Chapter 16
Drosophila guttifera as a Model System for Unraveling Color Pattern Formation

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Abstract A polka-dotted fruit fly, *Drosophila guttifera*, has a unique pigmentation pattern made of black melanin and serves as a good model system to study color pattern formation. Because of its short generation time and the availability of transgenics, it is suitable for dissecting the genetic mechanisms of color pattern formation. While the ecology and life history of *D. guttifera* in the wild are not well understood, it is known to be resistant to a mushroom toxin, and this physiological trait is under molecular scrutiny. Pigmentation around crossveins and longitudinal vein tips is common in closely related species of the *quinaria* group, in addition to which *D. guttifera* has evolved species-specific pigmentation spots around the campaniform sensilla. Regulatory evolution of the Wnt signaling ligand Wingless, which locally induces pigmentation in the developing wing epithelium, has driven the evolution of distinct aspects of wing and body pigmentation. A melanin biosynthesis pathway gene, *yellow*, is also involved in the elaboration of these traits, downstream of *wingless*. Unraveling the detailed mechanism of pigmentation pattern formation of this species sheds light on the general principles of morphological evolution and foreshadows potential parallels with other systems, such as the pigmented wings of butterflies.

Keywords *Drosophila guttifera* • Pigmentation • Color pattern • Evolution • Development • Transgenic • *Cis*-regulatory element • Phylogeny • Ecology • Life history • Taxonomy

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16.1 Introduction

Research on butterfly color patterns has greatly advanced in recent years. Knowledge of the characteristics of the genome, mechanisms of pattern formation, and the function and evolutionary mode of the pattern is rapidly growing. This was enabled by utilization of multiple model species, including species of *Bicyclus*, *Heliconius*, *Junonia*, *Vanessa*, *Papilio*, and others, and by the best use of characteristics of materials (Nijhout 1991; Carroll et al. 1994; Brakefield et al. 1996; Joron et al. 2011; Reed et al. 2011; The Heliconius Genome Consortium 2012; Martin et al. 2012; Kunte et al. 2014; Monteiro 2015; Nishikawa et al. 2015; Beldade and Peralta 2017).

In vertebrates, zebrafish (*Danio rerio*) has been a model of color pattern formation, and recently, domestic and wild cats and a four-striped mouse (*Rhabdomys pumilio*) were also used for research, making this an exciting time for color pattern studies (Singh and Nüsslein-Volhard 2015; Kaelin et al. 2012; Mallarino et al. 2016).

We have been using a dipteran insect, *Drosophila guttifera*, to study a mechanism of color pattern formation (Fig. 16.1). *D. guttifera* has a pattern on its wings, which is a commonality with butterflies; however, there are also some important differences. In contrast with the pigmented scales of butterflies and moths (as an exception, see Stavenga et al. 2010), *Drosophila* pigmentation is embedded in the cuticle layers of the wing membrane. This pigmentation is believed to be made of black melanin. A congeneric species, *Drosophila melanogaster*, is a model organism widely used in genetics and various biological researches, and we can utilize its knowledge, techniques, and resources to study *D. guttifera*. This phylogenetic proximity is an asset, as it is possible to transfer a part of the genetic system, such as an enhancer involved in pattern formation, into *D. melanogaster* and analyze its function in a heterologous context. *D. guttifera* has the potential to

Fig. 16.1 Adult male of *Drosophila guttifera*. The pigmentation pattern is very similar between the sexes

approach the same problem of color pattern as in butterflies but from a different angle. It also enables a good comparison, since its complex pigmentation patterns evolved independently from the ones seen in butterflies.

In this chapter, we present an overview of the biology of *D. guttifera*. Then we discuss differences in pattern formation between *D. guttifera* and butterflies and the advantage and potential of *D. guttifera* to contribute to the general understanding of animal color pattern formation.

16.2 Phylogenetic Position of *D. guttifera*

Fruit flies (drosophilid flies) belong to family Drosophilidae, order Diptera, and consist of 72 genera and more than 4000 described species (Yassin 2013). Among them, genus *Drosophila* includes more than 1160 described species (Markow and O'Grady 2006; Toda 2017). The best-studied species, *D. melanogaster*, also belongs to this genus. It should be noted, however, that the genus *Drosophila* is not monophyletic and potentially includes multiple genera within this clade, and there is ongoing debate on the proper taxonomic treatment of this genus (O'Grady 2010).

D. guttifera was described by an English entomologist, Francis Walker, based on a specimen collected in Florida (Walker 1849). This description consisted of 4 lines in Latin and 21 lines in English with no illustration and was one of many descriptions of a museum collection of the British Museum. In his taxonomic revision of North American drosophilids, Sturtevant (1921) examined multiple specimens of *D. guttifera* and redescribed the morphological features. Sturtevant (1942) established "species groups" to classify species within the genus *Drosophila*. *D. guttifera* was assigned to a monospecific *guttifera* group. He also established the *quinaria* group, which includes 11 species (*D. quinaria*, *deflecta*, *palustris*, *subpalustris*, *occidentalis*, *suboccidentalis*, *munda*, *subquinaria*, *transversa*, and possibly *phalerata* and *nigromaculata*). Patterson (1943) revised drosophilids of the Southwestern United States and Northern Mexico and redescribed many species with beautiful illustrations. *D. guttifera* was redescribed with illustrations of a pupa and internal organs of reproduction and a color illustration of the whole body. Patterson also described three new species in the *quinaria* group (*D. suffusca*, *tenebrosa*, and *innubila*). After that, many species were described in the *quinaria* group, and currently it includes 31 species (Markow and O'Grady 2006, Toda 2017).

The close relationship between *D. guttifera* and the *quinaria* group is almost certain at this time, based on molecular genetic evidence (Perlman et al. 2003; Izumitani et al. 2016). Morphological similarity between *D. guttifera* and the *quinaria* group was also noticed (Patterson and Stone 1952), and some authors even placed *D. guttifera* in the *quinaria* group (Throckmorton 1962, 1975; Markow and O'Grady 2006). Species-level relationships among *D. guttifera* and species of the *quinaria* group are not completely resolved; however, the commonly supported

result is bifurcation into two clades, one including mostly North American species and one including mostly Eurasian species (Perlman et al. 2003, Markow and O'Grady 2006, Izumitani et al. 2016).

There are species with pigment patterns on the thorax, abdomen, and wings to various degrees in the *quinaria* group (Patterson 1943; Werner and Jaenike 2017), but *D. guttifera* has distinctive vertical stripes on the thorax and a polka dot pattern on the abdomen and wings. Even when compared with the *quinaria* group species, *D. guttifera* has the most prominently pigmented appearance.

16.3 Food Habits, Poison Resistance, and Behavioral Ecology of *D. guttifera*

The life history and ecology of *D. guttifera* in the wild have not been well studied. There are many species of the *quinaria* group that utilize mushrooms as a food source. Sturtevant (1921) assumed *D. guttifera* is also a mushroom feeder based on the facts that *D. guttifera* was found around mushrooms and that he could rear *D. guttifera*, from eggs to adults, with mushrooms (he noted that both gill fungi and pore fungi can be utilized, but he did not describe mushroom species). Bunyard and Foote (1990a) studied what kind of dipteran insects emerged from mushrooms collected in the state of Ohio and reported that *D. guttifera* emerged from two mushroom species, *Psilocybe polytrichophila* and *Collybia dryophila*. They tested oviposition site preference among commercial *Agaricus bisporus*, banana, tomato, lettuce, and agar and found that *Agaricus* was the most preferred site (Bunyard and Foote 1990b). They also confirmed that *D. guttifera* can grow from eggs to adults with *Agaricus*. In laboratory conditions, however, we can keep strains of *D. guttifera* with artificial food containing sugar/corn meal/yeast/agar (sugar food) or molasses/corn meal/yeast/agar (molasses food) without adding mushrooms.

Some fungus-feeding drosophilids are known to have high tolerance to a mushroom toxin, alpha-amanitin, which is highly poisonous to most animals (Spicer and Jaenike 1996). *D. guttifera* has the potential to be a model system to study this phenomenon. Alpha-amanitin exerts its toxicity by binding to RNA polymerase II, an enzyme essential for transcription. A mutant strain of *D. melanogaster* with high alpha-amanitin tolerance had an amino acid substitution in RNA polymerase II (Chen et al. 1993). However, *D. guttifera* and other species with the tolerance do not have the same substitution, indicating that other mechanisms are involved (Stump et al. 2011). There are other strains of *D. melanogaster* with alpha-amanitin tolerance but without RNA polymerase II mutation. The responsible locus was mapped, and gene expression profiles were analyzed in these strains (Begum and Whitley 2000; Mitchell et al. 2014, 2015).

There are some other studies of *D. guttifera* behavior. Oviposition site preference of *D. guttifera* was affected by larval food condition, and this is known as a

classic example of olfactory conditioning of animals (Cushing 1941). The mating behavior of *D. guttifera* was also studied (Grossfield 1977). The ecological significance and function of pigmentation patterns of *D. guttifera* is not well understood. Some drosophilids are known to use wing pigmentation in courtship displays (Ringo and Hodosh 1978; Yeh et al. 2006; Fuyama 1979). Dombeck and Jaenike (2004) analyzed fitness effects of abdominal spot number in *D. falleni*.

16.4 The Evolution of Wing Pigmentation Pattern

Dombeck and Jaenike (2004) analyzed and illustrated the evolutionary path of wing and abdominal pigmentations of *D. guttifera* and seven species of the *quinaria* group. We summarize here the evolution of wing pigmentation pattern of *D. guttifera* and the *quinaria* group species based on molecular phylogenetics (Fig. 16.2). As previously explained, the *quinaria* group is divided into two major clades (Perlman et al. 2003; Markow and O'Grady 2006, Izumitani et al. 2016). We defined the clade with mostly North American species as "clade A" and the clade with mostly Eurasian species as "clade B." Species in clade A have relatively simple patterns; pigmentations are formed only around crossveins except in *D. innubila*, which has no pigmentation. The evolution of patterns in clade B is rather complicated. The relationships among basal species of clade B [*D. guttifera*, *nigromaculata*, and (*deflecta* + *palustris* + *subpalustris*)] have not been completely resolved, because the topologies of the phylogenetic trees depend on the analytical methods. These four species have pigmentations around crossveins and longitudinal vein tips. In addition, *D. guttifera* has pigmentations around the campaniform sensilla, which is unique to this species [at least unique among the clade of (*quinaria* group + *D. guttifera*) and probably among the genus *Drosophila*]. Among the rest of the species in clade B, *D. quinaria* has weak pigmentations on the tips of longitudinal veins in addition to crossveins. *D. recens* and many other species within this cluster have pigmentations around crossveins. *D. kuntzei*, which has a similar pattern to *D. quinaria*, branches from the most basal position of clade B according to Perlman et al. (2003), although the statistical support for this topology was low. Due to the lack of a robust phylogeny, it would be premature to propose a simple scenario stepwise pattern of gain and loss within the *quinaria* group. It is plausible that the instances of longitudinal vein tip pigmentation are the result of convergent evolution, perhaps via parallel mechanisms, although we cannot exclude the possibility of a single gain of the longitudinal vein tip pigmentation and a secondary loss in derived species of clade B. Nevertheless, the other dot-like patterns of *D. guttifera*, which overlap in position with innervated cupules known as campaniform sensilla (see below), are unique to this species and are assumed to form a true evolutionary novelty.

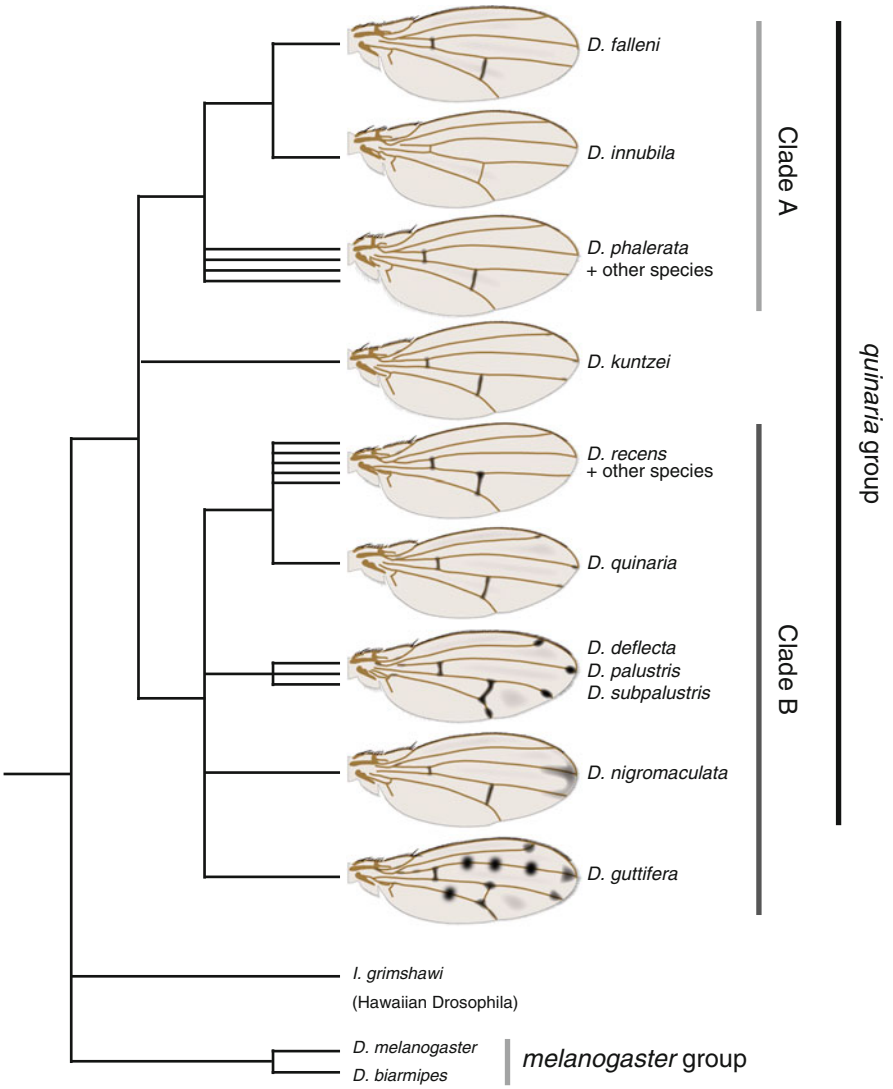

Fig. 16.2 Phylogenetic relationships of *D. guttifera* and species in the *quinaria* group. The topology was drawn from a consensus between Perlman et al. (2003) and Izumitani et al. (2016). See also Fig. 16.3 for interpretation of pigmentation

16.5 Wing Pigmentation Pattern Formation in *Drosophila*

The initial study of the mechanism of wing pigmentation pattern formation was done by True et al. (1999). They argued that patterns are formed through patterning by gene expression and subsequent elaboration by precursor trafficking through wing veins, based on experiments using *Drosophila grimshawi* (synonym of

Idiomyia grimshawi), *D. rajasekari* (synonym of *D. biarmipes*), and mutants and transgenics of *D. melanogaster*. Wittkopp et al. (2002) studied the function of *yellow* and *ebony* genes in the body trunk and wings of *D. melanogaster*. They also showed that the future spot position had more Yellow protein and less Ebony protein. Yellow is known to enhance black melanin synthesis, and Ebony is an enzyme that conjugates beta-alanine to dopamine and produces NBAD (N-beta-alanyldopamine) resulting in repression of black melanin synthesis. Gompel et al. (2005) analyzed the regulation of *yellow* gene expression in *D. biarmipes* and showed that evolution of an enhancer (a sequence that enhances expression of a nearby gene) was involved in the gain of pigmentation. In *D. biarmipes* and *D. guttifera*, they showed that Yellow protein was localized in future black spots and Ebony protein was localized in future transparent (no pigmentation) places. The *yellow* expression in the anteriodistal part of the wing in *D. biarmipes* results from regulation by at least two factors: posterior expression of *engrailed* repressing the *yellow* expression and anteriodistal expression of *Distal-less* enhancing expressions of *yellow* and other pigmentation genes (Gompel et al. 2005; Arnoult et al. 2013).

16.6 Features of Wing Pigmentation Pattern in *D. guttifera*

D. guttifera has prominent black polka dots on its wings, and these are believed to be made with melanin (Fig. 16.3). Pigmentations are formed around crossveins, longitudinal vein tips, and the campaniform sensilla. Weak pigmentations are also formed in intervein regions. As mentioned previously, crossvein pigmentation is widely observed in the *quinaria* group and also found in many species in other species groups. The crossvein pigmentation in *D. guttifera* is constricted in the center, forming an hourglass shape (or calabash shape), and this is unique to this species. Longitudinal vein tip pigmentations are observed in a few species, but the pigmentation area is largest in *D. guttifera*. Campaniform sensilla pigmentation is a trait unique to *D. guttifera*, although some species, such as a Hawaiian species, *Idiomyia grimshawi* (synonym of *Drosophila grimshawi*), have dappled spots all over the wings. The campaniform sensilla are lined on the third longitudinal vein in the same way as in other drosophilids, but in *D. guttifera*, one campaniform sensillum is also found on the fifth longitudinal vein, which is unique to this species. This campaniform sensillum is also surrounded by pigmentation (Sturtevant 1921; Werner et al. 2010). The wing pigmentation of *D. guttifera* starts to form in the pupal period, and it continues until one day old adult (Fukutomi et al. 2017).

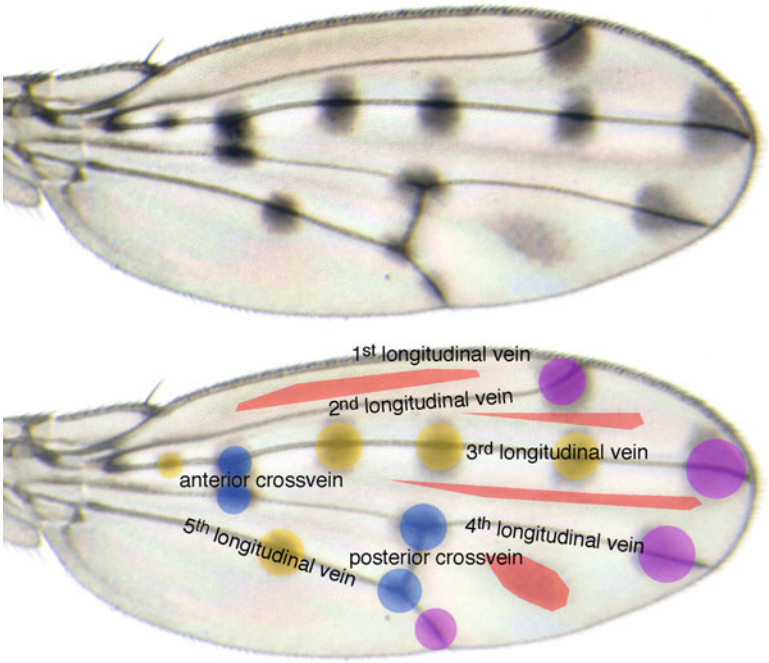

Fig. 16.3 *Top* Wing pigmentation of *D. guttifera*. *Bottom* Interpretation of the pigmentation pattern. Blue marks pigmentations around crossveins, purple marks longitudinal vein tips, yellow marks campaniform sensilla, and red marks intervein shading

16.7 *Wingless* Gene Induces Pigmentation Pattern Formation in *D. guttifera*

Werner et al. (2010) analyzed the *cis*-regulatory region of the *yellow* gene and identified *vein spot* CRE, which is an enhancer driving expression in all the polka dots, and *intervein shade* CRE, which is an enhancer driving expression in the intervein region. *Vein spot* CRE drove polka dots in *D. guttifera* but drove around crossveins and longitudinal vein tips if introduced in *D. melanogaster*. This difference means there is a difference in localization of a *trans*-regulatory factor that has an input to *vein spot* CRE. Gene expression patterns were known for several genes in *D. melanogaster*, and therefore they found candidate genes from genes showing similar expression with the *vein spot* CRE pattern. Among the candidate genes, *wingless*, a gene encoding a ligand of the Wnt signaling pathway, showed expression in the center of future spot positions (crossveins, longitudinal vein tips, and campaniform sensilla) in *D. guttifera*. There was no *wingless* expression in the campaniform sensilla in a closely related species, *D. deflecta*, which does not have pigmentation around them. A spontaneous mutant line of *D. guttifera*, *schwarzvier*, has additional pigmentation on the fourth longitudinal vein. In this mutant line, *wingless* was ectopically expressed on the fourth longitudinal vein. To obtain direct

functional evidence, they tried to make ectopic expressions of *wingless* by construction of the GAL4/UAS system in *D. guttifera*. Although they did not obtain optimal GAL4 lines, they found that one of the UAS-*wingless* lines had ectopic expression of *wingless*, probably caused by the enhancer trap mechanism. In this line, *wingless* was expressed ectopically on the second, third, and fourth longitudinal veins of pupal wings, and additional pigmentation was formed on these veins in adult wings. With these evidences, they concluded that *wingless* is the upstream *trans*-factor that induces pigmentation.

In *Heliconius* and *Limenitis* butterflies, the *WntA* gene, which also seems to encode a ligand of Wnt signaling, is involved in specifying wing pattern shapes, including in melanic elements (Martin et al. 2012; Gallant et al. 2014; Martin and Reed 2014). In *Junonia coenia* and some other butterfly species, *wingless* is known to be expressed in future pattern elements called basal (B), discal (DI and DII), and marginal (EI) elements (Carroll et al. 1994; Martin and Reed 2010, 2014; Huber et al. 2015) and was also identified at the center of eyespot patterns (Monteiro et al. 2006). The thoracic pattern of larval *Bombyx mori* is also regulated by *Wnt1* (homolog of *wingless*) (Yamaguchi et al. 2013). Evolutionary roles of secreted ligand genes such as *wingless* are reviewed in chapter 4 of this book (Martin and Courtier-Orgogozo 2017).

Werner et al. (2010) proposed a model of pigmentation pattern formation based on the assumption that Wingless protein diffuses from the source and serves as a long-range signal. There are a limited number of cells expressing *wingless*, and they are located in centers of future pigmented spots. In their model, secreted Wingless protein is diffused or transported to wider regions and transduces the signal. The signal is probably mediated by an unknown transcription factor and activates transcription of melanin synthesis-related genes, including *yellow*. Melanin should be synthesized by products of these genes and wings are consequently pigmented. This model should be validated by future research.

16.8 *Cis*-Regulatory Evolution of *Wingless*

The expression pattern of *wingless* evolved uniquely in *D. guttifera*. To examine how this unique expression pattern evolved, the genomic region around *wingless* was analyzed using a fluorescent reporter assay. As a result, three novel enhancer activities (in longitudinal vein tips, campaniform sensilla, and thoracic stripes) were found (Fig. 16.4). These novel enhancer activities are thought to have been involved in the evolution of the novel pigmentation pattern (Koshikawa et al. 2015). This study provided unique insights into the evolution of novel traits, illustrating how gains of novel enhancer activities at developmental regulatory gene were associated with derived expression domains and the emergence of novel traits (Rebeiz et al. 2011; Koshikawa et al. 2015; Rebeiz and Williams 2017).

We can generalize this concept as follows. In many organisms, gains of novel expression domains by gains of enhancer activities for a developmental regulatory

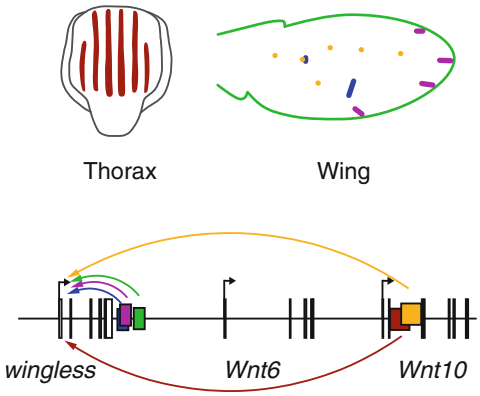

Fig. 16.4 Enhancers driving pupal wing and thoracic expressions of *wingless* in *D. guttifera*. Color code indicates correspondence of enhancer positions and expression domains. *Green*: wing margin. *Blue*: crossveins. *Purple*: longitudinal vein tips. *Yellow*: campaniform sensilla. *Brown*: thoracic stripes. Expressions in the wing margin and crossveins are ancestral (common in *D. melanogaster* and *D. guttifera*), and the longitudinal vein tips, campaniform sensilla, and thoracic stripes are novel (found in *D. guttifera* but not in *D. melanogaster*) (Modified from Koshikawa et al. (2015) and Koshikawa (2015))

gene could be a part of possible mechanisms of heterotopy (evolutionary duplication of a pre-existing trait in a different place on the body) (Gould 1977; West-Eberhard 2003; Rubinstein and de Souza 2013; Rebeiz et al. 2015; for more discussion see Koshikawa 2015).

16.9 Trials of Artificial Production of Pigmentation on *D. melanogaster* Wings

For now, only two genes, the upstream pattern inducer *wingless* and the melanin synthesis-related gene *yellow*, have been identified in the machinery required for pigmentation pattern formation in *D. guttifera*. In many cases, the Wingless signal is transduced through the so-called canonical pathway, where Pangolin/dTCF is an effector transcription factor regulating transcriptions of downstream genes. There were consensus sequences of Pangolin/dTCF binding sites in *vein spot* CRE, but replacement of these sequences by nonsense sequences did not change the expression pattern of the reporter gene (Werner et al. 2010). This means that the positional information of *wingless* does not directly regulate *yellow* through the canonical Wnt pathway. Involvement of another transcription factor is assumed, but so far it has not been identified. Furthermore, we know *yellow* is involved in pigmentation, but overexpression of *yellow* alone does not cause additional pigmentation in *D. melanogaster* (Gompel et al. 2005; Riedel et al. 2011). Proper expression or

repression of melanin synthesis-related genes and/or proper supply of melanin precursors, such as dopa and dopamine, could be required for artificial production of pigmentation in *D. melanogaster* wings.

16.10 Diversity and Generality in Color Pattern Formation

We summarized above what was revealed by studies of *D. guttifera*, but will it apply to pattern formation in other organisms? Due to the experimental strengths of this system, we can be optimistic that we will reach an integrated model for pigmentation pattern formation in *Drosophila*. Butterflies show interesting parallels with the *Drosophila* wing patterning genes, as *Wnt* genes and *Distal-less* are key players in both lineages (Werner et al. 2010; Martin et al. 2012; Brakefield et al. 1996; Arnoult et al. 2013). If we expand the comparison to vertebrates, there are large differences in genes involved in pattern formation and melanin synthesis (Kopp 2009; Kronforst et al. 2012; Kaelin et al. 2012; Mallarino et al. 2016). Still we assume we can find some common mechanisms, such as a way of measuring distance in a tissue, and a hierarchical regulatory architecture. Comparing comprehensive datasets will be instrumental in answering this question of fundamental interest for our understanding of the mechanisms that generate biodiversity on Earth.

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