they lack melanin in a structure that is required to grasp females for mating success. These results illustrate not only how a single gene mutation disrupts fly reproduction, but also why males possess sex combs.

Melanized appendages are ubiquitous in nature. From insect legs to bird talons to bear claws, animal species repeatedly rely on melanized structures to hunt food and capture mates. While the shapes of these structures and the behaviors that use them evolve rapidly (Emlen *et al.*, 2005; Bush and Hu, 2006; Fowler *et al.*, 2009; Kopp, 2011), their melanization state appears relatively conserved. Natural and sexual selection, therefore, likely filter out mutations that disrupt melanin synthesis in appendages used for foraging and reproduction, but mutations that change their shape and how they are used might be favored in certain environments. In *Drosophila*, for example, mutations causing within and between species differences in pigmentation are restricted to tissue-specific *cis*-regulatory regions of genes important for pigmentation development (Massey and Wittkopp, 2016). Previously, it has been hypothesized that this pattern was a consequence of pigmentation gene pleiotropy (Wittkopp and Beldade, 2009; Takahashi, 2013): Pigmentation enzymes also affect biogenic amine metabolism and brain function, so mutations affecting protein coding regions are likely deleterious. Results from chapter two, however, imply that Yellow protein evolution is more likely constrained due to its specific contribution in melanizing a secondary sexual structure. In fact, there is little evidence that Yellow functions in the fly brain at all (but see Drapeau *et al.*, 2003). Taken together, these data suggest that mutations impacting structural evolution can directly lead to behavioral differences, independent of the nervous system.

Recently, Matsuoka and Monteiro (2018) discovered that mutations disrupting pigmentation synthesis in the butterfly wing also altered wing scale morphology.
Similarly, in barn owls and snakes, changes in feather and scale pigmentation, respectively, are correlated with changes in structure (Roulin *et al.*, 2013; Spinner *et al.*, 2013). The behavioral role of animal color pattern evolution has often been discussed in the framework of mating signals and camouflage (Cuthill *et al.*, 2017), but application of scanning electron microscopy (SEM) and high-speed photography suggest that pigmentation-dependent structural changes themselves might influence how birds fly and snakes slither. In some cases, then, interactions between a species’ abiotic environment (e.g., wind speed or surface roughness) could be the primary force shaping color pattern evolution rather than sexual/social selection by mates or competitors. Swimming performance assays in water striders, for example, illustrate how anatomical evolution influences ecologically-relevant behavioral variation: Water striders possessing leg fans due to expression of a single gene swim more quickly upstream than water striders without fans (Santos *et al.*, 2017). These results again highlight the importance of characterizing the behavioral consequences of genes controlling structural evolution.

The precise mechanism that links gene function to phenotypic development and evolution is understood for only a few genes in a few organisms (e.g., Hoekstra *et al.*, 2006; Barrett *et al.*, 2008; Frankel *et al.*, 2011). It will remain difficult to describe why we see certain patterns of molecular evolution without this information. It will also remain difficult to understand the ecological purpose of certain phenotypes. In the case of the *yellow* gene, characterizing the behavioral consequences of a single loss-of-function mutation revealed both how *yellow* functions in behavior and why flies possess sex combs. While we were searching in the fly brain for clues, the genetic data led us to an unexpected result, teaching us something new about the biology of the fly. But, the *yellow* mutation taught us another lesson: The details connecting genes and mutations to fitness matter; they help explain the reason phenotypes are evolving (or not) in the first place.

**Cuticle structure and function: Consequences of pigmentation gene pleiotropy**
The reason *yellow* mutants were ever implicated in behavior was because an undergraduate student named Alfred Sturtevant carefully observed their abnormal mating patterns in fly bottles (Sturtevant, 1915). But, genes function in phenotypic development and evolution in numerous unobservable ways. Insects, for example, primarily communicate through chemical signaling (Blomquist and Bagnères, 2010). Sex, mating status, developmental stage, and behavioral state are all communicated through chemical pheromones in many fly, butterfly, and bee species (Blomquist and Bagnères, 2010). In *Drosophila*, pheromones are derived from lipid metabolism just beneath the cuticle that requires, in part, the function of Desaturase enzymes in specialized secretory cells called oenocytes along the abdomen (Makki *et al*., 2014). Lipids in the form of short- and long-chain hydrocarbons are secreted into the hardening cuticle after eclosion, coating the fly body in a complex mixture of compounds that elicit behavioral responses in hetero- and conspecifics (Billeter *et al*., 2009). In chapter three, I hypothesized that pigmentation enzymes, which are also secreted into the developing cuticle, might interact with lipid metabolism and pheromone production as a consequence of changes in the cuticle structure post eclosion. Surprisingly, we discovered that flies inheriting loss-of-function mutations in the enzymes Ebony and Tan showed reciprocal effects on short- versus long-chain cuticular hydrocarbon (CHC) abundance, suggesting that pigmentation and CHC synthesis might interact in the developing insect cuticle. Subsequent experiments also revealed that natural variation in *ebony* and *tan* expression co-varied with CHC synthesis in directions predicted by the mutants.

As in chapter two, results from chapter three suggest that structural changes themselves can change animal behavior. For example, in *D. melanogaster*, we observed significantly elevated levels of 7,11-heptacosadiene [a female derived pheromone important in species recognition (Billeter *et al*., 2009)] in *ebony* mutants and significantly decreased levels in *tan* mutants. Although we did not perform behavioral assays in these experiments, we speculate that changes in pigmentation intensity across the fly body could influence how well hetero- and conspecific animals perceive pheromone signals in nature. Since 7,11-heptacosadiene often inhibits courtship from species that do not synthesize it (Billeter *et al*., 2009), for example, flies evolving lighter body pigmentation as a consequence of
*ebony* expression might be harassed more often by heterospecific males due to pleiotropic changes in CHC production. Future experiments could test these hypotheses by quantifying social interactions among hetero- and conspecific flies varying in body pigmentation and 7,11-heptacosadiene abundance.

Pigmentation genes have also been implicated in vertebrate pheromone production. In mice, pleiotropic effects of alpha-melanocyte-stimulating hormone (*α*-MSH) stimulate both pigmentation and aggressive behaviors (Cone *et al*., 1996; Morgan *et al*., 2004a,b). *α*-MSH signaling at melanocortin receptor 1 (MC1R) in the epidermis promotes pigmentation, while signaling at MC5R in the preputial gland promotes aggression (Cone *et al*., 1996; Morgan *et al*., 2004a,b). Surprisingly, the mechanism that links *α*-MSH signaling to aggression is by stimulating the release of an aggression-promoting pheromone in male urine via MC5R (Morgan *et al*., 2004a,b). *α*-MSH acts pleiotropically on pigmentation and pheromone production, because it is capable of binding multiple receptors with tissue-specific expression patterns and physiological functions (Cone *et al*., 1996). It is possible that Ebony and Tan behave similarly. Rather than changing the structure of the cuticle, Ebony and Tan might instead influence CHC production through physiological changes. Since both enzymes participate in dopamine metabolism, for example, their effects on pigmentation and CHC synthesis might stem from changes in circulating dopamine levels in the hemolymph. Future studies should focus on dissecting how dopamine, Ebony, and Tan participate in lipid synthesis.

The pleiotropic impact of pigmentation genes in invertebrate and vertebrates is likely larger in magnitude than previously anticipated. Is this because pigmentation synthesis affects numerous other traits, or is it because pigmentation genes are also pheromone and behavior genes? In the case of *α*-MSH and its five receptors, the answer seems to be the latter, since *α*-MSH can affect multiple phenotypes by activating receptors in varied tissues. For Ebony and Tan, it is hard to say. Distinguishing between these two scenarios is important, however, because each implies different genetic and phenotypic modes for how behavior and pigmentation evolve. Mutations in the protein coding region of MC1R, for example, have repeatedly caused pigmentation to evolve but not behavior (Martin and
Orgogozo, 2013). Why is MC1R a hotspot for pigmentation evolution but not its ligand α-MSH? This is likely because α-MSH is capable of binding five different receptors in vertebrates, whereas the phenotypic effects of MC1R activation are restricted to the melanocytes (Cone et al., 1996). Mutations affecting α-MSH function must coordinate with numerous downstream processes. In humans, for example, loss-of-function mutations in the pre–pro–opiomelanocortin (POMC) gene, which generates α-MSH, causes a range of phenotypic effects from red hair color to obesity to adrenal insufficiency (Krude et al., 1998). These data suggest that protein coding changes at MC1R are repeatedly permissible because MC1R expression is restricted to a subset of α-MSH-positive tissue. As discussed above, protein coding changes at Ebony, Tan, and Yellow have never been implicated in pigmentation evolution in Drosophila, but there are numerous instances of tissue-specific, cis-regulatory changes affecting pigmentation at these genes (Massey et al., 2016). The biochemical function of Ebony, Tan, and Yellow likely restrict the position of new mutations influencing pigmentation evolution to non-coding regions of DNA. Unlike α-MSH, these enzymes do not rely on receptors expressed in pigmentation-specific cells to carry out their function. Instead, their synthesized products (dopamine derivatives) participate directly in pigmenting the cuticle (Wright 1987). As illustrated in chapters one and two, mutations disrupting their coding sequence impact phenotypes important in reproduction. Gene pleiotropy, therefore, manifests in different ways in different taxa depending on the function of the gene in question, which in turn influences how mutations can shape their contribution to phenotypic evolution.

How behavioral and anatomical divergence map onto the genome

While chapters two and three focused on the specific effects of single-gene loss-of-function mutations in behavior and anatomy, chapter four investigated how the genome more generally organizes phenotypic evolution at these traits. Surprisingly, QTL mapping identified a small region on the X chromosome that explains the majority of variation for wing spot and wing display divergence between D. elegans and D. gunungcola. These
results confirmed previous evidence suggesting a role for the X chromosome in divergence between these species (Yeh et al., 2006; Yeh and True, 2014). We then attempted to dissect the genetic basis of these QTL effects on both traits, discovering that, in isolation, the QTL controlling wing spot divergence did not control wing display divergence. Rather, the effects of the X-linked wing display QTL seemed to require the effects of QTL on the autosomes to be detected. These results, unexpectedly, implied that epistasis played an important role in behavioral divergence between D. elegans and D. gunungcola.

Multiple studies have investigated how correlated behavioral and anatomical evolution map onto the genome. In one example, QTL affecting schooling position in the threespine stickleback co-localized with QTL affecting the evolution of bony plates and the number of neuromasts in a locomotor sense organ (Greenwood et al., 2013). Similarly, in cavefish, QTL affecting eye size co-localized with QTL affecting locomotor behavior and sensory receptor number (Yoshizawa et al., 2012). And, in Heliconius butterflies, QTL affecting wing pigmentation co-localized with QTL affecting mate preference (Kronforst et al., 2006). Genetic dissections in these systems is exceedingly difficult, however, and to date no study has succeeded in disentangling whether physical linkage or gene pleiotropy underlie patterns of co-localized QTL for behavioral and anatomical divergence. But, preliminary results from multiple introgression analyses suggest a pattern: When behavioral QTL are repeatedly backcrossed from one genetic background into another, their effects tend to disappear (see results in chapter four; Jessica Cande, pers. comm; Yun Ding, pers. comm; Dolph Schluter, pers. comm). Behavioral QTL might often behave epistatically with loci that in combination build behavioral differences within and between species. Despite these “negative results” future studies should publish their attempts at fine-mapping behavioral QTL even if they ultimately fail, since these data will help determine if this is a common pattern.

How might epistatic interactions shape the evolution of behavior and its correlated anatomical traits? In the case of D. elegans and D. gunungcola divergence, previous work indicates that wing spots and wing displays were lost in D. gunungcola (Prud’homme et
al., 2006). Results from chapter four suggest that mutations disrupting the wing spot QTL on the X chromosome led to wing spot loss in *D. gunungcola,* but, these same (or physically linked) mutations did not by themselves cause wing display loss. Without genotype or phenotype information from more ancestral *D. gunungcola* populations, it is difficult to predict exactly how the genetic architecture for wing spots and wing displays evolved over time, however, field observations of wild *D. gunungcola* (all of which lack wing spots) courtship behavior suggest that elements of wing display behavior remain intact in the *D. gunungcola* genome. It is exciting to speculate that these observations reflect something about the epistatic nature of wing display divergence between these species. That is, data from chapter four suggest that multiple mutations on multiple chromosomes are required for wing display divergence in *D. gunungcola,* but mutations at a single locus are required for wing spot divergence. These preliminary results compel future work to study the genetic basis of natural phenotypic variation within and between *D. gunungcola* populations.

More generally, epistatic interactions between QTL affecting behavior have important implications for how behaviors might evolve. Results from epistatic analysis within individual proteins, for example, indicate that epistasis can limit the paths of mutations altering gene function, since the effects of new mutations change depending on their location within the protein (reviewed in Phillips, 2008). In a similar way, epistatic interactions among behavioral QTL might impact which genes or which types of mutations cause behaviors to evolve depending on their combined function in the nervous system. In addition, epistatic signatures in QTL mapping studies might reflect genetic robustness for the behavioral trait under investigation. In *D. elegans,* for example, advanced interspecific recombinants that inherited more than 5 Mb of *D. gunungcola* DNA on the X chromosome performed wing displays normally. If wing displays impact male mating success, sexual selection in the *D. elegans* genome could have created redundant mechanisms to maintain wing display performance in the face of new mutations. In any case, far more work needs to focus on the biological implications of epistatic interactions underlying the evolution of behavior and other complex traits.
Conclusions

In this thesis I performed a series of genetic analyses aimed at dissecting the mechanisms controlling correlations between mating behavior and pigmentation in *Drosophila*. Each chapter yielded surprising data about the interactions between these traits. Together, these results underscore a major theme: Understanding the specific function genes play in development and evolution helps reveal not only how and why phenotypes change within and between species but also new insights into the nature of phenotypes themselves.
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Appendices
Appendix A

Supplementary Figures and Tables for Chapter 2

Supplemental Figure S2-1  *yellow* expression in *dsx*-expressing cells is necessary and sufficient for male mating success

(A) Expressing *yellow-RNAi* in *dsx*-expressing cells using *dsx*\textsuperscript{GAL4} \cite{Rideout2010} significantly inhibited male mating success. (B) Expressing *yellow* in *dsx*-expressing cells using *dsx*\textsuperscript{GAL4} in a *y1* mutant background was sufficient to restore male mating success. (C) Expressing *yellow-RNAi* using *dsx*\textsuperscript{GAL4} \cite{Rideout2010} partially reduced black melanin levels in the male A5 and A6 abdominal tergites, consistent with prior work \cite{Williams2008,Rogers2014,Kalay2016}. (D) Expressing *yellow* using *dsx*\textsuperscript{GAL4} partially elevated black melanin levels in the male A5 and A6 abdominal tergites. Sample sizes are shown at the top of each barplot. Significance was measured using Chi-square tests with Bonferroni corrections for multiple comparisons. **$P<0.01$, ***$P<0.001$, ****$P<0.001$. 
**A**

Predicted dsx binding site
Clough et al. (2014)

Putative MRS enhancer
Drapeau et al. (2006)

**B**

wt  
\(\text{y1} \)  
\(\text{y1}:\text{wing-body-GAL4} > \text{UAS-yellow} \)  
\(\text{y1}:\text{wing-body-GAL4} > \text{UAS-yellow-RNAi} \)

**C**

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<tr>
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<tr>
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<td>+</td>
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<tr>
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**D**

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**E**

\(\text{wing-body-GAL4} > \text{UAS-myr-GFP} \)

**F**

-292 bp

\(-3xP3::\text{DsRed} \)

\(-\text{attP} \)

\(-\text{LoxP Site} \)

\(-\text{UTR} \)

**G**

1 kb ladder
-292 bp
-134 bp

**H**

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\(\text{myrGFP} \)
\(\text{nC82} \)
\(\text{♂} \)
\(\text{♀} \)
Supplemental Figure S2-2 The mating regulatory sequence (MRS) from Drapeau et al. (2006) does not affect male mating success

(A) Diagram of the yellow locus highlighting the putative “mating regulatory sequence” (MRS) (pink) region mapped in Drapeau et al. (2006) and a predicted dsx binding site (yellow) identified by ChIP-seq in Clough et al. (2014). The predicted binding site was identified based on in vivo Doublesex occupancy data (PWM score = 88.7) localized between 356,273 and 356,286 bp on the X chromosome (see Supplementary Table S2 in Clough et al., 2014). The wing-body enhancer region is indicated in blue, which was cloned upstream of GAL4 in Gilbert et al. (2006) to make the wing-body-GAL4 line. (B) Expressing yellow-RNAi using wing-body-GAL4 reduced black melanin to y1 levels, and expressing yellow in a y1 mutant background using wing-body-GAL4 restores black melanin synthesis to wild-type (wt) levels. (C) Expressing yellow-RNAi using wing-body-GAL4 did not inhibit male mating success. (D) Expressing yellow using wing-body-GAL4 in a y1 mutant background did not restore male mating success. (E) Brain and VNC of adult male and female flies stained with anti-GFP (green) antibody for myrGFP expressed using wing-body-GAL4 and counterstained with anti-nC82 (magenta) for neuropil. (F) Diagram illustrating the CRISPR/Cas9-facilitated homology-directed repair (HDR) strategy used to excise and replace the MRS (pink) with pHD-DsRed-attP (red) (Gratz et al., 2014). Two sgRNAs (pink letters) were designed towards target PAM sites (blue letters) at the most 5’ and 3’ bounds of the MRS (scissors). Sanger sequencing chromatograms illustrate the location of each cut site (black arrows) relative to the transcription start site. DsRed was removed using Cre-lox recombinase (Siegal and Hartl 1996). (G) PCR validation of DsRed removal and MRS deletion. (H) Excising the putative MRS did not inhibit male male mating success. Sample sizes are shown at the top of each barplot. Significance was measured using Chi-square tests with Bonferroni corrections for multiple comparisons.

***P<0.001. n.s., not significant.
Supplemental Figure S2-3 Expressing yellow-RNAi in subsets of CNS tissue does not affect male mating success

(A,B) Expressing yellow-RNAi using a series of CNS, dopaminergic, and serotonergic GAL4 drivers did not affect male mating success. Significance was measured using Chi-square tests with Bonferroni corrections for multiple comparisons. Sample sizes are shown at the top of each barplot. Significance was measured using Chi-square tests with Bonferroni corrections for multiple comparisons. n.s., not significant.

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<td>3740</td>
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<td>GAL4 expressed in neuroblasts and neurons.</td>
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<td>(ple-GAL4) Expresses GAL4 in dopaminergic cells (gift from Shinya Yamamoto)</td>
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<td>7010</td>
<td>(Ddc-GAL4) Expresses GAL4 in dopaminergic and serotonergic neurons under the control of Ddc (gift from Shinya Yamamoto)</td>
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<td>TPH-GAL4</td>
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\[
\text{Percent mated in 60 min}\]

\[
\text{ns}
\]
Supplemental Figure S2-4 Expression pattern of 42D04-GAL4

(A,B) Brain and VNC of adult female fly stained with anti-GFP (green) antibody for myrGFP expressed using 42D04-GAL4 and counterstained with anti-nC82 (magenta) for neuropil. (C) L3 larval female genital disc stained with anti-GFP (green) antibody for cytGFP expressed using 42D04-GAL4, anti-Dil (red) for Distal-less expression, and counterstained with DAPI (blue) for DNA (courtesy of Janelia Fly Light). (D) Adult female genitalia native cytGFP (green) expressed using 42D04-GAL4. (E) L3 CNS native cytGFP (green) expressed using 42D04. (F) L3 larval male genital disc stained with anti-GFP (green) antibody for cytGFP expressed using 42D04-GAL4, anti-Dil (red) for Distal-less expression, and counterstained with DAPI (blue) for DNA (courtesy of Janelia Fly Light). (G) Adult male genitalia did not show native cytGFP expression using 42D04-GAL4. (H) L3 larval posterior spiracle (white arrowhead) native cytGFP (green) expression. (I) L3 larva whole body highlighting native cytGFP (green) expression in the genital disc (white arrowhead). (J) Expressing yellow-RNAi using 42D04-GAL4 does not affect body pigmentation relative to wild-type (wt) flies.
Supplemental Figure 2-5 yellow EGFP reporters localize yellow sex comb expression to the intronic bristle enhancer

(A) Diagram of the yellow locus highlighting two D. melanogaster enhancer regions [5’ up including the wing, body, and putative MRS enhancers reported in Geyer and Corces (1987), Martin et al., (1989), and Drapeau et al., (2006); and intron, including the bristle and putative sex comb enhancer reported in Geyer and Corces (1987) and Martin et al., (1989)] that were cloned upstream of an EGFP reporter in Kalay and Wittkopp (2010). (B) Confocal image of a 96 h old (APF) pupal sex comb expressing cytGFP under the control of the 5’ up enhancer region. (C) Confocal image of a 96 h APF pupal sex comb expressing cytGFP under the control of the intronic enhancer region, highlighting expression in bristle sockets, sex comb sockets, and sex comb teeth.
Supplemental Figure S2-6 Genetic dissection of the 42D04-GAL4 enhancer confirms the specific role of sex comb melanization, and not the aedeagus, in male mating success

(A) Expressing Laccase2-RNAi using 42D04-GAL4 blocked melanin synthesis in the aedeagus. (B) Diagram of the male exon structure of the dsx locus highlighting the strategy used to dissect the 42D04-GAL4 expression pattern. Five new GAL4 lines were created by synthesizing different sized sub-fragments of the 42D04-GAL4 enhancer fragment and cloning them upstream of GAL4 (see Supplemental Materials and Methods). Note, 42D04_B-GAL4 could not be maintained, since female flies expressing GAL4 using this enhancer region were all sterile and showed necrotic growths on their genitalia. (C) Expression pattern of 42D04_A,C,D, and E-GAL4 lines. Expressing cytGFP using 42D04_A-GAL4 showed GFP (green) localized to bristle sockets, and 42D04_E-GAL4 shows bright GFP in the sex comb and lower leg region. 42D04_C-GAL4 and 42D04_D-GAL4 did not show GFP expression in the legs. Expressing Laccase2-RNAi using 42D04_A-GAL4 and 42D04_E-GAL4 blocked melanin synthesis in the sex combs but not the aedeagus. (D) Expressing Laccase2-RNAi using 42D04_A-GAL4 and 42D04_E-GAL4 inhibited male mating success. Sample sizes are shown at the top of each barplot. Significance was measured using Chi-square tests with Bonferroni corrections for multiple comparisons. **P<0.01, ***P<0.001, ****P<0.001. n.s., not significant.
Supplemental Figure S2-7 *Drosophila* species with varying sex comb morphology used for high-speed video assays

Supplementary Figure S2-8 Sex comb melanization is required for male mating success with y1 females

(A) y1 males showed increased male mating success with y1 females. (B) Expressing Laccase2-RNAi using 42D04-GAL4 in males significantly inhibited male mating success with y1 females. Significance was measured using Chi-square tests with Bonferroni corrections for multiple comparisons. **P<0.01, ***P<0.001. n.s., not significant.
Chapter 2 Graphical Abstract
Appendix B

Supplementary Figures and Tables for Chapter 3

Supplemental Figure S3-1 Effects of CRISPR/Cas9 gene editing on *ebony* coding region

(A) Schematic of the *ebony* gene in *Drosophila melanogaster*, highlighting the sites of two gRNAs (red lines) targeted to the first coding exon. (B) DNA target sequences used to synthesize two gRNAs to direct Cas9 to *ebony*'s first coding exon. Both target sequences were previously published in Ren et al. (2014), showing the highest heritable germline transformation rate. (C) *ebony<sub>CRISPR(1,2)</sub>* contains a 55 bp deletion in the first coding exon that caused a frame-shift in *ebony*'s coding sequence. (D) *ebony<sub>CRISPR(3)</sub>* contains an in-frame 3 bp deletion in the first coding exon. (E) *ebony<sub>CRISPR(4)</sub>* contains an in-frame 3bp deletion in the first coding exon, shift 1 bp upstream of the *ebony<sub>CRISPR(3)</sub>* deletion.
Supplemental Figure S3-2 *ebony*<sup>CRISPR(3)</sup> and *ebony*<sup>CRISPR(4)</sup> show darker body pigmentation but no CHC lengthening effect.

(A) Photographs highlighting the effects of *ebony*<sup>CRISPR(3)</sup> and *ebony*<sup>CRISPR(4)</sup> mutations on body pigmentation. (B) Difference in log-contrast of relative CHC intensity between *ebony*<sup>CRISPR(3)</sup> and un-injected vasa-Cas9 control flies. (C) Difference in log-contrast of relative CHC intensity between *ebony*<sup>CRISPR(4)</sup> and un-injected vasa-Cas9 control flies.
Female GAL4 lines were crossed with either the male UAS-ebony-RNAi effector line or a w1118 CS control line, and males from the UAS-ebony-RNAi effector line were crossed to the same w1118 CS control line for comparison. (A) Difference in log-contrast of relative CHC intensity between females expressing ebony-RNAi under the control of the dsxGAL4 and dsxGAL4/+ control females. (B) Difference in log-contrast of relative CHC intensity between females expressing ebony-RNAi under the control of dsxGAL4 and UAS-ebony-RNAi/+ control females. (C) Difference in log-contrast of relative CHC intensity between females expressing ebony-RNAi under the control of the oenocyte driver OK72-GAL4 and UAS-ebony-RNAi/+ control females.
Difference in log-contrast of relative CHC intensity between *ebony*<sub>CRISPR(4)</sub> females fed 36 mM L-AMPT and *ebony*<sub>CRISPR(4)</sub> females fed a solvent control.

Spearman’s rho = -0.57

\[ P = 0.009 \]
Supplemental Figure S3-5 Feeding WT (\textit{w}^{1118} \textit{CS}) females L-DOPA precursor causes a slight CHC shortening effect

(A) Difference in log-contrast of relative CHC intensity between \textit{wild-type} (\textit{w}^{1118} \textit{CS}) fed 1mM methyl L-DOPA hydrochloride (L-DOPA precursor) and \textit{w}^{1118} \textit{CS} fed a solvent control. (B) Difference in log-contrast of relative CHC intensity between \textit{wild-type} (\textit{w}^{1118} \textit{CS}) fed 10mM L-DOPA precursor and \textit{w}^{1118} \textit{CS} fed a solvent control.
Supplemental Figure S3-6 DGRP lines with lightly pigmented A6 abdominal tergites show a CHC shortening effect

Pigmentation scores and CHC data were obtained from Dembeck et al. (2015a, b). (A) Difference in log-contrast of relative CHC intensity between DGRP females with darkly-pigmented 6th abdominal tergites (A6) (3 < score, N = 51) and the 155 line average. (B) Difference in log-contrast of relative CHC intensity between DGRP females with intermediately-pigmented A6 (2 < score ≤ 3, N = 55) and the 155 line average. (C) Difference in log-contrast of relative CHC intensity between DGRP females with lightly-pigmented A6 (score ≤ 2, N = 49) and the 155 line average.
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†Br: methyl branched; T: tricosene; P: pentacosene; H: heptacosene; TD: tricosadiene; PD: pentacosadiene; ND: nonacosadiene

Supplemental Table S3-1 Common CHCs in female *D. melanogaster*
Supplemental Figure S4-1 ImageJ procedure for measuring maximum wing display angles

Screenshots of each wing display were captured for every recombinant courtship video. The maximum wing display bout was identified for each fly by quickly comparing screenshots that varied in wing display angles (from wing tip to wing tip) and picking by eye the display with the largest angle. Next, for each fly, the maximum wing display angle was quantified in ImageJ by using the 1) Find Edges function, 2) polygon tool to Fit Ellipse around the fly body, 3) Ellipse Macros (Supplemental File S2) to fit the major and minor axes of the ellipse, and 4) draw Angle tool, fitting the angle vertex at the major and minor axes intersection to calculate the wing display angle from wing tip to wing tip.
Supplemental Figure S4-2 LOD scores estimated from a two-dimensional, two QTL scan of maximum wing display angles

(A) For the *D. elegans* backcross, the Interaction LODi (see Supplementary Table S1) is displayed in the upper left triangle; the Full LODf (see Supplementary Table S1) is displayed in the lower right triangle. The color scale on the right indicates LOD values for LODi (left) and LODf (right). (B) For the *D. gunungcola* backcross, the Interaction LODi (see Supplementary Table S2) is displayed in the upper left triangle; the Full LODf (see Supplementary Table S2) is displayed in the lower right triangle. The color scale on the right indicates LOD values for LODi (left) and LODf (right).
Supplemental Figure S4-3 Genome-wide frequency and distribution of recombination breakpoints

Each histogram summarizes the frequency and distribution of recombination breakpoints for every Muller Element. The x-axis represents the physical map of each Muller Element measured in bp. On average, there were only two breakpoints per genome per individual recombinant.

Total breaks = 2083
Avg. # breaks per indiv. = 2.19

X: 645 breaks
0.68 per indiv.

B: 445 breaks
0.47 per indiv.

C: 371 breaks
0.39 per indiv.

D: 375 breaks
0.39 per indiv.

E: 234 breaks
0.25 per indiv.

F: 13 breaks
0.01 per indiv.
Supplemental Figure S4-4 QTL analysis for wing spot size, excluding spotless individuals

Wing pigmentation QTL map for the *D. elegans* (red) and *D. gunungcola* (blue) backcross, excluding spotless recombinants. LOD (logarithm of the odds) is indicated on the y-axis. The x-axis represents the physical map of Muller Elements X, B, C, D, E, and F based on the *D. elegans* assembled genome. Individual SNP markers are indicated with black tick marks along the x-axis. Horizontal red and blue lines mark p = 0.01 for the *D. elegans* and *D. gunungcola* backcross, respectively.
Supplemental Figure S4-5 Representative genome-wide ancestry assignments for seven individuals from the X chromosome introgression experiment

The ancestry states are shown for each Muller element for representative male individuals from F4-F9 backcross generations. The posterior probability that a region is homozygous (Andolfatto et al., 2011) for *D. elegans* (red) or *D. gunungcola* (blue) is plotted along the y-axis. Individual SNP markers are indicated with red or blue tick marks along the x-axis. Crossover positions are indicated by a switch from red to blue or blue to red along each chromosome, representing a shift from *D. elegans* to *D. gunungcola* ancestry or *D. gunungcola* to *D. elegans* ancestry, respectively.
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*P < 0.05, ** P < 0.01, *** P < 0.001

<sup>a</sup> Maximum LOD score for the full model with interactions allowed

<sup>b</sup> Difference between the Full LOD and the maximum single-QTL LOD for the chromosome pair

<sup>c</sup> Difference between the maximum Full and Full Additive LODs

<sup>d</sup> Maximum LOD score for two QTLs with only additive interactions allowed

<sup>e</sup> Difference in LODs between the Full Additive model and the maximum single QTL model for the chromosome pair

Supplemental Table S4-1 Results of two-QTL scan for max wing display angle in D. elegans backcross
<table>
<thead>
<tr>
<th>Chromosomes</th>
<th>Full&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Two QTL&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Interaction&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Full Additive&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Two Additive&lt;sup&gt;e&lt;/sup&gt;</th>
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<tr>
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<td>0.00193</td>
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<td>X:D</td>
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<td>1.37202</td>
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*P < 0.05, ** P < 0.01, *** P < 0.001

<sup>a</sup> Maximum LOD score for the full model with interactions allowed

<sup>b</sup> Difference between the Full LOD and the maximum single-QTL LOD for the chromosome pair

<sup>c</sup> Difference between the maximum Full and Full Additive LODs

<sup>d</sup> Maximum LOD score for two QTLs with only additive interactions allowed

<sup>e</sup> Difference in LODs between the Full Additive model and the maximum single QTL model for the chromosome pair

Supplemental Table S4-2 Results of two-QTL scan for max wing display angle in D. gunungcola backcross
<table>
<thead>
<tr>
<th>Trait</th>
<th>Backcross</th>
<th>Chromosome</th>
<th>QTL interval (bp)$^a$</th>
<th>QTL peak (bp)</th>
<th>LOD</th>
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<td>Wing spot size</td>
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<td>E</td>
<td>10,907-4,009,870</td>
<td>12,292</td>
<td>6.85</td>
</tr>
</tbody>
</table>

$^a$ LOD drop 1.5 support interval

Supplemental Table S4-3 QTLs detected for wing spot size, excluding spotless individuals
Appendix D

Book chapter: The genetic basis of pigmentation differences within and between species
The Genetic Basis of Pigmentation Differences Within and Between Drosophila Species

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Abstract

In Drosophila, as well as in many other plants and animals, pigmentation is highly variable both within and between species. This variability, combined with powerful genetic and transgenic tools as well as knowledge of how pigment patterns are formed biochemically and developmentally, has made Drosophila pigmentation a premier system for investigating the genetic and molecular mechanisms responsible for phenotypic evolution. In this chapter, we review and synthesize findings from a rapidly growing body of case studies examining the genetic basis of pigmentation differences in the abdomen, thorax, wings, and pupal cases within and between Drosophila species. A core set of genes, including genes required for pigment synthesis (eg, yellow, ebony, tan, Dat) as well as developmental regulators of these genes (eg, bab1, bab2, omb, Dil, and wg), emerge as the primary sources of this variation, with most genes having been shown to contribute to pigmentation differences both within and between species. In cases where specific genetic changes contributing to pigmentation divergence...
were identified in these genes, the changes were always located in noncoding sequences and affected cis-regulatory activity. We conclude this chapter by discussing these and other lessons learned from evolutionary genetic studies of Drosophila pigmentation and identify topics we think should be the focus of future work with this model system.

1. INTRODUCTION

Heritable changes in DNA sequence within and among species explain much of life’s diversity. Identifying these changes and understanding how they impact development to generate phenotypic differences remains a major challenge for evolutionary biology. A growing number of case studies have localized the specific genes involved in trait variation both within and among species, and some have described how individual mutations affect the developmental pathways underlying phenotypic differences. With a catalog of studies describing more than 1000 alleles contributing to morphological, physiological, or behavioral evolution of diverse traits in diverse species now available, researchers have begun to synthesize the genetic and developmental mechanisms underlying phenotypic evolution in search of genetic and molecular patterns that underlie the evolutionary process (Carroll, 2008; Kopp, 2009; Martin & Orgogozo, 2013; Stern & Orgogozo, 2008; Streisfeld & Rausher, 2011).

One finding from this synthesis is that different types of traits tend to evolve through different molecular mechanisms. For example, changes in cis-regulatory DNA sequences that regulate gene expression contribute to morphological differences within and among species more often than they contribute to differences in physiological traits, while the converse is true for changes in the amino acid sequence of proteins (Stern & Orgogozo, 2008, 2009). Another finding to emerge from this synthesis is that some traits have evolved multiple times independently using the same genetic changes (eg, xenobiotic resistance), whereas other traits have evolved similar changes using different mutations in the same gene (eg, coat color) or using different genes (Martin & Orgogozo, 2013). Differences in the genetic basis of phenotypic diversity also seem to exist within and between species, with changes in cis-regulatory sequences playing a larger role in interspecific than intraspecific differences (Coolon, Mcmanus, Stevenson, Graveley, & Wittkopp, 2014; Stern & Orgogozo, 2008; Wittkopp, Haerum, & Clark, 2008).
In this chapter, we examine patterns in the genetic and molecular mechanisms responsible for phenotypic evolution that emerge from focusing on a collection of studies investigating changes in a single trait within and among species in the same genus. Specifically, we review and synthesize the collection of case studies dissecting the genetic basis of body color (pigmentation) in *Drosophila*, emphasizing a comparison of genetic and molecular mechanisms that vary within and among *Drosophila* species. *Drosophila* pigmentation is an ideal trait for such an analysis because (i) pigmentation is one of the most variable traits within and among species \( (\text{Kopp, 2009; Wittkopp, Carroll, & Kopp, 2003}) \), (ii) much is known about the genes involved in pigment synthesis as well as those that control expression of these genes during *Drosophila* development \( (\text{Kopp, 2009; Takahashi, 2013; True, 2003; Wittkopp, Carroll, et al., 2003}) \), and (iii) specific genes and genetic changes have been identified as contributing to differences in *Drosophila* pigmentation that have evolved over multiple timescales and in multiple lineages \( (\text{Table 1}) \). These differences in pigmentation that have been dissected genetically include examples of trait divergence, convergent evolution, and evolutionary novelty.

## 2. DEVELOPMENT OF DROSOPHILA PIGMENTATION

In *Drosophila* (as well as in many other insects; \( \text{True, 2003; Wittkopp & Beldade, 2009; Zhan et al., 2010}) \), body color results from a combination of dark black and brown melanins as well as light yellow-tan and colorless sclerotins \( (\text{True, 2003; Wittkopp, Carroll, et al., 2003; Wright, 1987}) \). These four types of pigments are produced by a branched biochemical pathway that processes tyrosine obtained from the diet \( (\text{Fig. 1}) \). Tyrosine is first converted into DOPA (\( l\)-3,4-dihydroxyphenylalanine) by a tyrosine hydroxylase (TH) encoded by the *pale* gene. This DOPA is then converted into dopamine through a reaction catalyzed by the dopa decarboxylase enzyme encoded by the *Ddc* gene. Prior reviews have suggested that DOPA can also be polymerized into a black melanin through a process involving the Yellow protein \( (\text{Kopp, 2009; Wittkopp, Carroll, et al., 2003}) \), but recent data show that the formation of black pigment requires the function of *Ddc* \( (\text{J.-M. Gibert, personal communication}) \) and is thus likely produced from dopamine rather than DOPA, as has also been previously suggested \( (\text{Riedel, Vorkel, & Eaton, 2011; Walter et al., 1996}) \). Dopamine can then have one of four fates: it can be converted into a black melanin through a
Table 1 The Loci of Pigmentation Evolution in *Drosophila*

<table>
<thead>
<tr>
<th>Level of Variation</th>
<th>Trait</th>
<th>Species</th>
<th>Gene(s)</th>
<th>Gene Function</th>
<th>Type of Mutation(s)</th>
<th>References</th>
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<tbody>
<tr>
<td>Within species</td>
<td>Abdominal</td>
<td><em>D. melanogaster</em></td>
<td>bab</td>
<td>TF</td>
<td>cis-Regulatory</td>
<td>Rogers et al. (2013)</td>
</tr>
<tr>
<td>Within species</td>
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<td>TF</td>
<td>cis-Regulatory</td>
<td>Bastide et al. (2013)</td>
</tr>
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<td>Within species</td>
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<td><em>D. melanogaster</em></td>
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<td>TF</td>
<td>cis-Regulatory</td>
<td>Endler, Betancourt, Nolte, and Schlötterer (2016)</td>
</tr>
<tr>
<td>Within species</td>
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<td>bab</td>
<td>TF</td>
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<td>Kopp, Graze, Xu, Carroll, and Nuzhdin (2003)</td>
</tr>
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<td>bab1</td>
<td>TF</td>
<td>cis-Regulatory</td>
<td>Bickel, Kopp, and Nuzhdin (2011)</td>
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<td>Enzyme</td>
<td>cis-Regulatory</td>
<td>Pool and Aquadro (2007)</td>
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<td>ebony</td>
<td>Enzyme</td>
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<th>Species</th>
<th>Gene(s)</th>
<th>Gene Function</th>
<th>Type of Mutation(s)</th>
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This table summarizes case studies that have examined the genetic basis of pigmentation differences within and between species of *Drosophila*. 
process involving the Yellow protein and phenol oxidases (POs); converted into a brown melanin through a process involving POs, but not yellow; into a yellow-tan sclerotin through the activity of Ebony converting dopamine into beta-alanyl dopamine (NBAD) and POs polymerizing it into NBAD sclerotin, or into a colorless pigment through the activity of dopamine-acetyl-transferases (DATs) converting dopamine into N-acetyl dopamine (NADA) and POs polymerizing it into NADA sclerotin. One of these reactions, the conversion of dopamine into NBAD, is reversible, with the reverse reaction catalyzed by the Tan protein. Disruption of the tan gene reduces the production of dark melanins, indicating that the conversion of NBAD back into dopamine is a necessary step in the development of pigmentation. Changing relative expression levels of yellow, ebony, and/or tan can shift the balance between dark (black, brown) and yellow-tan pigments.
as this branched biochemical pathway produces more of one type at the expense of the other (Wittkopp et al., 2009; Wittkopp, True, & Carroll, 2002).

Pigments produced by this biochemical pathway are deposited into the developing cuticle during late pupal and early adult stages (Kraminsky et al., 1980; Sugumaran, Giglio, Kundzicz, Saul, & Semensi, 1992; Walter et al., 1996; Wittkopp, Carroll, et al., 2003). The spatial distribution of these pigments is determined in a nearly cell autonomous manner by spatially regulated transcription of genes such as yellow, tan, and ebony. As discussed in detail later, changes in the expression patterns of these genes often underlie evolutionary changes in pigmentation. Genes regulating expression of these pigment synthesis genes are thus also potential targets for genetic divergence contributing to pigmentation diversity. Five transcription factors (bric-a-brac (bab), abdominal-B (Abd-B), doublesex (dsx), Distal-less (Dll), and Engrailed (en)) have been shown to regulate expression of pigment synthesis genes (yellow, ebony, tan) in Drosophila either directly (by binding to transcription factor binding sites located in enhancers controlling the gene’s expression) or indirectly (by influencing abundance, activity, or binding of direct regulators; Fig. 1; Arnoult et al., 2013; Gompel et al., 2005; Jeong et al., 2006; Kopp, Duncan, Godt, & Carroll, 2000; Williams et al., 2008). For example, in at least one Drosophila species each, En (Gompel et al., 2005), Dll (Arnoult et al., 2013), and Abd-B (Jeong et al., 2006) have all been shown to directly bind to yellow enhancers, whereas Abd-B and Dsx (including both the male (dsxM) and female (dsxF) forms of dsx) have been shown to directly bind to enhancers of the bab gene (Williams et al., 2008). It is not yet known whether Bab proteins directly bind to enhancers of any pigment synthesis genes, but it is clear that Bab proteins affect expression of pigment synthesis genes in some manner (Kopp, 2009). Similarly, Wingless (Wg, a ligand for a signal transduction pathway) (Koshikawa et al., 2015; Werner, Koshikawa, Williams, & Carroll, 2010) has also been shown to influence expression of at least one pigment synthesis gene (Fig. 1), although questions remain about the precise molecular mechanisms by which it does so. Additional transcription factors with effects on abdominal pigmentation in Drosophila melanogaster have been identified in recent RNAi screens (Kalay, 2012; Rogers et al., 2014), but the ways in which they alter expression of pigment synthesis genes remain unknown. Elucidating the structure and complexity of the gene network regulating expression of pigment synthesis genes (and hence pigmentation) remains one of the biggest challenges for understanding the development and evolution of Drosophila pigmentation within and between species.
3. TISSUE-SPECIFIC REGULATION OF PIGMENTATION

Null mutations disrupting the function of proteins required for pigment synthesis such as TH, DDC, Yellow, Tan, Ebony, DATs, and POs alter pigmentation throughout the fly, whereas mutations in specific enhancers of these genes and mutations affecting transcriptional regulators of these genes typically alter pigmentation in only some parts of the fly. Evolutionary changes in pigmentation are often restricted to specific body parts, suggesting that such changes are likely to result from these latter types of mutations. One reason for this may be that null mutations in pigment synthesis genes often also alter behavior and/or other phenotypes in addition to pigmentation (Takahashi, 2013; True, 2003; Wittkopp & Beldade, 2009), making null mutations unlikely to survive in natural populations. The presence of tissue-specific enhancers for pigment synthesis genes coupled with differences in the sets of regulators that interact with each enhancer provide genetic mechanisms for overcoming these pleiotropic constraints and altering pigmentation independently in different body parts. Because the developmental control of pigmentation in different body regions often involves different regulatory genes, we have chosen to structure our review of the genetic mechanisms underlying pigmentation differences within and between Drosophila species by body part, examining the evolution of abdominal pigmentation, thorax pigmentation, wing pigmentation, and pupal pigmentation in Drosophila separately below.

4. ABDOMINAL PIGMENTATION

Abdominal pigment patterns (especially those on the dorsal side of the abdomen) are conspicuous and highly variable within and among species (Wittkopp, Carroll, et al., 2003; Rebeiz, Pool, et al., 2009; Rebeiz, Ramos-Womack, et al., 2009). It is not surprising then that most studies of genetic mechanisms underlying pigmentation differences in Drosophila have attempted to explain differences in intra– and interspecific abdominal pigmentation. These abdominal pigment patterns are displayed in a series of overlapping tergites that can vary in pigment color, pattern, and intensity among individuals and sexes in the same population, different populations, and different species (Kronforst et al., 2012; Wittkopp, Carroll, et al., 2003). For example, in D. melanogaster females, the most prominent abdominal tergites (A2–A6) show a “stripe” of dark melanins at the posterior edge of the
segment as well as a peak of this dark color along the dorsal midline (Fig. 2, left). In male *D. melanogaster*, this pattern is seen in the A2, A3, and A4 tergites, but A5 and A6 are much more completely covered by dark melanins (Fig. 2, right). Sexually dimorphic pigmentation is absent in many species, however, with both sexes showing the same pigmentation pattern in all segments (Kopp et al., 2000). The pattern of pigmentation within each segment can also vary, with modifications to the shape of the stripe, unique patterns such as spots, and melanins distributed evenly throughout the abdomen as seen in different species (Wittkopp, Carroll, et al., 2003). Differences in abdominal pigmentation are generally assumed to result from adaptation, but the selection pressures responsible for the evolution of a particular pattern in a particular species remain unclear. Potential selection pressures proposed for divergent abdominal pigmentation include sexual selection resulting from mate choice as well as environmental factors that differ across gradients of altitude, latitude, temperature, humidity, and UV radiation (Bastide, Yassin, Johanning, & Pool, 2014; Brisson, De Toni, Duncan, & Templeton, 2005; Capy, David, & Robertson, 1988; Clusella-Trullas & Terblanche, 2011; Kopp et al., 2000; Matute & Harris, 2013; True, 2003; Wittkopp et al., 2011).

### 4.1 Genetic Basis of Abdominal Pigmentation Differences Within a Species

In *D. melanogaster*, the most studied of all *Drosophila* species, abdominal pigmentation often varies within and among populations. For example, in sub-Saharan Africa, *D. melanogaster* collected from low elevations showed lighter
abdominal pigmentation than *D. melanogaster* collected from high elevations (Pool & Aquadro, 2007); these differences persisted when rearing these flies in the lab, demonstrating that the differences in pigmentation were caused by genetic differences rather than phenotypic plasticity. Genetic analysis implicated one or more loci on the X and 3rd chromosomes in this pigmentation difference, and analysis of the pigment synthesis gene *ebony*, which is required for the synthesis of yellow–tan pigments and is located on the 3rd chromosome, revealed distinct haplotypes in populations from different altitudes that correlated with these differences in abdominal pigmentation. Nucleotide diversity levels within this region suggested that natural selection has elevated the frequency of dark *ebony* alleles in one of the populations sampled from Uganda, possibly facilitating adaptation to different altitudes (Pool & Aquadro, 2007). Further analysis identified a cis–regulatory element in this region that controls *ebony* expression in the abdomen and showed that the allele of this sequence from a lightly pigmented fly drives higher levels of *ebony* expression than the allele of this sequence found in a more darkly pigmented fly (Rebeiz, Pool, et al., 2009), consistent with Ebony’s function in the synthesis of light-colored sclerotin (Walter et al., 1996). This region was also found to have recently accumulated multiple mutations in the Uganda population that appear to have given rise to an allele of large effect that contributes to divergence of abdominal pigmentation (Rebeiz, Pool, et al., 2009).

Genetic differences in *ebony* cis–regulatory sequences also appear to contribute to variable abdominal pigmentation in other populations of *D. melanogaster* and other species (Bastide et al., 2013; Dembeck, Huang, Magwire, et al., 2015; Endler et al., 2016; Johnson et al., 2015). For example, an association study using the Drosophila Genetic Reference Panel (DGRP) of *D. melanogaster* strains isolated from a population in Raleigh, North Carolina (Mackay et al., 2012) found a significant correlation between a noncoding variant located within a known cis–regulatory element of *ebony* and pigmentation variation within this population (Dembeck, Huang, Magwire, et al., 2015). Weak associations with noncoding SNPs in *ebony* cis–regulatory elements were also observed for European populations of *D. melanogaster* (Bastide et al., 2013; Dembeck, Huang, Carbone, & Mackay, 2015; Endler et al., 2016), with the most highly ranked SNP associated with *ebony* in Bastide et al. (2013) located in a sequence that inhibits *ebony* expression in male abdominal segments during development (Rebeiz, Pool, et al., 2009; Rebeiz, Ramos–Womack, et al., 2009). Outside of *D. melanogaster*, genetic variation linked to *ebony* has been shown to be
associated with polymorphic abdominal pigmentation within *Drosophila americana* (Wittkopp et al., 2009) and *Drosophila auraria* (Johnson et al., 2015). In this latter species, specific alleles of *ebony cis*-regulatory sequences were identified in light and dark individuals, and transgenic analyses of reporter genes were used to demonstrate the effects of these variable sites on *ebony* expression (Johnson et al., 2015). These *cis*-regulatory changes in *D. auraria* are located in a sequence that represses pigmentation in males (Johnson et al., 2015), but does not overlap with the male-specific enhancer (MSE) identified previously in *D. melanogaster* (Rebeiz, Pool, et al., 2009; Rebeiz, Ramos-Womack, et al., 2009).

The *tan* gene, which plays the opposite role of *ebony* in pigment synthesis, promoting production of dark brown melanin at the expense of yellow–tan sclerotin, also contributes to pigmentation variation within *Drosophila* species. In fact, the study that found evidence of an association between *ebony* genotype and abdominal pigmentation within the DGRP collection also identified multiple SNPs within noncoding regions near *tan* that were associated with differences in abdominal pigmentation in this population of *D. melanogaster* (Dembeck, Huang, Magwire, et al., 2015). Three of these noncoding SNPs were also found to be associated with abdominal pigmentation in European populations and an African population of *D. melanogaster* (Bastide et al., 2013; Endler et al., 2016). These SNPs were located within a *cis*-regulatory element known as the MSE (Jeong et al., 2008) that drives expression in *D. melanogaster* in the abdominal stripes as well as throughout the A5 and A6 abdominal segments with male–specific pigmentation. *tan cis*-regulatory evolution at the MSE was also recently implicated in a sex-specific color dimorphism involving abdominal pigmentation differences within *Drosophila erecta* (Yassin et al., 2016). This final case study is particularly interesting because ancient balancing selection was shown to likely be responsible for maintaining alternative alleles at the *tan* MSE and thus both light and dark morphs of female *D. erecta* (Yassin et al., 2016).

Genetic changes contributing to polymorphic pigmentation are not always caused by pigmentation synthesis genes such as *ebony* and *tan*; changes in regulatory genes upstream of the pigmentation synthesis pathway contribute to pigmentation differences segregating within a species as well. These sources of variation include genetic changes at the *bab* locus, a locus originally discovered to be an important regulator of abdominal pigmentation differences between sexes in *D. melanogaster* (Kopp et al., 2000). Null mutations in *bab* cause the development of a male–like pigmentation pattern in the A5 and A6 abdominal segments of female *D. melanogaster*, suggesting that
bab acts to repress male-specific abdominal pigmentation in females (Kopp et al., 2000). Using quantitative trait locus (QTL) mapping coupled with quantitative complementation tests to examine the genetic basis of abdominal pigmentation differences in a population of D. melanogaster from Winters, California, Kopp et al. (2003) found genetic variation at bab had a major effect on abdominal pigmentation differences in females. The bab locus includes two genes, bab1 and bab2, each of which acts as a transcriptional regulator, and it was unclear in Kopp et al. (2003) if variation affecting bab1 and/or bab2 was responsible for variation in abdominal pigmentation. To address this uncertainty, Bickel et al. (2011) sequenced the bab region in multiple inbred lines from the California population and found that non-coding SNPs at both bab1 and bab2 were associated with abdominal pigmentation differences. Specifically, SNPs associated with pigmentation were found in the first intron of bab1 and near the promoter region of bab2. In the DGRP collection, European populations, and an African population of D. melanogaster, only SNPs in the first intron of bab1 were associated with abdominal pigmentation variation (Bastide et al., 2013; Dembeck, Huang, Carbone, et al., 2015; Dembeck, Huang, Magwire, et al., 2015; Endler et al., 2016). A cis-regulatory element controlling sex-specific expression of bab1 in the A5–A7 segments in D. melanogaster males (repression) and females (induction) was also identified in the first intron of bab1 (Williams et al., 2008) and overexpression of bab1 during late pupal development was shown to be sufficient to suppress dark pigmentation (Salomone, Rogers, Rebeiz, & Williams, 2013), suggesting that the associated sites might alter pigmentation by altering expression of bab1. Indeed, Rogers et al. (2014) found that different alleles of this element were present in lightly and darkly pigmented D. melanogaster that drove different patterns of gene expression that correlate with pigmentation in the manner expected given bab’s role as a repressor of dark pigmentation. A small number of derived sequence changes were found to be responsible for these differences in cis-regulatory activity (Rogers et al., 2014). Genetic variation linked to another regulator of pigmentation, omb, has also been found to be associated with polymorphic body color in Drosophila polymorpha, but much less is known about this association, including whether coding or noncoding changes are more likely to be responsible for the association (Brisson et al., 2004).

Together, the studies described earlier demonstrate that genetic variation contributing to variable abdominal pigmentation within a species has repeatedly accumulated at noncoding regions near the ebony, tan, and bab1 genes.
In fact, in the European *D. melanogaster* population studied in Bastide et al. (2013), 79% of the most strongly associated SNPs mapped to noncoding regions linked to *ebony*, *tan*, and *bab1*. Other loci also clearly contribute to polymorphic abdominal pigmentation, however (Dembeck, Huang, Carbone, et al., 2015; Dembeck, Huang, Magwire, et al., 2015; Ng et al., 2008), and some of these loci have recently begun to be identified in *D. melanogaster* (Dembeck, Huang, Carbone, et al., 2015; Dembeck, Huang, Magwire, et al., 2015). The developmental role that these newly identified genes (eg, *pinstripe*, *triforce*, *plush*, and *farmer*) play in pigment patterning remains unknown.

### 4.2 Genetic Basis of Abdominal Pigmentation Differences Between Species

Differences in pigmentation between species have evolved over longer timescales than differences in pigmentation within a species, suggesting that even phenotypically similar changes in pigmentation might have a distinct genetic basis within and between species (Orr, 2001). For example, different genes and/or different types of changes in the same genes might tend to contribute to phenotypic differences that have evolved over longer evolutionary timescales (Orr, 2001; Stern & Orgogozo, 2009). By directly comparing the genetic basis of intra- and interspecific pigmentation differences, we can better understand how the variants underlying polymorphism within a species give rise to divergence between species. In this section, we review what is known about the genetic basis of abdominal pigmentation differences between species.

The genetic basis of pigmentation differences between species can be dissected genetically using the same methods used to identify genes contributing to intraspecific polymorphism if two species with differences in pigmentation are closely related enough that they can still be crossed and produce viable offspring in the laboratory. One such species pair is *D. yakuba* and *D. santomea*, which are estimated to have begun diverging ~400,000 years ago (Cariou, Silvain, Daubin, Da Lage, & Lachaise, 2001) (Fig. 3). *D. yakuba* exhibits stripes of dark melanins in A2–A6 in both sexes as well as more complete dark pigmentation in segments A5 and A6 of males similar to *D. melanogaster* (Fig. 2B), whereas *D. santomea* lacks dark melanin in these regions in both sexes (Jeong et al., 2008). QTL mapping was used to identify regions of the genome contributing to abdominal pigmentation divergence between these two species. In Llopart et al. (2002), five QTLs were identified, one of which was on the X chromosome and
explained nearly 90% of the species differences. Using a slightly different phenotyping procedure, Carbone et al. (2005) identified four QTL, two located on the X chromosome (one with a much larger effect on pigmentation than the other) and two located on autosomes.

To identify the specific gene(s) that might be responsible for the X-linked QTLs contributing to pigmentation differences between *D. yakuba* and *D. santomea*, Jeong et al. (2008) took a candidate gene approach. Specifically, they examined the pigment synthesis genes *tan* and *yellow*, which were located within the large and small effect X-linked QTLs,
respectively, and found differences in expression of both genes that correlated with differences in abdominal pigmentation between \textit{D. yakuba} and \textit{D. santomea} (Jeong et al., 2008). Analysis of \textit{yellow} and \textit{tan} expression in F1 hybrids from reciprocal crosses showed that only the expression difference in \textit{tan} was caused by \textit{cis}-acting genetic changes on the X chromosome; the difference in \textit{yellow} expression appeared to be caused by one or more \textit{trans}-acting autosomal loci (Jeong et al., 2008). To further localize the genetic changes responsible for divergent \textit{tan} expression and presumably pigmentation, transgenic reporter genes were used to compare enhancer activity of sequences from \textit{D. yakuba} and \textit{D. santomea} in \textit{D. melanogaster}. Three distinct mutations within an MSE located 5' of \textit{tan} in the genome, each of which reduces \textit{tan} expression, were found to have likely caused, in part, loss of abdominal pigmentation in \textit{D. santomea} (Jeong et al., 2008). This role of \textit{tan} in pigmentation divergence between \textit{D. santomea} and \textit{D. yakuba} was further supported by introgressing the \textit{D. yakuba} allele of \textit{tan} into \textit{D. santomea} and directly demonstrating this gene's contribution to the evolution of abdominal pigmentation differences between these two species (Rebeiz, Ramos-Womack, et al., 2009).

A similar story has emerged for pigmentation differences between the interfertile sister species \textit{D. americana} and \textit{D. novamexicana}, which are also thought to have diverged approximately 400,000 years ago (Morales-Hojas, Vieira, & Vieira, 2008) (Fig. 3). \textit{D. americana} has an overall dark body color typical for a member of the virilis species group, whereas \textit{D. novamexicana} displays a derived light body color with greatly reduced abundance of dark melanins (Wittkopp, Williams, Selegue, & Carroll, 2003). Analysis of F1 hybrids from reciprocal crosses again showed a large contribution of the X chromosome to pigmentation divergence (Wittkopp, Williams, et al., 2003), at least some of which was attributable to loci linked to the \textit{tan} gene (Wittkopp et al., 2009). Fine-scale genetic mapping confirmed that divergence at \textit{tan} was indeed a contributor to pigmentation divergence and localized the functionally divergent sites within \textit{tan} to the first intron (Wittkopp et al., 2009). Subsequent work has shown small, but significant differences in \textit{cis}-regulatory activity of the \textit{D. americana} and \textit{D. novamexicana tan} alleles that presumably contribute to pigmentation differences (Cooley, Shefner, McLaughlin, Stewart, & Wittkopp, 2012). The contribution of \textit{tan} to pigmentation divergence between these two species was further confirmed when the \textit{D. americana tan} allele caused darker pigmentation than the \textit{D. novamexicana tan} allele when each was put into a common \textit{D. melanogaster} genetic background using transgenes.
Variation linked to the *ebony* gene is also an important source of pigmentation divergence between these two species, with introgression of chromosomal regions containing *tan* and *ebony* from *D. americana* into *D. novamexicana* together explaining 87% of the difference in abdominal pigmentation seen between *D. americana* and *D. novamexicana* (Wittkopp et al., 2009). Effects of *ebony* have yet to be separated from linked loci, however, because *ebony* is located within a region of the genome inverted between these two species, recombination-based mapping is not possible. In all, genetic mapping between *D. americana* and *D. novamexicana* has identified five regions of the genome that contribute to the difference in abdominal pigmentation (Wittkopp et al., 2009; Wittkopp, Williams, et al., 2003).

Variation at *ebony* also appears to be important for abdominal pigmentation differences between the montium subgroup species *D. auraria* and *D. serrata* in the melanogaster group (Johnson et al., 2015), which last shared a common ancestor approximately as long ago as *D. melanogaster* and *D. simulans* (Nikolaidis & Scouras, 1996), that is ~1.5 million years ago (Cutter, 2008) (Fig. 3). In *D. auraria*, males have a stripe of pigment in each abdominal segment similar to *D. melanogaster*, but the more complete pigmentation of male abdominal segments is seen only on A6 rather than in A5 and A6 (Johnson et al., 2015). By contrast, males of *D. serrata* have an abdomen that is more yellow in color overall and lacks dark melanins almost completely in both A5 and A6 (Johnson et al., 2015). Using in situ hybridization, expression of *ebony* was found to be higher in the A5 and A6 segments of *D. serrata* than *D. auraria*, consistent with the role of *ebony* in the formation of yellow-tan sclerotins at the expense of dark melanins (Wittkopp, True, et al., 2002). This evolutionary change in expression appears to have resulted from changes in a *cis*-regulatory element located upstream of *ebony* that controls its expression in the A5 and A6 abdominal segments of males (Johnson et al., 2015).

Another montium subgroup species, *D. kikkawai*, which is estimated to have diverged from *D. melanogaster* ~20 million years ago (Prud’homme et al., 2006) (Fig. 3), has also lost the dark male-specific pigmentation in A5 and A6, but in this case, changes in a MSE of *yellow* that reduce its expression in these segments seem to have played a role (Jeong et al., 2006). Changes in *yellow* expression caused by *cis*-regulatory divergence have also been implicated in an expansion of male-specific abdominal pigmentation to include segments A3 and A4 in *D. prostipennis* relative to *D. takahashi*, two members of the oriental lineage in the melanogaster subgroup (Ordway et al., 2014) (Fig. 3). Interestingly, *D. prostipennis* also showed changes in
ebony and tan expression that correlated with the expanded male-specific pigmentation (decrease in ebony expression and increase in tan expression), but these changes in gene expression were found to be caused by divergence of trans-acting loci rather than cis-regulatory changes at ebony and tan (Ordway et al., 2014). Differences in the activity of yellow cis-regulatory sequences from D. melanogaster, D. subobscura, D. willistoni, D. mojavensis, D. virilis, and D. grimshawi, much more distantly related species (Fig. 3), that correlate with species-specific pigmentation were also observed when these cis-regulatory sequences were assayed in D. melanogaster (Kalay & Wittkopp, 2010; Wittkopp, Vaccaro, & Carroll, 2002) (Fig. 3). Observing these changes in cis-regulation and gene expression that correlate with divergent abdominal pigmentation for pigment synthesis genes yellow, tan, and ebony strongly suggests that these changes have contributed to pigmentation divergence, although their relative contributions in any individual case remain unknown.

Pigment synthesis genes are not the only source of abdominal pigmentation divergence between species; divergence in a transcription factor regulating expression of pigmentation genes, bab1, also plays a role in interspecific differences. In D. melanogaster, Bab1 expression represses development of dark pigmentation in segments A5 and A6 of males (Kopp et al., 2000). By contrast, in D. willistoni, a species without sexually dimorphic pigmentation in which males and females both have only a stripe of dark melanin near the posterior edge of each tergite, bab1 is expressed in segments A2–A6 in both sexes (Kopp et al., 2000; Williams et al., 2008). Sex-specific differences in abdominal bab1 expression seen in D. melanogaster were found to be controlled by a dimorphic cis-regulatory element containing binding sites for the transcription factors Abd-B and Dsx (Williams et al., 2008). Changes in the binding sites for these transcription factors as well as other changes in the cis-regulatory sequence were found to be responsible for the differences in bab1 cis-regulatory activity between D. melanogaster and D. willistoni (Williams et al., 2008). Divergence in this sexually dimorphic cis-regulatory element was also found to contribute to interspecific differences in bab expression that correlate with differences in female abdominal pigmentation among D. melanogaster, D. yakuba, D. frayamai, and D. auraria (Rogers et al., 2013).

5. THORAX PIGMENTATION

Like abdominal pigmentation, thorax pigmentation varies widely in intensity and patterning within and among Drosophila species. Species like
D. guttifera, for example, possess distinctive stripes of black melanin along their thorax that D. melanogaster and most other Drosophila species lack (Koshikawa et al., 2015). In D. melanogaster populations, individuals often vary in the intensity of black and brown melanins that fill a “trident” pattern on the thorax, and variation in this pattern tends to follow altitudinal or latitudinal clines around the world (David & Capy, 1988; Parkash & Munjal, 1999; Telonis-Scott et al., 2011). Intensity of UV radiation was also recently shown to be a good predictor of thorax pigmentation in D. melanogaster for clinal variation in Africa, with more darkly pigmented flies found to inhabit regions with higher levels of UV radiation (Bastide et al., 2014). This finding suggests that increased levels of melanin in the thorax may play a protective role for D. melanogaster in the wild; however, D. yakuba shows the opposite relationship between the intensity of UV radiation and abdominal pigmentation (which is often correlated with thorax pigmentation; Matute & Harris, 2013; Rajpurohit & Gibbs, 2012), indicating that this is not a general relationship for all Drosophila. Regardless of the selective forces driving diversity of thorax pigmentation in Drosophila, the variety of pigment patterns seen within and among species provides the raw material needed to further investigate the genetic basis of phenotypic evolution.

5.1 Genetic Basis of Thorax Pigmentation Differences Within a Species

In natural populations of D. melanogaster and D. simulans, variation in a pigmented thorax trident pattern is often seen in which individuals differ in the intensity of darkness in trident shape and size (Capy et al., 1988; David & Capy, 1988). A similar darkening of this trident pattern is also readily observed in D. melanogaster ebony loss-of-function mutants (Lindsley & Zimm, 1992), suggesting that variation in ebony expression and/or activity might underlie this intraspecific diversity. Consistent with this hypothesis, Takahashi et al. (2007) found that a chromosomal region containing the ebony locus was most strongly associated with differences in trident pigmentation intensity between inbred lines of D. melanogaster isolated from West Africa and Taiwan. Complementation tests combined with differences in ebony expression levels between strains further suggested that regulatory changes at ebony contributed to these differences in trident pigmentation (Takahashi et al., 2007). Natural variation in trident intensity within a D. melanogaster population collected from Japan was also found to be associated with genetic variants in ebony enhancer regions located on the cosmopolitan inversion, In(3R)Payne (Takahashi & Takano-Shimizu, 2011).
Interestingly, none of the 19 nucleotide sites found to be in complete association with trident pigment intensity in this study overlapped with sites associated with differences in abdominal pigmentation in African populations described earlier (Pool & Aquadro, 2007; Rebeiz, Pool, et al., 2009). Genetic variants associated with thoracic pigmentation in this Japanese population do still appear to affect cis-regulation of ebony, however, because differences in relative allelic expression were observed for ebony in F1 hybrids produced by crossing lightly and darkly pigmented lines of D. melanogaster from this population (Takahashi & Takano-Shimizu, 2011). Variable sites located within an enhancer that drives expression in both the thorax and abdomen (Rebeiz, Ramos-Womack, et al., 2009) failed to cause differences in cis-regulatory activity when tested in a common genetic background using reporter genes, however (Takahashi & Takano-Shimizu, 2011). cis-Regulatory variation affecting ebony expression also seems to contribute to variable thoracic pigmentation observed among the DGRP lines of D. melanogaster used in the Dembeck, Huang, Magwire, et al. (2015) study of abdominal pigmentation, with the most strongly associated SNPs again unique to this population (Miyagi et al., 2015). Significant associations were also observed between genetic variants in known enhancers of tan and allele-specific tan expression levels, but not with variation in thoracic pigmentation (Miyagi et al., 2015). Taken together, these studies indicate that ebony cis-regulatory sequences are often variable in natural populations of D. melanogaster, with different genetic variants contributing to differences in thoracic pigmentation in different populations.

5.2 Genetic Basis of Thorax Pigmentation Differences Between Species

The best-studied difference in thoracic pigmentation between species is that seen between D. guttifera and D. melanogaster. In D. guttifera, a member of the quinaria species group (Fig. 3), males and females possess a distinct pattern of darkly pigmented stripes along their thorax in addition to the “polka-dot” deposits of black melanin seen on their abdomen and wings. To identify genes involved in the evolution of D. guttifera thoracic pigmentation, Koshikawa et al. (2015) examined the regulation of wingless expression, which was previously shown to be spatially correlated with the black polka-dots in the wings during development (Werner et al., 2010). After testing many noncoding sequences in and around wingless for activity in the thorax, an enhancer driving expression in this part of the body was finally located in an intron of the Wnt10 gene, two genes away from wingless (Koshikawa
et al., 2015). This enhancer, called “gutTS” for D. guttifera thorax stripes, was sufficient to activate wingless expression during pupal stages of D. guttifera that mirrors the thoracic pigment stripes seen in adult D. guttifera (Koshikawa et al., 2015). In D. melanogaster, this D. guttifera cis-regulatory element drove weaker thoracic stripes, indicating that some trans-acting regulators of this wingless enhancer had diverged between species (Koshikawa et al., 2015). The orthologous enhancer from D. melanogaster was also tested for activity in both D. melanogaster and D. guttifera and failed to drive expression in thoracic strips in either species, indicating that cis-regulatory divergence had occurred between D. melanogaster and D. guttifera within the gutTS wingless enhancer (Koshikawa et al., 2015). These results suggest that the evolution of a novel cis-regulatory element affecting wingless expression contributes to the derived thoracic stripe pigment pattern seen in D. guttifera.

6. WING PIGMENTATION

D. melanogaster wings are evenly pigmented throughout the wing blade, but many other species of Drosophila (especially Hawaiian and Oriental species) have wing spots of dark melanins that vary in size, shape, and position on the wing (Edwards, Doescher, Kaneshiro, & Yamamoto, 2007; O’Grady & DeSalle, 2000; Prud’homme et al., 2006; Wittkopp, Carroll, et al., 2003). These darkly pigmented wing patterns are often sexually dimorphic and thought to be the result of sexual selection. Males that possess wing spots in the Oriental melanogaster species group, for example, perform an elaborate wing display behavior in front of females during courtship, whereas males without wing spots tend to perform courtship from behind the female (Yeh & True, 2006). Developmentally, these complex wing pigment patterns result from a two-step process in which (i) spatial prepatterns of enzymes involved in the pigmentation synthesis pathway are laid down in the developing wing during the Drosophila pupal stage and (ii) precursors for melanin such as dopa and dopamine are transported to the wing through the hemolymph and diffuse from the wing veins post-eclosion, polymerizing to form black and/or brown melanins in the shape of the enzymatic prepatterns (True, Edwards, Yamamoto, & Carroll, 1999). The precise size and shape of wing spots often varies within species, but the genetic basis of this variation has yet to be determined. Several studies have, however, elucidated genetic mechanisms underlying interspecific differences in wing spot size and patterning, and these are reviewed below.
6.1 Genetic Basis of Wing Pigmentation Differences Between Species

In the melanogaster group of *Drosophila*, several species possess a darkly pigmented male-specific spot at the distal tip of their wing. Phylogenetic reconstructions suggest that the common ancestor of the melanogaster group lacked a wing spot and that the current distribution of this trait in this species resulted from at least one gain followed by multiple losses in independent lineages (Prud’homme et al., 2006). The best studied of these spotted species is *D. biarmipes*, a member of the Oriental lineage within the melanogaster species group (Fig. 3), that has a single spot of dark pigmentation at the distal tip of the wing in males. This spot has been shown to be prefigured by expression of the Yellow protein and the absence of the Ebony protein during pupal stages (Wittkopp, True, et al., 2002). For *yellow*, the novel pattern of expression is caused by cis-regulatory changes in a pre-existing wing enhancer of *yellow*, suggesting that cis-regulatory evolution at *yellow* contributed to the evolution of the wing spot pattern (Gompel et al., 2005). Further investigation revealed that cis-regulatory changes affecting *yellow* expression had arisen independently in multiple lineages, with different preexisting wing enhancers coopted to create the novel patterns of wing spot expression (Prud’homme et al., 2006). In the case of spot divergence between the two sister species *D. elegans* (spotted) and *D. gunungcola* (spotless), which are also members of the Oriental lineage of the melanogaster group (Fig. 3), the spot of *yellow* expression present in *D. elegans* is controlled by sequences orthologous to the spot enhancer in *D. biarmipes* and divergence of only a few nucleotides in this sequence is responsible for the loss of this *yellow* expression pattern (and presumably at least part of the wing spot) in *D. gunungcola* (Prud’homme et al., 2006). In another spotted species, however, *D. tristis*, which is a member of the obscura group, a wing spot prefigured by *yellow* expression has evolved using a novel cis-regulatory element that coopted a different preexisting wing enhancer of *yellow* (Prud’homme et al., 2006). Taken together, these studies suggest that the cis-regulatory sequences of *yellow* have evolved repeatedly to cause changes in gene expression that contribute to the gain and loss of wing spots in multiple *Drosophila* species (also reviewed in Monteiro & Das Gupta, 2016).

To better understand how *yellow* expression is regulated and evolves, Arnoult et al. (2013) performed an RNAi screen in a strain of *D. melanogaster* that carried a reporter gene reflecting activity of the *D. biarmipes* spot enhancer. Among the ~350 screened transcription factors,
five candidates emerged as potential activators of the D. biarmipes spot enhancer. One of these genes was Distal-less (Dll), which has previously been shown to be important for Drosophila wing development (Cohen, Wimmer, & Cohen, 1991). Using RNAi knockdown, overexpression, and electrophoretic mobility shift assays, Dll was shown to be both necessary and sufficient for driving activity of the yellow spot enhancer in the wings of D. melanogaster (Arnoult et al., 2013). Moreover, manipulating Dll expression in D. biarmipes itself lead to a gain and loss of wing pigmentation when Dll was over- and underexpressed, respectively (Arnoult et al., 2013). Neither changes in wing pigmentation nor yellow expression were observed when Dll expression was modified in D. ananassae, a species without a wing spot, indicating that the regulatory connection between Dll and yellow had evolved in the lineage leading to D. biarmipes since it last shared a common ancestor with D. ananassae (Arnoult et al., 2013). This regulatory link does not appear to be restricted to D. biarmipes, however, as correlations between Dll expression, yellow expression, and wing spots were also observed in D. pulchrella, D. elegans, D. rhopaloa, and D. prolongata (Arnoult et al., 2013). These data suggest an evolutionary trajectory in which Dll regulation of yellow was gained and then changes in Dll expression evolved to produce a variety of wing spot patterns. While the second step of this model remains to be tested, it is clear from these data that divergent expression patterns of Dll (as well as potentially other transcription factors) have contributed to the divergence of wing pigment patterns through the direct (and likely also indirect) modulation of genes in the pigmentation synthesis pathway (Monteiro & Das Gupta, 2016).

In other Drosophila species, wing pigmentation is not limited to males and involves more than a single spot. For example, in D. guttifera, both males and females develop a polka-dot pattern of 16 dark melanin spots and 4 melanized areas across their wings (Koshikawa et al., 2015; Werner et al., 2010). Yellow expression during pupal stages again mirrors the final adult wing pigment pattern (Werner et al., 2010), as does expression of Ebony expression, which is reduced in regions with wing spots (Gompel et al., 2005). To identify cis-regulatory regions of yellow responsible for this spotted expression pattern, noncoding regions surrounding yellow were tested for cis-regulatory activity using a reporter gene introduced into D. melanogaster. Unlike in other studies of yellow cis-regulatory elements (Arnoult et al., 2013; Gompel et al., 2005; Kalay & Wittkopp, 2010; Prud’homme et al., 2006; Wittkopp, Vaccaro, et al., 2002), the unique expression pattern of D. guttifera yellow could not be recapitulated by reporter genes in
D. melanogaster, indicating that changes in trans-regulatory factors controlling yellow expression in D. guttifera had diverged between these two species. Transforming these reporter genes into D. guttifera did, however, drive spotted patterns of expression similar to those seen for endogenous yellow (Werner et al., 2010). Through careful examination of the reporter constructs assayed in D. melanogaster, phenotypes observed in a spontaneous D. guttifera mutant, and prior knowledge of wing development, Werner et al. (2010) identified wingless as a potential regulator of D. guttifera yellow. Ectopic expression of wingless in D. guttifera resulted in ectopic wing pigmentation, providing evidence that wingless does indeed regulate wing spot pigmentation in D. guttifera (Werner et al., 2010). Additional reporter gene experiments using an orthologous spot enhancer from a closely related species lacking wing spots, D. deflecta, also showed that D. guttifera had evolved a novel pattern of wingless expression that contributed to the evolution of its polka-dotted wings (Werner et al., 2010).

The novel expression pattern of wingless in D. guttifera could have evolved through changes in its cis-regulatory sequences, changes in one or more trans-acting regulators of wingless, or both. To determine whether cis-regulatory changes were responsible for divergent wingless expression, Koshikawa et al. (2015) tested noncoding sequences in and around the wingless gene for cis-regulatory activity in pupal wings. A cis-regulatory element located 3' of D. guttifera wingless was found to drive expression in D. guttifera-like spots near the distal tip of the wing, an activity that seems to have evolved by coopting activity of preexisting cis-regulatory elements driving expression in the cross-veins and/or wing margin (Koshikawa et al., 2015). Two more cis-regulatory elements that appear to drive novel patterns of wingless expression in D. guttifera were also identified more than 69 kb away from wingless in introns of the Wnt10 gene (Koshikawa et al., 2015). Testing the activity of these cis-regulatory regions using transgenes inserted into D. melanogaster showed that changes in the cis-regulatory elements of wingless were largely sufficient to explain divergent wingless expression and presumably thus contribute to the evolution of novel wing pigmentation in D. guttifera (Koshikawa et al., 2015).

Because of the candidate gene approaches used to study the evolution of wing spots in the species described earlier, the contribution of cis-regulatory changes observed in yellow and wingless relative to changes that likely exist at other loci in the genome remain unknown. Two studies investigating the genetic basis of a difference in wing spot between interfertile species in the Oriental lineage of the melanogaster subgroup, D. elegans and
D. gunungcola (Fig. 3), begin to address this issue (Yeh & True, 2006, 2014). D. elegans has a male-specific wing spot of dark pigment similar to that seen in D. biarmipes, whereas its sister species D. gunungcola has no spots of dark pigment on its wing (Prud’homme et al., 2006). The similarity of wing spots seen in D. biarmipes and D. elegans is consistent with the proposed inheritance from a common ancestor that also had a wing spot (Prud’homme et al., 2006), suggesting that the roles of yellow (Prud’homme et al., 2006) andDll (Arnoult et al., 2013) in the development of the D. biarmipes wing spot described earlier are likely conserved in D. elegans. Genetic mapping of loci contributing to the difference in wing spot between D. elegans and D. gunungcola identified three QTL affecting the wing spot (Yeh & True, 2006, 2014). Although each of these QTL encompasses many genes, the inclusion of yellow in one QTL and Dll in another is consistent with prior studies suggesting that divergence at these loci contributes to the loss of the wing spot in D. gunungcola (Arnoult et al., 2013; Prud’homme et al., 2006). The QTL overlapping yellow provides more circumstantial evidence that the cis-regulatory divergence of yellow identified between D. elegans and D. gunungcola using reporter genes (Prud’homme et al., 2006) impacts pigmentation. In addition, the QTL overlapping Dll suggests that differences in Dll expression might exist between D. elegans and D. gunungcola and be caused by cis-regulatory changes at Dll itself, similar to observations for divergent wingless expression in D. guttifera (Koshikawa et al., 2015). The third QTL does not include any obvious candidate genes.

7. PUPAL PIGMENTATION

In addition to the highly variable pigment patterns of the Drosophila abdomen, thorax, and wings, differences in pigmentation are also seen among some species in the pupal cases from which the adult flies emerge. For example, in the virilis group of Drosophila (Fig. 3), D. virilis has a distinctly darker pupal case color than its closest relatives, D. americana, D. lummei, and D. novamexicana (Stalker, 1942). The D. virilis pupal case appears almost completely black, whereas pupal cases in the other species are lighter shades of brown and tan (Ahmed-Braimah & Sweigart, 2015). The virilis species group is amenable to genetic dissection of this trait because D. americana, D. novamexicana, and D. virilis all produce fertile hybrids when crossed with each other (Heikkinen, 1992). Early studies investigating the genetic basis of this difference in pupal color between D. virilis and D. americana suggested that it was due to a large effect locus on chromosome
5 as well as other loci, possibly linked to chromosomes 2 and 3 (Stalker, 1942). To identify the molecular basis of pupal color divergence between *D. virilis* and *D. americana* more precisely, Ahmed-Braimah and Sweigart (2015) analyzed a backcross population between these two species and scored more than 30,000 recombinant offspring for pupal case color. This experimental design allowed them to identify an ~11-kb sequence on chromosome 5 that contributes to the difference in pupal case color. This region contains the first exon and noncoding regions of the *Dat* gene (Ahmed-Braimah & Sweigart, 2015). *Dat*, as described earlier and in Fig. 1, is required for the conversion of dopamine to NADA, which is then polymerized into a colorless pigment. Expression differences were observed for *Dat* at the onset of pupation between *D. americana* (high expression) and *D. virilis* (low expression; Ahmed-Braimah & Sweigart, 2015) that suggest reduced expression of *Dat* in *D. virilis* creates an excess of dopamine that allows production of more dark melanins and thus a much darker pupal case. Pupal expression of *Dat* in *D. novamexicana*, which has a lighter body color than *D. americana* but a similarly colored pupal case, was similar to that observed for *D. americana* (Ahmed-Braimah & Sweigart, 2015). Genetic variation linked to *Dat* did not explain any of the difference in body color between *D. americana* and *D. novamexicana*, consistent with prior work identifying *ebony* and *tan* as the primary drivers of divergent body color between these two species (Wittkopp et al., 2009).

### 8. LESSONS LEARNED FROM *DROSOPHILA* PIGMENTATION

With the rapid growth of studies identifying genes and genetic changes contributing to pigmentation differences within and between *Drosophila* species during the last 10 years, the time is ripe to step back and take an integrative look at the findings from these case studies. What have we learned about the genetic basis of pigmentation evolution and hopefully phenotypic evolution more generally? What questions remain unanswered?

First and foremost, we have learned that the same handful of genes have been modified over and over again in different lineages to give rise to polymorphic pigmentation within a species as well as divergent pigmentation between species (Table 1). A similar pattern has also been seen for other types of evolutionary changes (Martin & Orgogozo, 2013; Stern & Orgogozo, 2009), suggesting that evolutionary trajectories are sometimes
predictable. For pigmentation, genes harboring polymorphism and divergence that affects body color include genes that encode developmental regulators (blue in Fig. 4) as well as enzymes required for pigment biosynthesis (red in Fig. 4). The apparent reuse of these genes has likely been biased by the use of candidate gene approaches that limited analysis to these genes in some studies (Gompel et al., 2005; Johnson et al., 2015; Prud’homme et al., 2006; Werner et al., 2010; Wittkopp, Vaccaro, et al., 2002); however, the same conclusion emerges if only studies using unbiased genetic mapping approaches are considered (Bastide et al., 2013; Dembeck, Huang, Carbone, et al., 2015; Dembeck, Huang, Magwire, et al., 2015; Endler et al., 2016; Pool & Aquadro, 2007; Wittkopp et al., 2009). Despite this repeatability, the set of nine genes implicated in pigmentation diversity thus far is clearly not exhaustive; Dembeck, Huang, Carbone, et al. (2015) found SNPs in 84 loci that had significant associations with variable abdominal pigmentation in a single population of D. melanogaster.

A second lesson results from the striking consistency seen in the types of functional genetic changes observed in genes contributing to pigmentation diversity: cis-regulatory changes in noncoding sequences appear to be responsible for a gene’s effects on pigmentation in all cases where the type of mutation is known (Table 1). This observation holds for both developmental regulators and genes in the pigment synthesis pathway (Table 1). cis-Regulatory changes have been proposed to be the predominant source of evolutionary change in genes with pleiotropic effects on multiple traits because they allow one function of the gene to be modified without affecting others (Carroll, 2008; Stern & Orgogozo, 2008; Wray et al., 2003). All of the genes implicated in pigmentation diversity thus far are indeed pleiotropic.

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Fig. 4 The loci of pigmentation evolution. A summary of genes implicated in pigmentation differences within and/or between species is shown. Genes labeled in blue (bab1, bab2, Dll, omb, and wg) are regulators of pigmentation development. Genes labeled in red (ebony, Dat, tan, and yellow) are involved in the pigment biosynthesis pathway. All genetic changes identified as likely to be contributing to a pigmentation difference either within or between species thus far affect cis-regulatory sequences.
and are regulated by multiple cis-regulatory elements that subdivide their functions. In addition to pigmentation, bab1 and bab2 also affect development of mechanosensory organs (Godt, Couderc, Cramton, & Laski, 1993; Kopp et al., 2000); ebony, tan, yellow, and Dat also impact behavior (Drapeau, Radovic, Wittkopp, & Long, 2003; Shaw, Cirelli, Greenspan, & Tononi, 2000; True et al., 2005), and Dll, omb, and wg have widespread effects on development (Drysdale & FlyBase Consortium, 2008). The genetic basis of pigmentation differences in vertebrates reveals a different pattern, however, with changes in pigmentation attributed more equally to cis-regulatory changes and changes in amino acid sequence affecting protein function (Hubbard, Uy, Hauber, Hoekstra, & Safran, 2010).

A final message emerging from these studies is that intra- and interspecific sources of pigmentation diversity share some properties but not others. For example, nearly all genes shown to contribute to differences in abdominal pigmentation within a species also contribute to pigmentation differences that exist between species (Fig. 4). One notable exception is yellow. Changes in yellow expression often accompany changes in pigmentation between Drosophila species, but they have yet to be implicated in intraspecific variation. This might be because overexpression of yellow has more subtle effects on pigmentation than overexpression of ebony, tan, or bab1 (Jeong et al., 2008; Salomone et al., 2013; Wittkopp et al., 2009; Wittkopp, True, et al., 2002), such that changes in yellow expression arising alone within a species are insufficient for altering pigmentation in most populations (but see Wittkopp, Vaccaro, et al., 2002). Genetic changes in the same cis-regulatory regions have been observed within and between species, but the scope of these changes differs. Within a species, genetic variants typically modulate activity of existing cis-regulatory elements, with different variants affecting cis-regulatory activity in different populations. By contrast, divergent sites that differ between species are much more likely to have given rise to a novel enhancer that coopts preexisting developmental regulators. Differences between alleles contributing to intra- and interspecific pigmentation variation are not always apparent, however, as the alleles of tan and ebony contributing to divergent pigmentation in D. novamexicana were found to also contribute to clinal variation in pigmentation within D. americana (Wittkopp et al., 2009).

As illustrated in this chapter, detailed studies of pigmentation divergence within and among Drosophila species have provided an unprecedented look at the genetic mechanisms underlying phenotypic evolution over various timescales. There is still much more to be learned from studying this system,
however. For example, many QTLs contributing to pigmentation differences within and between species have been identified for which the causative genes remain unknown. Identifying these genes might alter our view of the types of genes most likely to harbor genetic changes affecting pigmentation. Many direct and indirect regulators of genes in the pigment synthesis pathway are also yet to be identified. Knowing the identity of these factors and the sequences they bind to will help us understand why some noncoding changes alter pigmentation while others do not. Important questions also remain about whether the complementary changes in expression of pigmentation genes such as *yellow* and *ebony* that are often observed between species have evolved through independent genetic changes or a single change affecting a shared regulator. Finally, improving our understanding of both the ecological functions of pigmentation in specific taxa and the pleiotropic effects of pigmentation genes will help us better understand the role natural selection might play in shaping the genetic basis of pigmentation evolution. Ultimately, understanding the genetic and molecular mechanisms underlying pigmentation diversity has the potential to answer questions not only about evolution but also about ecology, biochemistry, and neuroscience.

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