Part V
Color Patterns of Larva and Other Insects

Chapter 15
Molecular Mechanisms of Larval Color Pattern Switch in the Swallowtail Butterfly

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Abstract In lepidopterans (butterflies and moths), larval body color pattern, which is an important mimicry trait involved in prey–predator interactions, presents a great diversity of pigmentation and patterning. Unlike wing patterns, larval body color patterns can switch during development with larval molting. For example, in the Asian swallowtail butterfly *Papilio xuthus*, a younger larva (first–fourth instar) has a white/black color pattern that mimics bird droppings, whereas the final instar (fifth) larva drastically changes to a greenish pattern that provides camouflage on plants. Insect mimicry has interested scientists and the public since Darwin's era. Broadly, mimicry is an antipredation strategy whereby one creature's color, shape, or behavior resembles another creature or object. In this review, I address basic knowledge about larval cuticular pigmentation and advanced understanding of its regulatory mechanism in *P. xuthus*; I also discuss larval body color patterns among members of the genus *Papilio*, followed by conclusions and prospects for further research.

Keywords Larval pigmentation • Lepidoptera • Mimicry • Cuticular melanization • Ecdysteroid • Juvenile hormone • *Papilio xuthus* • *Papilio polytes* • *Papilio machaon*

15.1 Introduction

About 150 years ago, following H.W. Bates' report on mimicry in insects (Bates 1862), Charles Darwin wrote to Bates and said: "In my opinion, it is one of the most remarkable & admirable papers I ever read in my life. The mimetic cases are truly marvelous. . ." (Darwin 1863). Today, the mimicry phenomenon remains as an interesting evolutionary theme as ever, attracting the interest of both scientists and the public. To better understand the molecular mechanisms behind insect mimicry, we need to understand how wing and body color patterns evolve.

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Lepidopterans (butterflies and moths) show highly diverse wing colors and patterns and are considered to be an ideal model system for examining color pattern formation and evolution. In lepidopterans, evidence for wing color and pattern evolution has been frequently reported (Nijhout 1991; Reed et al. 2011; Heliconius Genome 2012; Kunte et al. 2014), whereas our knowledge of larval body coloration and pattern formation is relatively limited.

Generally, lepidopteran larvae are soft bodied and cannot escape by flight from predators. Natural selection has led to the development of many chemical and morphological devices in larvae that aid survival in the wild (Scoble and Scoble 1992). Among those, body color pattern is particularly interesting because it is important in visual recognition. Two different strategies are commonly used for predator defense. Toxic larvae tend to warn predators with their colorful markings which act as warning signals. The majority of larvae, which are palatable and nonpoisonous, mimic an item in the surroundings (such as a bud, a twig, or even a moss) or conceal their bodies in the environmental background (Pasteur 1982).

In the case of *P. xuthus*, a larva switches its body color pattern with larval molting (Fig. 15.1). A younger larva (first–fourth instar) mimics bird droppings with a black/white body color (denoted mimetic pattern, Fig. 15.1a). The fifth (final) instar larva dramatically switches to a greenish body pattern with a pair of eyespots on the metathorax, which allows it to blend in with the color and pattern of its host plant (denoted cryptic pattern, Fig. 15.1b). A similar switching of body color pattern is observed in other *Papilio* species (Prudic et al. 2007) and is considered to be a successful survival strategy for this genus (Tullberg et al. 2005). Recent studies have reported that two critical insect hormones, ecdysone (Fig. 15.2a, b) and juvenile hormone (JH), directly regulate pigmentation and color pattern switch in the larva of *P. xuthus* (Futahashi and Fujiwara 2007, 2008a).

In this chapter, I review recent progress in understanding the molecular mechanisms underlying cuticular melanization and the hormonal regulation of pigmentation in the larva of *P. xuthus*. I also discuss possible evolutionary changes among three *Papilio* species, followed by conclusions and prospects for further research.

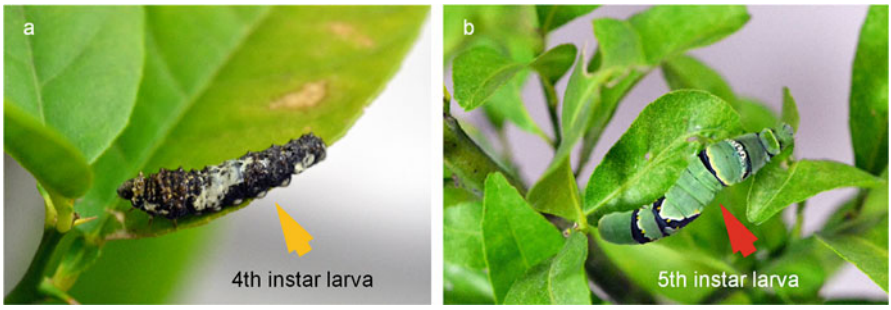

Fig. 15.1 (**a**) Fourth instar larva with bird-dropping body pattern; (**b**) Fifth instar larva with *green* body pattern of *P. xuthus*

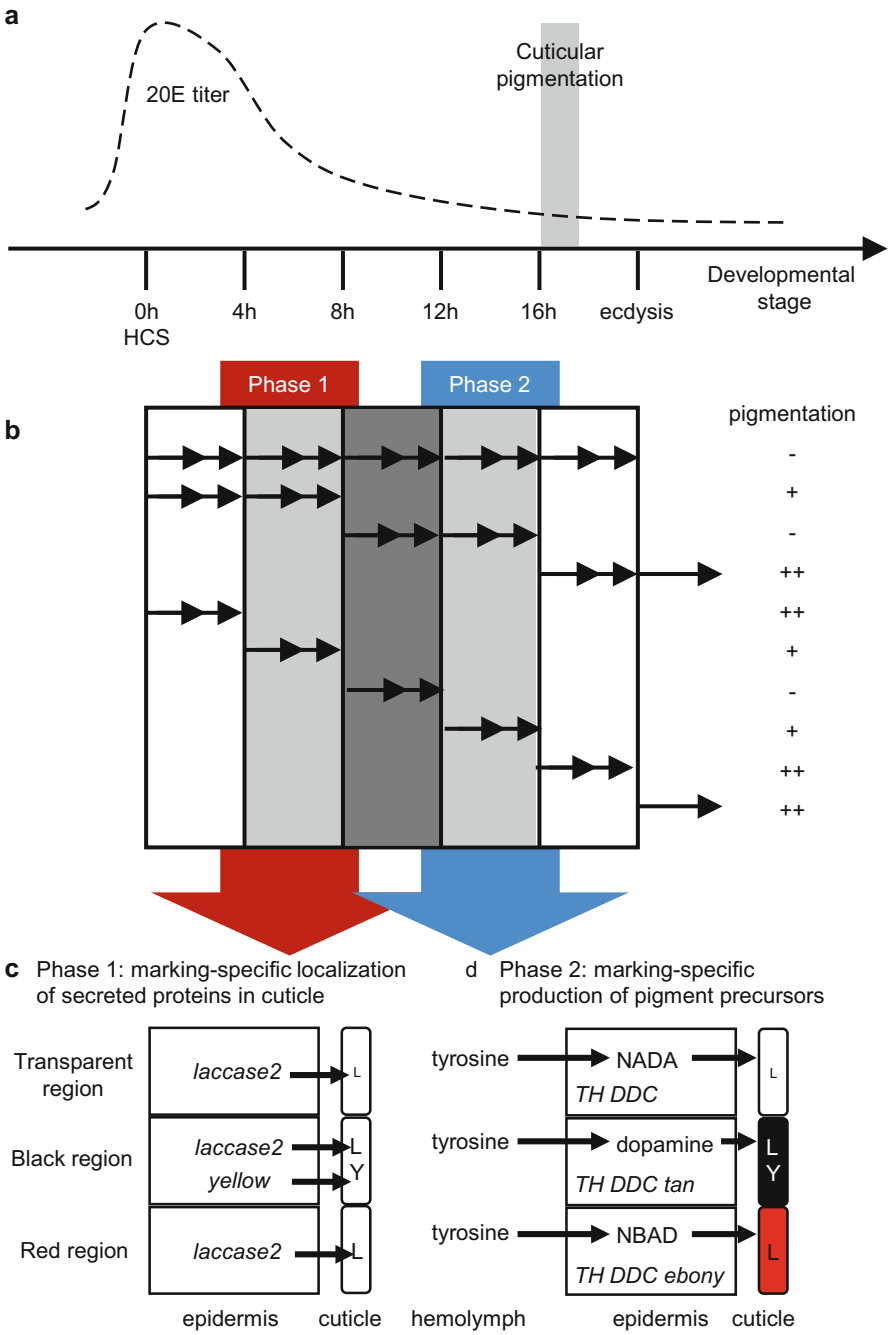

Fig. 15.2 A working model for the two-phase cuticular pigmentation in larvae of *P. xuthus*. (**a**) 20E titer in hemolymph during the fourth molt; (**b**) The timing effect of 20E on *black* pigment synthesis. The intervals of 20E applications (*arrows*, every 2 h); (**c**) phase 1; (**d**) phase 2. N-b-alanyldopamine (NBAD). N-acetyldopamine (NADA). L and Y in cuticle indicate laccase 2 and Yellow proteins (Modified from Futahashi et al. 2010)

15.2 Pigmentation of Larval Cuticle in *P. xuthus*

An insect cuticle is a hardened exoskeleton composed of chitin and proteins. In lepidopteran larvae, black cuticular pigments mainly comprise melanin, which is produced by the oxidization of dopamine or L-3,4-dihydroxyphenylalanine (DOPA) (Kramer and Hopkins 1987; Hiruma and Riddiford 2009; Wright 1987). In both *P. xuthus* and *Manduca sexta* (tobacco hornworm), the pigmentation procedure of larval cuticle can be summarized in two steps: localization of secreted proteins (Fig. 15.2c, phase 1) and production of pigment precursors (Fig. 15.2d, phase 2) (Hiruma and Riddiford 2009; Futahashi et al. 2010; Walter et al. 1991). These steps occur in the cuticle and the epidermal cell, respectively (Fig. 15.2).

In phase 1, laccase 2 (Lac2), which is a phenol oxidase (PO), and other pigment-related proteins (such as Yellow) are synthesized and deposited into the newly forming cuticle (Fig. 15.2c) (Futahashi and Fujiwara 2007; Kramer and Hopkins 1987; Hiruma and Riddiford 1988, 2009). Lac2 catalyzes the oxidation of dopamine to dopamine–melanin in many species (Hiruma and Riddiford 2009; Noh et al. 2016; Futahashi et al. 2011). In *Tribolium castaneum*, laccase 2 (coded by *TmLac2*) is the major PO involved in the tanning of larval, pupal, and adult cuticles (Arakane et al. 2005).

In *P. xuthus*, Futahashi et al. (2010) found that *Pxlaccase2* (*Pxlac2*) expression is strongly associated with the presumptive black pigment (11 h after head capsule slippage (HCS) at the fourth molt) (Futahashi et al. 2010). Typically, expression of Lac2 begins in the middle period of molting, and the deposited Lac2 is on standby until the pigment precursors reach the cuticular surface. These events precede the expression of melanin synthesis genes at mRNA levels and the production of pigment precursors (Walter et al. 1991; Hiruma and Riddiford 1988; True et al. 1999; Futahashi and Fujiwara 2005). Another pigmentation-related protein, Yellow (coded by *Pxyellow*), shows an expression pattern similar to that of Lac2 in phase 1. However, the precise function of *Pxyellow* gene remains unclear (Futahashi et al. 2010; Noh et al. 2016). It is inferred that PxYellow may be secreted into the cuticle and probably acts as a cofactor (Futahashi and Fujiwara 2005).

In phase 2, the precursors of melanin compounds are synthesized from phenolic amino acids (mainly tyrosine) (Fig. 15.2c). The dopamine–melanin synthesis pathway is conserved in many insects (Hiruma and Riddiford 2009; Noh et al. 2016; Futahashi and Fujiwara 2005; Massey and Wittkopp 2016). First, tyrosine is converted to DOPA by tyrosine hydroxylase (TH), and then dopamine is synthesized from DOPA by DOPA decarboxylase (DDC) (Futahashi and Fujiwara 2005). Dopamine is a prominent black pigment precursor in many insects (Hiruma et al. 1985). After its synthesis in an epidermal cell, dopamine is incorporated into the cuticle and converted to dopamine–melanin by PO and other proteins. However, it also can be converted to a reddish brown pigment by ebony or to a transparent pigment called N-acetyldopamine (NADA) by dopamine N-acetyltransferase (DAT) activity (Futahashi et al. 2010; Futahashi and Fujiwara 2005; Massey and Wittkopp 2016; Wittkopp et al. 2002).

In *P. xuthus*, spatially specific localization of melanin synthesis genes contributes to the color pattern (Futahashi and Fujiwara 2005). Futahashi and Fujiwara (2005) showed that the spatial expression of melanin synthesis genes (*TH*, *DDC*, and *tan*) perfectly corresponds with the presumptive black pigment (Futahashi et al. 2010; Futahashi and Fujiwara 2005) and that the expression of *ebony* is limited to the red area within the eyespot (Futahashi and Fujiwara 2005). They also demonstrated that the addition of excess tyrosine did not promote pigmentation, whereas the application of DOPA with 3-iodotyrosine (3IT, a competitive inhibitor of TH protein) led to a clear color pattern with an overall pigmentation in vitro (Futahashi and Fujiwara 2005). Their results indicate that cuticle color patterns form from spatially specific localization of melanin synthesis genes rather than the differential uptake of melanin precursors into individual epidermal cells.

Cuticular pigmentation occurs in the latter half of the molting period just before ecdysis (16–18 h after HCS during the fourth molting period). When Futahashi and Fujiwara (2005) examined the timing of expression of *PxTH*, *PxDDC*, *Pxebony*, and *Pxtan*, they noticed that the expression of these melanin synthesis genes precisely coincides with melanization onset. Therefore, cuticular pigmentation is predictably strictly controlled by ecdysteroid, the molting hormone (Futahashi and Fujiwara 2005, 2007; Futahashi et al. 2010).

15.3 Hormonal Regulation of Larval Pigmentation

Ecdysone and juvenile hormone are directly and indirectly involved in larval pigmentation in insects (Futahashi and Fujiwara 2008a; Hiruma and Riddiford 1990, 2009; Hwang et al. 2003).

15.3.1 Ecdysone-Induced Cuticular Pigmentation

Ecdysone is a steroid hormone and the central regulator in insect development and reproduction (Kopec 1926). The periodic release of ecdysone triggers larval molting and pupal metamorphosis (Yamanaka et al. 2013).

The first evidence of ecdysone-regulated pigmentation was reported by Karlson and Sekeris in 1976. They showed that ecdysone causes elevated activity of DDC in *Calliphora* (Hiruma and Riddiford 2009; Karlson and Sekeris 1976). In *M. sexta*, regulation of DDC expression requires exposure of 20-hydroxyecdysone (also known as 20E, an active form of ecdysone), followed by its withdrawal during larval molting (Hiruma and Riddiford 1986, 1990; Hiruma et al. 1995; Hiruma and Riddiford 2007). Hiruma et al. (1995) found continuous exposure of 20E insufficient for DDC expression, unless there is a 20E-free period (Hiruma et al. 1995).

In *P. xuthus*, Futahashi and Fujiwara (2007) successfully tested the effect of 20E exposure on larval pigmentation using a topical application method in vivo.

Consistent with the results in *M. sexta*, they demonstrated that cuticular melanization and epidermal pigmentation are inhibited through 20E treatment during the molt and confirmed that the removal of ecdysone is necessary for the onset of normal coloration. Moreover, they showed that 20E inhibited pigmentation if it was applied at the middle of the molt when native ecdysone titers decline (Fig. 15.2b) (Futahashi and Fujiwara 2007). As expected, the expression of melanin synthesis genes, including *TH*, *DDC*, and *ebony*, was repressed by high 20E concentration (Futahashi and Fujiwara 2007). Unexpectedly, the expression of *Pxyellow* was promoted by a high concentration of 20E. This led Futahashi and Fujiwara to hypothesize that PxYellow must function as a cofactor for other melanin synthesis enzymes since it alone is not sufficient for melanization (Futahashi and Fujiwara 2007).

Like the pigment synthesis genes, some upstream regulatory factors are also controlled by ecdysone. In the ecdysone signaling pathway, 20E acts as a hormonal signal and regulates the expression of downstream transcription factors (Yamanaka et al. 2013; Yao et al. 1992). Hiruma and Riddiford (2007) found that two nuclear transcription factors, E75B and MHR4, are 20E-induced inhibitors of *Msddc* in vitro (Hiruma and Riddiford 2007). Evidence also showed that there is at least one other suppressive protein other than E75B and MHR4 that binds to a specific sequence (GGCTTATGCGCTGCA) in the DDC promoter when the ecdysone titer decreases (Hiruma et al. 1995). In *Drosophila melanogaster*, *DmDDC* is directly modulated by an ecdysone response element (EcRE), located at position −97 to −83 bp relative to the transcription initiation site (Chen et al. 2002). In *D. melanogaster*, Yellow is known to be a prepattern factor as well as a pigmentation factor in adult body patterning (Massey and Wittkopp 2016). Recently, comprehensive yeast one-hybrid and RNAi screens were carried out by Kalay et al. (2016). They screened and identified four ecdysone-induced nuclear reporters (*Hr78*, *Hr38*, *Hr46*, and *Eip78C*) that showed a statistically significant interaction with at least one Yellow enhancer. In an RNAi experiment, all four caused altered pigmentation when knocked down (Kalay et al. 2016). In *Bombyx mori*, Yamaguchi et al. (2013) used a type of *L* (multi lunar) mutant with twin-spot markings on the sequential segments and proved that the gene responsible for this phenotype (*BmWnt1*) can be induced by high concentrations of 20E in vitro (Yamaguchi et al. 2013).

In *P. xuthus*, Futahashi et al. (2012) used a microarray EST dataset to recognize *E75A* and *E75B*, which are transcription factors involved in ecdysone signaling, as candidates involved in specific marking-specific patterning (Futahashi et al. 2012). The expression of *E75A* and *E75B* is specifically localized at the eyespot marking region, and temporal expression patterns are similar to those of *Pxyellow*, as described before. It is known that *E75* is active early in ecdysone signaling (Palli et al. 1995; Jindra et al. 1994; Jindra and Riddiford 1996). Taken together, this suggests that 20E-induced *E75A* and/or *E75B* expression may regulate both the prepattern of marking and the stage specificity of several black marking-associated genes (Futahashi et al. 2012). Interestingly, 3-dehydroecdysone 3β-reductase (coded by the *3DE 3β-reductase* gene) has a clear marking-specific expression in

the presumptive black region, similar to TH or DDC. Since its function is converting inactivated 3-dehydroecdysone to ecdysone, localized marking-specific ecdysone synthesis may be critical for complex cuticular pigmentation and patterning (Futahashi et al. 2012).

The evidence above shows that there is a complicated relationship between ecdysone signaling and larval cuticular pigmentation and patterning. However, because only some of the regulatory genes have been identified, the detailed regulatory mechanisms remain to be uncovered.

15.3.2 Juvenile Hormone Directly Regulates Larval Color Pattern Switch

Juvenile hormone (JH) is a group of acyclic sesquiterpenoids secreted from the corpora allata (CA), which is an endocrine gland near the brain (Jindra et al. 2013). Like ecdysteroids, JH plays a critical role in molting, metamorphosis, reproduction, and other physiological processes in insects (Jindra et al. 2013). JH is also known as "*status quo* hormone," because the presence of JH prevents insect metamorphosis (Riddiford 1996). In a simplified model, a lepidopteran progresses through a larva-to-larva molt when JH is present and a larva-to-pupa metamorphosis when JH is absent at the final molting stage. It has been hypothesized that JH modulates the action of ecdysteroid-molting hormones, but the detailed mechanisms of the modulation are still unclear (Jindra et al. 2013; Urena et al. 2014; Kayukawa et al. 2016).

There is some evidence that JH has an effect on larval pigmentation. Lack of sufficient JH (caused by the artificial removal of the CA from the larva) causes black larvae in the tobacco hornworm, *M. sexta*. In addition, when the larvae of the black strain are treated with JH, they revert to their normal green color (Riddiford 1975).

As described above, *P. xuthus* larvae markedly switch from a black/white body pattern to a greenish one after the fourth–fifth larval ecdysis (Fig. 15.1). Futahashi (2006) found that when 20E was injected at the early fourth instar stage, precociously molted fifth larva appeared with a black/white mimetic pattern instead of the normal green pattern (Futahashi 2006). It is known and proven that JH controls the action of ecdysteroid at least through direct inhibition of *Broad–Complex* (*BR-C*) activity (Kayukawa et al. 2016; Nijhout and Wheeler 1982; Ogihara et al. 2015). To define the role of JH in facilitating larval color pattern regulation, Futahashi and Fujiwara (2008a) performed experiments using three types of JH analogs (JHA), which they artificially applied on the integument of fourth instar larvae (Fig. 15.3). Their results showed that some individuals failed to switch color patterns, either completely or partially, after the fourth molt. The larvae treated with fenoxycarb (JHA) kept a fourth instar-like black/white pattern or developed an intermediate color pattern with elements of both fourth and fifth instars (Fig. 15.3).

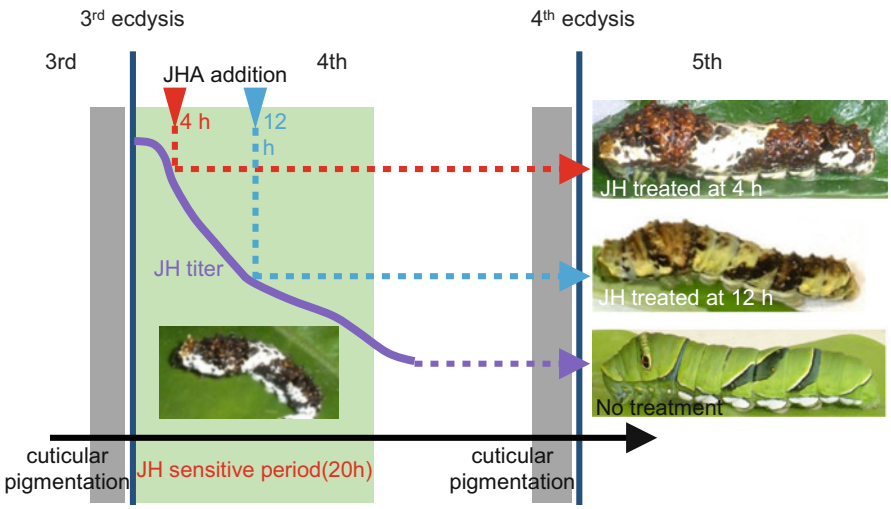

Fig. 15.3 Treatment of juvenile hormone analogs during the JH-sensitive period (Modified from Futahashi and Fujiwara 2008a)

Furthermore, they noticed that the epidermis is only sensitive to JHA during the first 20 h of the fourth instar stage. Exposure after this relatively short time frame did not prevent the color pattern switch. Hence, they named that specific time window "JH-sensitive period." In nontreated species, JH titer in the hemolymph was measured and found to be decreasing continuously during the early days of the fourth instar stage. Taken together, this evidence indicates that the decline of JH titer within a restricted developmental stage regulates the body color pattern switch in *P. xuthus* larvae (Futahashi and Fujiwara 2008a).

Because of our fragmentary knowledge of JH pathways, the molecular mechanisms underlying how JH alters color patterning and controls pigment synthesis are still under investigation (Jindra et al. 2013). Jin et al. have found some candidate genes involved in the larval color pattern switch by RNAi screening using the latest genomic information of *P. xuthus* (unpublished data). In my opinion, future studies may shed light on the downstream regulation of the JH cascade in larval pigmentation.

15.4 Species-Specific Color Patterns in the *Papilio* Genus

15.4.1 A Combination of Yellow and Blue Makes the Larval Body Green

A greenish body pattern follows the bird-dropping pattern in many *Papilio* species, making us wonder what the identity of the "green" pigment is. Green body

coloration seems to be a beneficial adaptation for the final instar larvae of *Papilio*, which helps them conceal themselves in the host plant. The chemical nature of caterpillar's green pigment was once misunderstood as chlorophyll derived from the plant because of the strong color resemblance (Meldola 1873). However, studies show that larval green pigmentation is instead formed by a particular combination of yellow and blue pigments (Przibram and Lederer 1933). Przibram and Lederer (1933) proposed that the yellow pigments are carotenoids and that most of the blue pigments are biliverdins (Przibram and Lederer 1933). Later investigations led to a model that postulates that pigments are intimately associated with specific proteins and that the complex of pigment-conjugated proteins presents the visible coloration (Kawooya et al. 1985).

The blue pigment-binding protein (or bilin-binding protein, BBP) has been isolated and identified in various lepidopterans (Riley et al. 1984; Huber et al. 1987; Saito and Shimoda 1997; Kayser et al. 2009). In *M. sexta*, insecticyanin (INS) was identified to be a bilin-binding protein. Riddiford et al. (1990) found that INS is synthesized in the epidermis and is mainly stored in epidermal pigment granules or secreted into the hemolymph and cuticle (Riddiford et al. 1990). Other pigment-binding proteins are less well known. Although carotenoid-binding protein (CBP) has been well studied in vertebrates (Bhosale and Bernstein 2007), few homologs have been recognized among the Lepidoptera. In Lepidoptera, the *yellow-blood* mutant (Y) of *B. mori* (which produces yellow cocoon) was identified (Tsuchida and Sakudoh 2015); however, the expression of *BmCBP* was not detected in the epidermis. Using next-generation sequencing (NGS) technology, whole genomes of several lepidopteran species were recently released (Suetsugu et al. 2013; Li et al. 2015; Nishikawa et al. 2015; Kanost et al. 2016). Putative BmCBP homologs in other lepidopteran species can be found by BLAST search. Nonetheless, no biological experiment has been performed, and the molecular functions of these putative CBPs are largely unknown.

In *P. xuthus*, two related genes, *bilin-binding protein 1* (*BBP1*) and *yellow-related protein* (*YRG*), were identified to be associated with greenish epidermal coloration by Futahashi and Fujiwara (2008a, b) and Shirataki et al. (2010), respectively. In addition, two *putative carotenoid-binding proteins* (*PCBP1*, *PCBP2*) and other members of BBP family were later identified, which proved to be specifically expressed in the green epidermal regions during the final larval ecdysis (Futahashi et al. 2012).

15.4.2 Species-Specific Color Pattern Among Papilio *Species*

Another vital question in adaptive evolution is how the larval body pattern evolves among closely related species. There are about 200 species included in the genus *Papilio*, and these cover more than one-third of all Papilionidae (Prudic et al. 2007). In the genus *Papilio*, all the larvae share a similar bird-dropping coloration (mimetic pattern) until the fourth or fifth (final) instar (Prudic et al. 2007). The

color pattern in the final instar stage is divided into three patterns: bird-dropping mimetic pattern, green cryptic pattern, and aposematic pattern with orange or black spots and black or white stripes (Prudic et al. 2007; Yamaguchi et al. 2013).

Shirataki et al. (2010) investigated the larval color pattern formation using three *Papilio* species: *P. xuthus*, *P. machaon*, and *P. polytes* (Shirataki et al. 2010). In 2015, whole-genome sequences of those three species were released and made freely accessible (Li et al. 2015; Nishikawa et al. 2015). The last instar larvae of *P. xuthus* and *P. polytes* exhibit similar green cryptic body patterns, with a pair of eyespots on the metathorax and a V-shaped marking on the abdomen, whereas the fourth and fifth instar larvae of *P. machaon* have aposematic color patterns, with a greenish epidermis covered by black bands and an orange twin-spot marking. However, *P. xuthus* and *P. machaon* are more closely related to each other than either is to *P. polytes* (Fig. 15.4) (Zakharov et al. 2004).

Shirataki et al. (2010) cloned several pigmentation-related genes, including *TH*, *DDC*, *yellow*, *BBP1*, and *YRG*, from all three species and compared their expression patterns using in situ hybridization (Fig. 15.4). The results showed a perfect correlation between gene expression and pigmentation among species. Expression of TH, DDC, and yellow matched the black regions in the eyespot, the V-shaped markings of *P. xuthus* and *P. polytes*, and the black bands of *P. machaon*. Regardless of the universal expression of BBP1 and YRG in the green regions among all the three species, BBP1 was specifically expressed in the blue spots in *P. polytes*, and YRG was tightly associated with the orange spots in *P. machaon*. Notably, a unique expression pattern of *ebony* was only detected in the red area within the eyespot region in *P. xuthus*. This work led to the model described in Fig. 15.3.

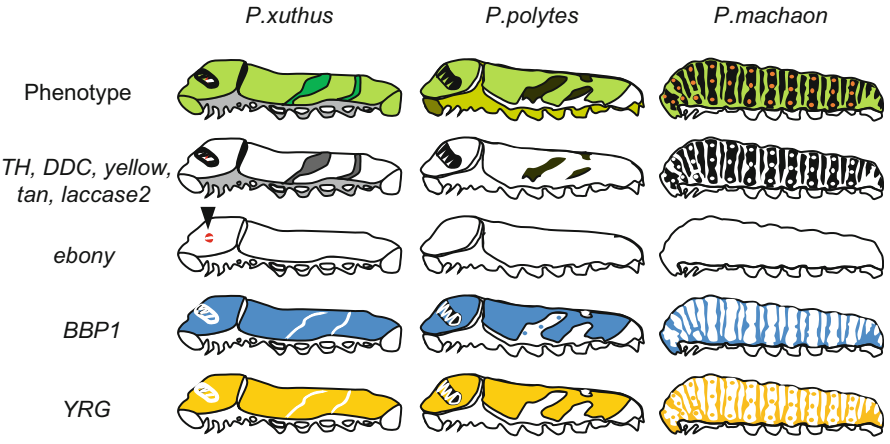

Fig. 15.4 Schema of species-specific body color pattern among three *Papilio* species (Modified from Shirataki et al. 2010)

15.4.3 *Trans-regulation of YRG in the Genus* Papilio

Morphological and phenotypic differences arising from evolutionary change, particularly using large-scale genetic information, have been recently identified in lepidopterans (Kunte et al. 2014; Nishikawa et al. 2015; Wallbank et al. 2016). Some studies have examined the genetic basis underlying intraspecific differences among members of the genus *Drosophila (*Massey and Wittkopp 2016; Wittkopp et al. 2009*)*. F1 hybrids allow researchers to understand regulation changes between close species (Wittkopp et al. 2003, 2008; Wittkopp and Kalay 2012).

Although hybrids of *Papilio* species are difficult to breed under laboratory conditions (Watanabe 1968), Shirataki et al. (2010) successfully bred an F1 hybrid by hand-pairing a *P. xuthus* male with a *P. polytes* female (Clarke and Sheppard 1956), and the fifth instar larvae showed intermediate characteristics between parents (Shirataki et al. 2010). One pigment-related gene, *YRG*, was selected to study expression patterns in the F1 hybrid because both the nucleotide and amino acid sequences had diverged enough to include species-specific regions. Species-specific YRG probes (PxYRG and PpYRG) were designed, and the spatial expression pattern was detected in the last instar larvae of the F1 hybrid. Both the PxYRG and PpYRG probes showed similar expression patterns, indicating that changes in expression of the *YRG* gene are mainly caused by trans-regulatory changes (Shirataki et al. 2010).

15.5 Conclusion and Future Prospects

In the swallowtail butterfly, the larval body color pattern is a vital ecological trait that affects prey–predator interactions. It is precisely regulated by ecdysteroid and juvenile hormone. In *P. xuthus*, pigmentation mechanisms and pathways have been recently elucidated. However, the details of hormonal regulation need to be understood, and the molecular mechanism underlying larval body color patterning has not been studied. New information from next-generation whole-genome sequencing projects will provide a valuable resource that can be used to gain insight into the genetic basis underlying those questions. Moreover, pioneering functional analysis methods, like electroporation-mediated transgenic methods (Ando and Fujiwara 2013) and the CRISPR/Cas9 system (Li et al. 2015), may also lead to new approaches for examining gene functions in non-model species, such as *P. xuthus*.

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

Shigeyuki Koshikawa, Yuichi Fukutomi, and Keiji Matsumoto

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.

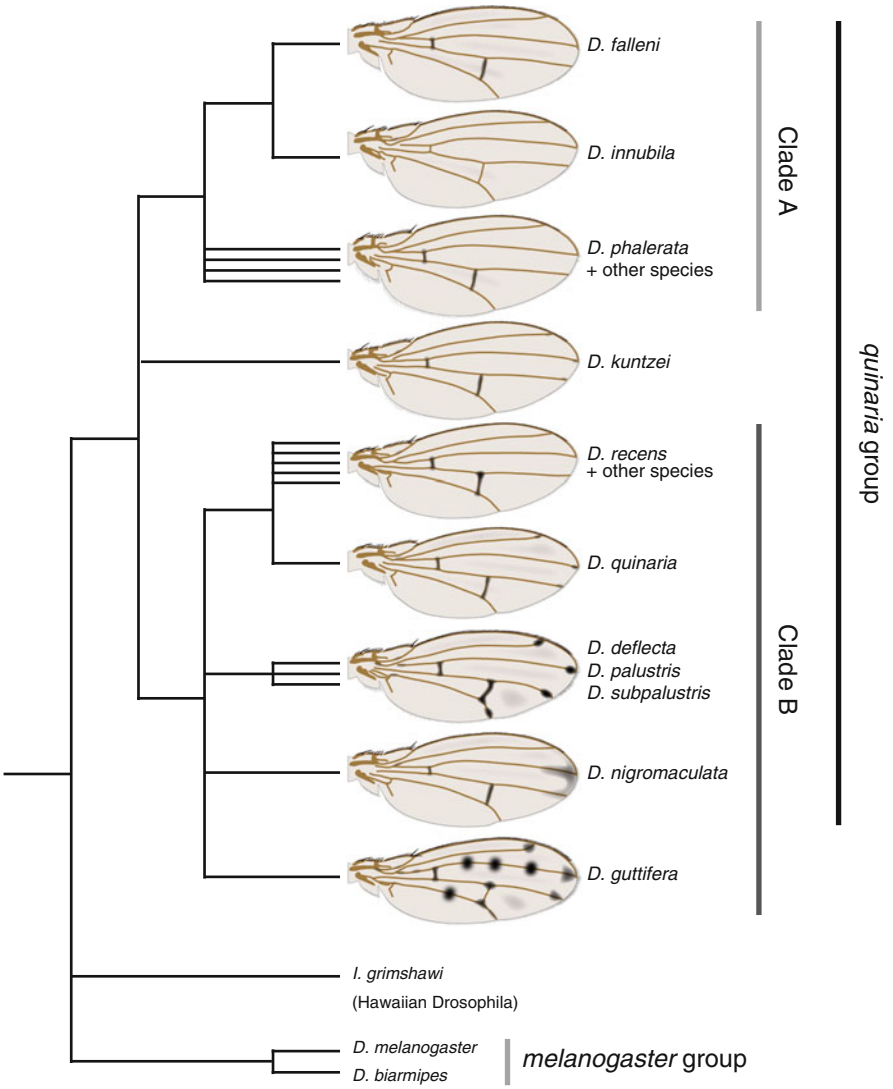

Fig. 16.2 Phylogenetic relationships of *D. guttifera* and species in the *quinaria* group. The topology was drawn from a consensus between Perlman et al. (2003) and Izumitani et al. (2016). See also Fig. 16.3 for interpretation of pigmentation

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).

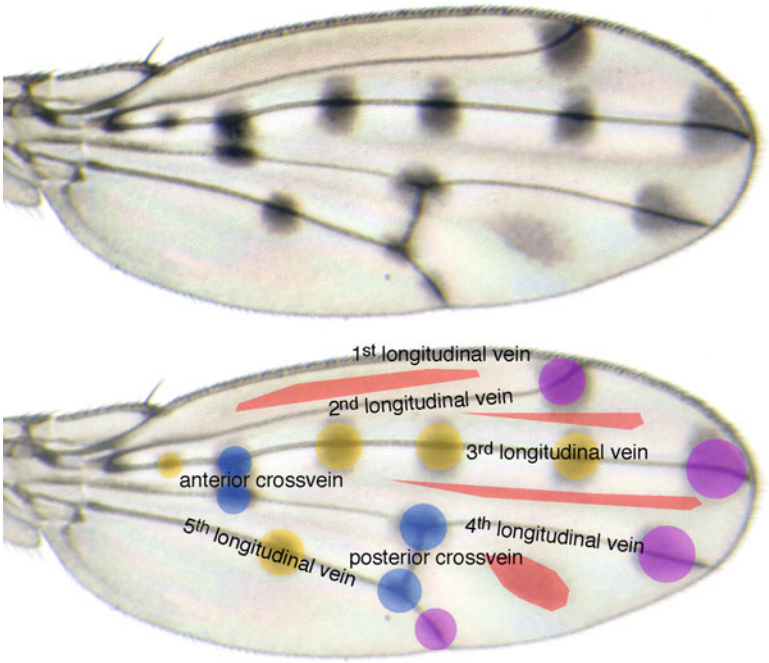

Fig. 16.3 *Top* Wing pigmentation of *D. guttifera*. *Bottom* Interpretation of the pigmentation pattern. Blue marks pigmentations around crossveins, purple marks longitudinal vein tips, yellow marks campaniform sensilla, and red marks intervein shading

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

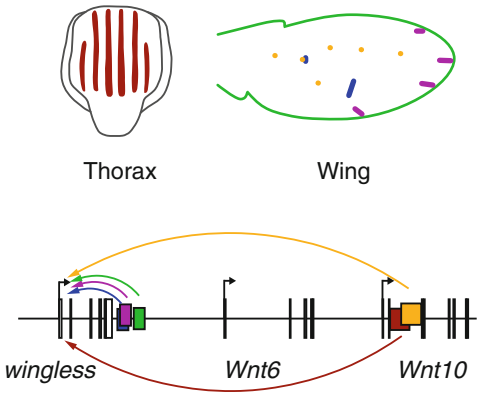

Fig. 16.4 Enhancers driving pupal wing and thoracic expressions of *wingless* in *D. guttifera*. Color code indicates correspondence of enhancer positions and expression domains. *Green*: wing margin. *Blue*: crossveins. *Purple*: longitudinal vein tips. *Yellow*: campaniform sensilla. *Brown*: thoracic 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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Chapter 17
Molecular Mechanisms Underlying Color Vision and Color Formation in Dragonflies

Ryo Futahashi

Abstract Dragonflies are colorful diurnal insects with large compound eyes. Because they visually recognize conspecific and heterospecific individuals, their body color plays essential roles in ecology and reproductive biology. Here I introduce the recent topics of molecular mechanisms underlying color vision and color formation in dragonflies. Complex wing color polymorphism is recognized among the two closely related Japanese *Mnais* species, presumably due to stepwise character displacement to avoid interspecific mating. We discovered an extraordinary large number of visual opsin genes by RNA sequencing of 12 dragonfly species. Manual correction after de novo assembly was crucial for determining the exact number and sequence of opsin genes. Each opsin gene was differentially expressed between the adult and larva, as well as between dorsal and ventral regions of adult compound eyes, highlighting the behavior, ecology, and adaptation of aquatic larva to terrestrial adult. The repertoire of opsin genes differed among dragonfly species, plausibly involved in the diversity of the habitat and behavior of each species. We also found that sex-specific yellow-red color transition in red dragonflies is regulated by redox changes in ommochrome pigments, which unveils a previously unknown molecular mechanism underlying body color change in animals. Establishment of the methods of gene functional analyses in dragonflies is desired for future studies.

Keywords Dragonfly • Color polymorphism • Character displacement • Opsin • Color vision • Pigment • Redox • Ommochrome

17.1 Introduction

Like butterflies, dragonflies (including damselflies, Insecta: Odonata) are one of the most colorful insects, and their color patterns have been focused from ecological and evolutionary aspects for a long time (Tillyard 1917; Corbet 1999; Bybee et al.

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2016). Dragonflies are well-known insects, and almost all Japanese people know the songs Aka-tombo (= red dragonflies; a symbol of autumn in Japan) and Tombo-no-megane (= eye glasses of dragonflies; a metaphor for the colorful compound eyes of dragonflies) (Ueda 2004; Inoue and Tani 2010). Despite the fact that the detailed genetic analyses of pattern formation in butterfly adult wing and larval body have progressed greatly in recent years as described in this book, the molecular biological study of dragonfly's color pattern formation has just started.

17.2 Important Role of Color Pattern for Partner Recognition in Dragonflies

Unlike most insects, drastic adult color transitions are widely recognized among dragonflies, resulting in conspicuous sexual dimorphism. In red dragonfly species, body colors of males turn from yellow to red in the course of sexual maturation, whereas females are yellowish throughout their adult lives in general (Fig. 17.1a–c). Moreover, many dragonfly species have color polymorphism even in the same sex (Fig. 17.1c–f, i–n), which is genetically controlled at least in several species (Futahashi 2016a). Considering that gynandromorph specimens display discontinuous male/female mosaicism in their coloration (Fig. 17.1g, h), sex-specific color formation is regulated cell-autonomously in dragonflies.

In many dragonfly species, adult body color plays important roles in partner recognition (Corbet 1999; Svensson et al. 2007; Córdoba-Aguilar 2008; Svensson et al. 2014; Takahashi et al. 2014; Beatty et al. 2015; Drury et al. 2015). Interspecific or male-male connection has sometimes been observed in the field between similar-colored individuals (Fig. 17.2a–c), and interspecific hybrids has been reported occasionally (Fig. 17.2d–f) (Corbet 1999; Futahashi 1999; Futahashi and Futahashi 2007; Moriyasu and Sugimura 2007; Ozono et al. 2012; Sánchez-Guillén et al. 2014; Futahashi 2016a). Parent combination of hybrid specimen can be determined by biparentally inherited nuclear DNA and maternally inherited mitochondrial DNA analyses (Fig. 17.2g), and it has been reported that males of *Sympetrum eroticum* are apt to catch females of other species (Futahashi 1999; Futahashi and Hayashi 2004a), suggesting that the direction of gene flow with hybridization is nonreciprocal in some cases. These misidentifications in dragonflies may be attributed to their poor sense of audition and olfaction; dragonflies lack the auditory organs, and their antennae are less developed (Yager 1999; Cocroft and Rodríguez 2005).

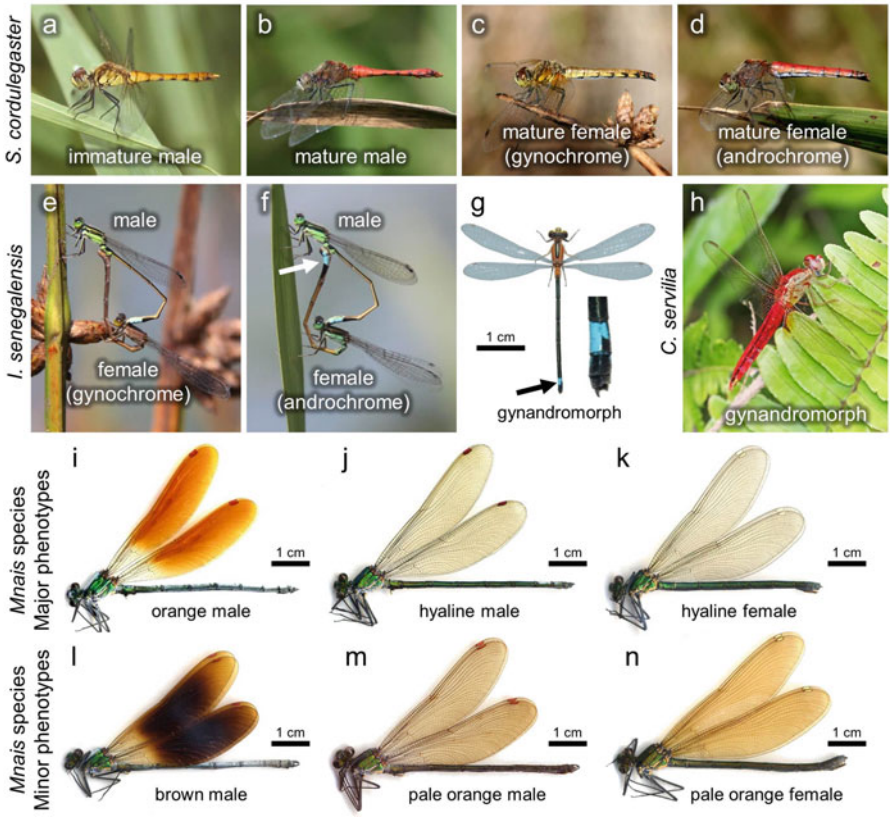


Fig. 17.1 Intraspecific color pattern diversity in dragonflies. (**a**–**d**) Sexual dimorphism, male color transition, and female color polymorphism of *Sympetrum cordulegaster*. (**e**–**f**) Female color polymorphism of *Ischnura senegalensis*. *Arrow* indicates a blue spot existed in males and androchrome females. (**g**) Gynandromorph of *I. senegalensis* showing the main region with female coloration and the posterior left side (*arrow*) with male coloration and appendage (Photo courtesy of Mitsutoshi Sugimura). (**h**) Gynandromorph of *Crocothemis servilia* showing the main region with male coloration and the anterior right side with female coloration (Photo courtesy of Kohji Tanaka). (**i**–**n**) Wing color polymorphism of *Mnais* species. Territorial males have orange (**i**) or brown (**l**) wings, whereas female mimicking males have hyaline (**j**) or pale orange (**m**) wings. Females have hyaline (**k**) or pale orange (**n**) wings. (**i**–**k**, **m**, **n**) *M. costalis*. (**l**) *M. pruinosa* (See also Fig. 17.3)

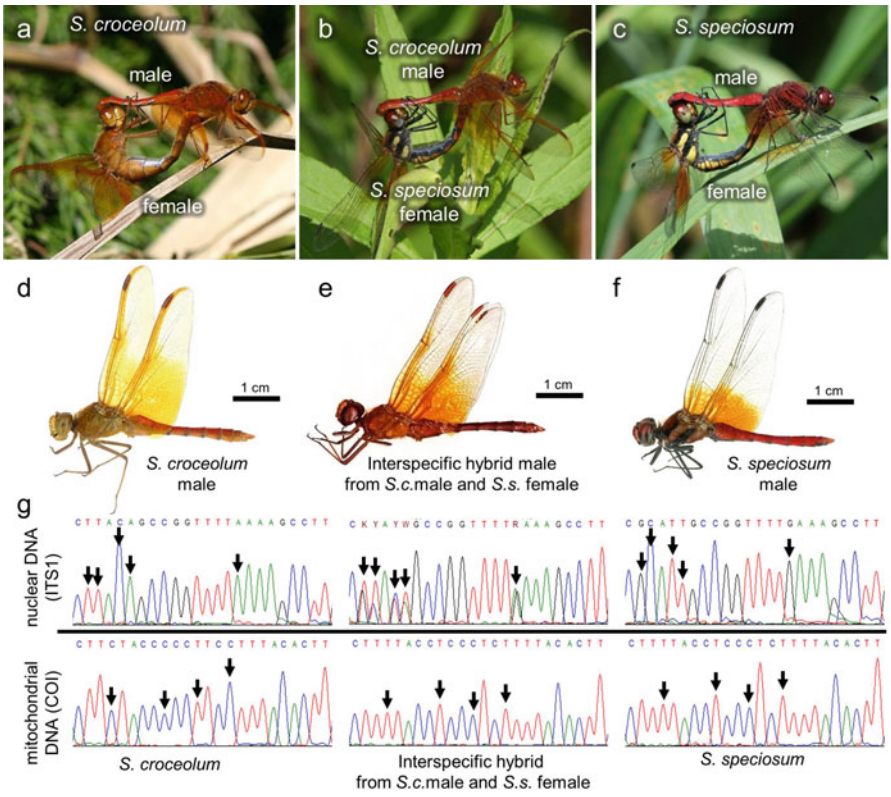


Fig. 17.2 Interspecific copulation and hybrid of dragonflies. (**a**) Normal copulation of *Sympetrum croceolum*. (**b**) Interspecific copulation between *S. croceolum* male and *Sympetrum speciosum* female (Figure modified from Ozono et al. 2012). (**c**) Normal copulation of *S. speciosum*. (**d**) Male of *S. croceolum*. (**e**) Interspecific hybrid male from *S. croceolum* male and *S. speciosum* female. (**f**) Male of *S. speciosum*. (**g**) Nuclear and mitochondrial DNA analyses of *S. croceolum, S. speciosum*, and interspecific hybrid between *S. croceolum* male and *S. speciosum* female. The internal transcribed spacer 1 (ITS1) or cytochrome c oxidase subunit I (COI) region were used for nuclear or mitochondrial DNA marker, respectively. *Arrows* indicate species specific nucleotides

17.3 Wing Color Polymorphism and Presumptive Character Displacement in Japanese *Mnais* Species

In order to avoid interspecific mating or aggression, presumptive character displacement has been reported in some species, wherein interspecific color differences are larger in sympatric populations than in allopatric populations (Waage 1975; Suzuki 1984; Tynkkynen et al. 2004; Hayashi et al. 2004b; Hassall 2014; Drury and Grether 2014; Tsubaki and Okuyama 2016). Here I introduce an interesting example of wing color polymorphism in the two closely related *Mnais* species, *M. costalis* and *M. pruinosa*, in Japan (Hayashi et al. 2004a, b; Ozono

et al. 2012). These two species can be distinguished by nuclear ITS1 sequences, relative length of wing to head in adult males, shape of adult wing pterostigma, and the shape of larval caudal gill (Hayashi et al. 2004a, b). On the other hand, interspecific hybrids have been discovered occasionally and multiregional introgression of mitochondrial DNA is recognized between these two species (Hayashi et al. 2004a, 2005; Futahashi and Hayashi 2004b). Both species exhibit complex wing color polymorphism (Figs. 17.1i–n and 17.3), in which orange-winged males, hyaline-winged males, and hyaline-winged females appear widely in Japan (Fig. 17.1i–k, Asahina 1976; Hayashi et al. 2004b; Ozono et al. 2012). Male orange/hyaline wing polymorphism of *M. costalis* can be explained by a single autosomal locus, and the orange-winged phenotype is dominant (Tsubaki 2003). Previous ecological studies have shown that orange-winged males are territorial, whereas hyaline-winged males are female-mimics and usually non-territorial sneakers (Nomakuchi et al. 1984; Tsubaki et al. 1997; Hayashi et al. 2004b). In addition to these three major phenotypes, the following three phenotypes are recognized in some populations: brown-winged males of *M. pruinosa*, pale orange-winged males and females of *M. costalis* (Fig. 17.1l–n, Asahina 1976; Hayashi et al. 2004b; Ozono et al. 2012). Thus, in *M. costalis*, there are three (orange, pale orange, and hyaline) and two (pale orange and hyaline) wing color forms for males and females, respectively, whereas in *M. pruinosa,* three (brown, orange, and hyaline) and one (hyaline) wing color forms exist in males and females, respectively. Interestingly, wing polymorphism is associated with abdominal body coloration: whitish in mature territorial males (orange or brown wings) (Fig. 17.1i, l) and metallic green in female-mimicking males and females (hyaline or pale orange wings) (Fig. 17.1j, k, m, n, Asahina 1976; Hayashi et al. 2004b; Ozono et al. 2012). Exceptional untransparent white-winged phenotypes have been reported in the Boso Peninsula population of *M. pruinosa* (Asahina 1976), although these white-winged phenotypes are now almost extinct (Futahashi and Hayashi 2004b; Ozono et al. 2012).

Geographic variation of wing color polymorphism is associated with cohabitation (Suzuki 1984; Hayashi et al. 2004b; Tsubaki and Okuyama 2016). In allopatric regions, males of both *M. costalis* and *M. pruinosa* show orange/hyaline wing color polymorphism, whereas females are all hyaline-winged and monomorphic in both species (Fig. 17.3a). In central Japan where both species cohabit, males of *M. costalis* show only orange wings, while males of *M. pruinosa* show only hyaline wings in general (Fig. 17.3a). In *M. costalis*, pale orange-winged females appear prominently in the southern area, where both males and females can be distinguished solely by wing coloration (Fig. 17.3a). In addition to wing color polymorphism, sympatric populations of *M. costalis* show larger body size and prefer sunnier habitats than *M. pruinosa* and allopatric populations of *M. costalis*, suggesting that multiple character displacements emerged in central Japan to avoid interspecific mating (Tsubaki and Okuyama 2016). In western Japan, however, males of both species are polymorphic even in sympatric regions. It should be noted that two forms of female-mimicking males (pale orange and hyaline) appear in accordance with female color polymorphism in *M. costalis* (Fig. 17.3). In the

southwestern area, territorial males of *M. pruinosa* have brown wings instead of orange, where both males and females can be distinguished solely by wing coloration (Fig. 17.3b, rightmost). Although it is not clear why wing color polymorphism is maintained in western Japan even in sympatric region, it has been reported that

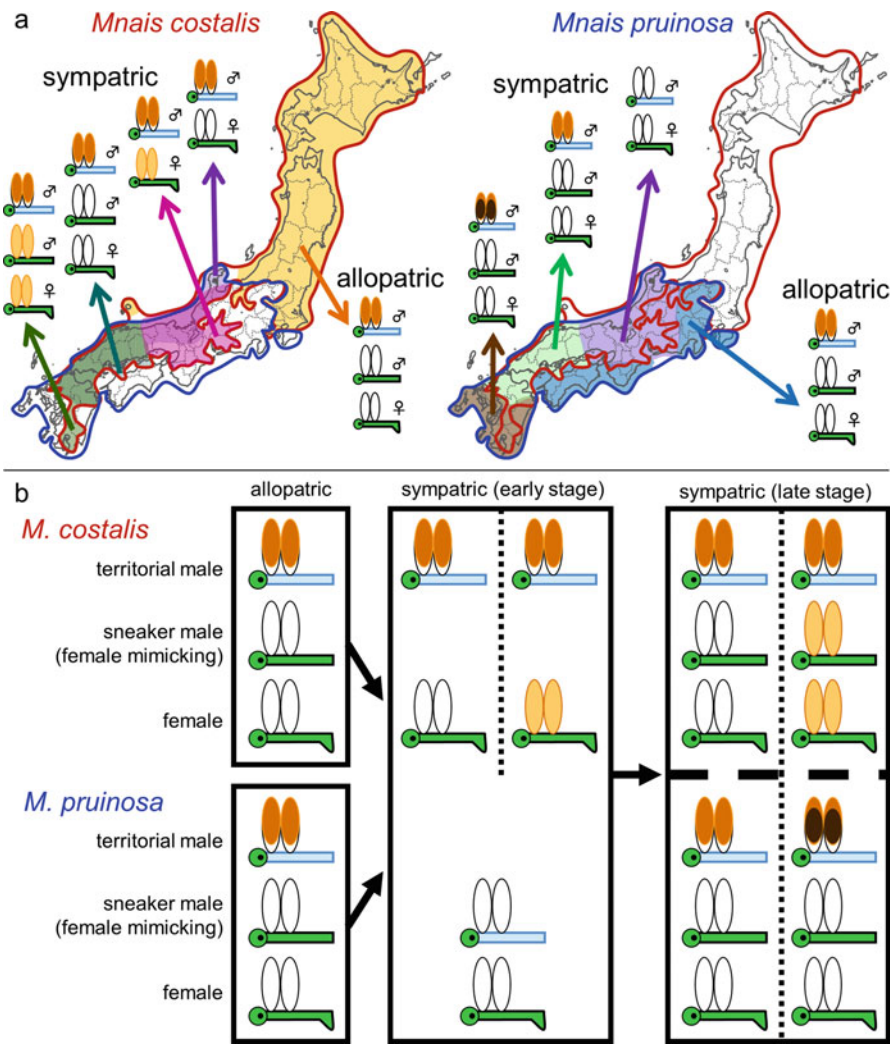


Fig. 17.3 Wing color polymorphism of two Japanese *Mnais* species. (**a**) Geographical variation of *M. costalis* and *M. pruinosa*. (**b**) Hypothetical evolutionary model of wing color polymorphism. The photos of each form are shown in Fig. 17.1i–n. As described Fig. 17.3a, wing color polymorphism varied among populations in sympatric region. Pale orange-winged males and females often appear together with hyaline-winged males and females, respectively. For example, three male forms (orange, pale orange, and hyaline) and two female forms (pale orange and hyaline) emerge simultaneously in some populations of *M. costalis*. Figure modified from Hayashi et al. 2004b; Ozono et al. 2012

degrees of mitochondrial introgression are smaller in western Japan than in central Japan (Hayashi et al. 2005), suggesting that reproductive isolating mechanism between the two *Mnais* species is more robust in western Japan. Hayashi et al. (2004b) proposed the evolutionary model of *Mnais* wing polymorphism, in which the following three stages are hypothesized (Fig. 17.3b):

1. In allopatric populations (eastern or southeastern Japan), males exhibit orange (territorial) and hyaline (female-mimicking) wing color polymorphism, while females are monomorphic (hyaline) in both species.
2. In the early stage of cohabitation (central Japan), males become monomorphic (only orange in *M. costalis* and only hyaline in *M. pruinosa*), and pale orange-winged females of *M. costalis* emerge in some places whereas all females are hyaline-winged in *M. pruinosa*. It should be noted that hyaline-winged males of *M. pruinosa* in central Japan show whitish abdomen like territorial males and have flexible territorial strategy (Fig. 17.3b middle, Siva-Jothy and Tsubaki 1989; Hayashi et al. 2004b).
3. In the late stage of cohabitation (western Japan), both territorial (orange or brown) and female-mimicking males (hyaline or pale orange) appear in both species once again.

According to this scenario, pale orange-winged males and brown males are likely to appear secondarily. The brown/orange color difference may have occurred because the wing color was lost in early sympatry and was reconstructed without models.

Orange wing color of *M. costalis* is derived from tyrosine, suggesting that pigments of orange wing are kinds of melanin (Hooper et al. 1999). Considering that dopamine, a melanin precursor, is also known as a neurotransmitter, genes involved in melanin synthesis pathway are strong candidates for analysis of pleiotropic effects on wing color formation and territoriality. Genetic mechanisms underlying wing color polymorphism deserve future studies.

17.4 Identification of Remarkable Number of Opsin Genes in Dragonflies

Because dragonflies visually recognize environment, foods, enemies, rivals, and mates, their sense of vision has been studied using electrophysiological approach. Critical flicker frequency test has revealed that dragonflies can discriminate beyond 300 Hz, suggesting that they have keen dynamic vision (McFarland and Lowe 1983). Meanwhile, based on anatomical studies, it has been hypothesized that dragonflies have approximately 20/2000 vision (Kirschfeld 1976). In addition to temporal and spatial resolution, wavelength discrimination capability (i.e., color vision) of dragonflies has been investigated, and previous studies have shown that they have three to five classes of photoreceptors (Autrum and Kolb 1968; Eguchi

1971; Meinertzhagen et al. 1983; Yang and Osorio 1991; Bybee et al. 2012; Huang et al. 2014).

Evolution of animal color vision is strongly correlated with the diversity of opsin genes (Briscoe and Chittka 2001; Terakita 2005; Briscoe 2008, Shichida and Matsuyama 2009; Hering et al. 2012; Cronin et al. 2014). Specific types of opsin gene produce light sensors sensitive to specific wavelength light. For example, human beings possess three opsin genes sensitive to blue, green, or red light and can perceive light from purple to red but not ultraviolet (UV). On the other hand, honey bees and fruit flies possess an opsin gene for UV light but not for red light, which allows them to recognize UV light, instead of discriminating red from gray. It has been thought that 2–5 opsin proteins are involved in color vision in most animals (Cronin et al. 2014).

Recently, we discovered that dragonflies possess surprisingly many opsin genes by RNA sequencing (RNA-seq) analyses using adult and larval visual organs (Futahashi et al. 2015). First we surveyed the visual transcriptomics of the red dragonfly *Sympetrum frequens* (Libellulidae). After de novo assembly using Trinity software, we obtained 60 contigs with high similarity to insect opsin proteins. When we aligned these contigs, many of them seemed to be partial or chimeric (gray and blue arrows in Fig. 17.4a). We also obtained 144 opsin gene-like contigs from the white-tailed skimmer dragonfly *Orthetrum albistylum* (Libellulidae) and found that chimeric pattern was different between these two species (Fig. 17.4a). We often encountered similar problem of chimeric contigs in de novo assembly when we focused on paralogous genes. To overcome this problem, we carefully checked and manually corrected each of the contig sequences using Integrative Genomics Viewer (Thorvaldsdóttir et al. 2013) (Fig. 17.4b). Through this manual correction, we also found that partial sequence information is often lost in automatically assembled contigs among highly paralogous genes, due to merging of several similar sequences into one (Futahashi 2016b). We verified the revised sequences by RT-PCR and DNA sequencing. Consequently, we obtained the presumably full length sequences of 20 opsin genes, consisting of 4 nonvisual opsin genes and 16 visual opsin genes of 1 UV, 5 short wavelength (SW), and 10 long wavelength (LW) type from both *S. frequens* and O. *albistylum* (Fig. 17.4c). Next we inspected the draft genome data of the scarce chaser dragonfly *Ladona fulva* (Libellulidae) and identified the same set of 20 opsin genes (Fig. 17.4c). No other opsin genes could be found in the genome. Molecular phylogenetic analysis revealed that the 20 opsin genes of these three species formed distinct 20 monophyletic clusters (Fig. 17.4c), indicating that the common ancestor of the libellulid dragonflies had these 20 genes.

Opsin genes of dragonflies are extraordinarily large in number compared with other insects (Fig. 17.5a). Why do dragonflies have so many opsin genes? In dragonflies, the structure and function of compound eyes are markedly different between not only adult and larva but also dorsal and ventral regions of adult eyes (Fig. 17.5b–c) (Labhart and Nilsson 1995). Electrophysiological analysis of *S. frequens* revealed that the dorsal eye region was sensitive to a short wavelength range from UV (300 nm) to blue-green light (500 nm), whereas the ventral eye

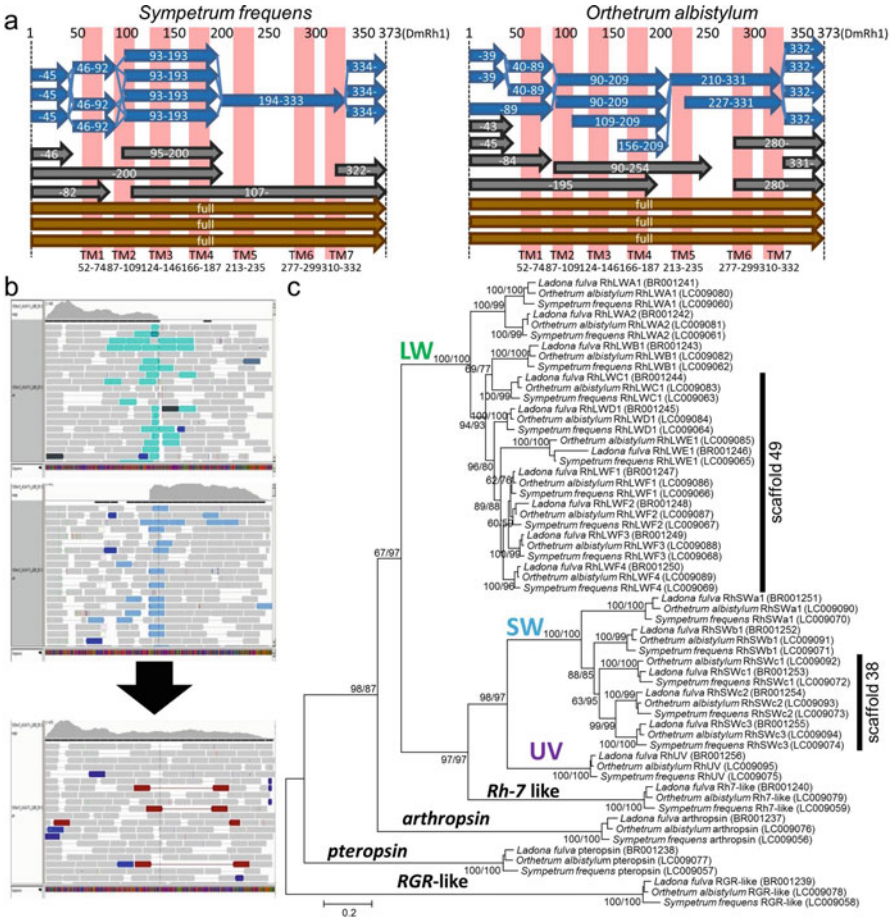


Fig. 17.4 Identification and manual assembly of 20 opsin genes in three libellulid dragonflies. (**a**) Results of de novo assembly by Trinity before manual correction. LW opsin gene-like contigs were aligned based on the similarity of *ninaE*/*Rh1* gene of *Drosophila melanogaster*. The presumptive seven transmembrane regions are shaded by *red*. *Blue* and *gray arrows* indicate chimeric and partial contigs, respectively. (**b**) Manual correction of the contig sequences using Integrative Genomics Viewer. *Cyan* and *sky-blue colors* mean paired end reads mapped on the different contigs. (**c**) Molecular phylogeny of 20 opsin genes of three libellulid dragonflies inferred from 795 aligned amino acid sites. On each node, bootstrap values are indicated in the order of neighbor-joining method/maximum-likelihood method. Accession numbers are shown in *parentheses*. On the genome of *L. fulva*, seven LW opsin genes (*LWC1*, *LWD1*, *LWE1*, and *LWF1–F4*) and three SW opsin genes (*SWc1-c3*) were located in tandem, respectively (Figure modified from Futahashi 2016b)

region was sensitive to a broader wavelength range from UV to red light (620 nm) (Fig. 17.5d). Interestingly, most of the opsin genes were expressed only at a specific life stage and in a specific region (Fig. 17.6). Although many opsin genes were expressed in adults, relatively small number of opsin genes was expressed in larvae, reflecting their lifestyle under water with less visual dependence. In adult

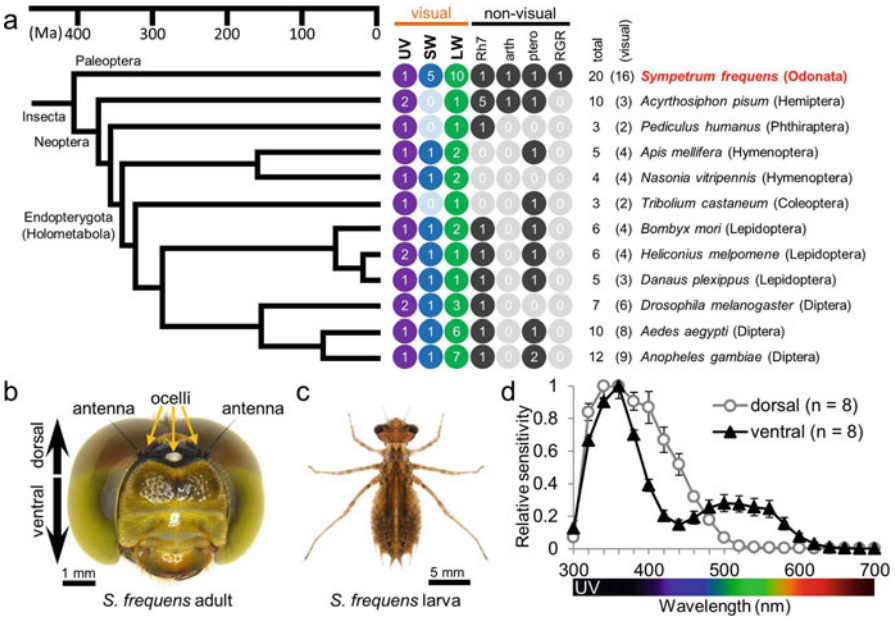
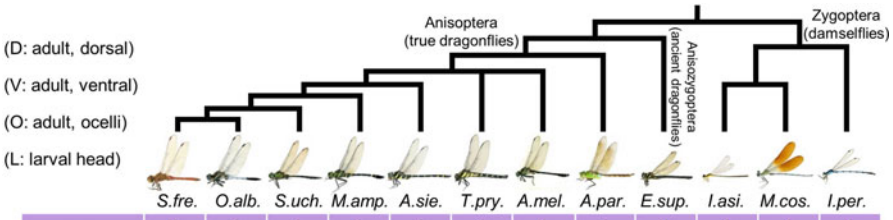


Fig. 17.5 Insect opsin genes and spectral sensitivity of adult compound eyes of *Sympetrum frequens*. (**a**) Numbers of opsin genes of ultraviolet type (UV), short wavelength type (SW), long wavelength type (LW), *rhodopsin7*-like (Rh7), *arthropsin* type (arth), *pteropsin* type (ptero), and *retinal G protein-coupled receptor*-like (RGR) are mapped on the insect phylogeny (Misof et al. 2014). (**b**) Frontal view of adult head of *S. frequens*. (**c**) Larva of *S. frequens* (Photo courtesy of Akira Ozono). (**d**) Spectral sensitivity of the dorsal and ventral regions of adult eyes of *S. frequens* measured by electroretinography (Figure modified from Futahashi et al. 2015)

compound eyes, most SW and LW opsin genes were, respectively, expressed in the dorsal and ventral regions in accordance with their spectral sensitivity, reflecting that dorsal eyes mainly perceive the SW-rich light directly from the sky, whereas the ventral eyes perceive reflected light from objects on the ground.

17.5 Diversity of Opsin Genes among Dragonflies

Body and wing color pattern, behavior, and microhabitats of dragonflies are variable among the families (Corbet 1999; Ozono et al. 2012). To investigate the opsin gene repertoire across dragonflies, comparative RNA-seq analyses were performed in additional 10 species representing 10 different dragonfly families: *Somatochlora uchidai* (Corduliidae), *Macromia amphigena* (Macromiidae), *Anotogaster sieboldii* (Cordulegastridae), *Tanypteryx pryeri* (Petaluridae), *Asiagomphus melaenops* (Gomphidae), *Anax parthenope* (Aeshnidae), *Epiophlebia superstes* (Epiophlebiidae), *Ischnura asiatica* (Coenagrionidae), *Mnais costalis*



		S.fre.	O.alb.	S.uch.	M.amp.	A.sie.	T.pry.	A.mel.	A.par.	E.sup.	I.asi.	M.cos.	I.per.
UV		1 (DV)	1 (DV)	1 (DV)	1 (DV)	1 (DVO)	1 (DV)	1 (DV)	1 (DVO)	1 (DV)	1 (DV)	1 (DV)	1 (DVO)
SW	a	1 (L)	1 (L)	1 (L)	1 (L)	0	0	0	2 (VL)	1 (VL)	0	0	1 (L)
	b	1 (V)	1 (V)	1 (V)	1 (V)	1 (V)	1 (DV)	1 (DV)	1 (V)	0	2 (DVL)	1 (DVL)	1 (DV)
	c	3 (D)	3 (D)	4 (D)	4 (DO)	3 (D)	2 (D)	2 (D)	5 (DO)	6 (DV)	0	0	0
LW	A	2 (VL)	2 (VL)	2 (VL)	2 (VL)	2 (DV)	2 (VL)	2 (DV)	2 (VL)	2 (VL)	2 (DVL)	2 (DVL)	2 (DVL)
	B	1 (L)	1 (L)	1 (L)	1 (L)	1 (L)	0	0	2 (L)	1 (L)	0	0	0
	C	1 (L)	1 (L)	1 (L)	1 (L)	2 (OL)	2 (L)	1 (L)	1 (OL)	1 (OL)	1 (L)	1 (L)	1 (L)
	D	1 (O)	1 (O)	1 (O)	1 (O)	0	1 (O)	0	0	1 (O)	1 (O)	1 (O)	2 (O)
	E	1 (D)	1 (D)	3 (D)	4 (D)	1 (DO)	0	1 (DO)	16 (DVO)	1 (D)	1 (O)	1 (DO)	2 (DV)
	F	4 (V)	4 (V)	5 (V)	5 (V)	5 (DV)	5 (DV)	6 (DV)	0	5 (DV)	4 (DV)	4 (DV)	1 (DV)
Non-visual		4	4	4	4	4	4	4	3	4	4	4	4
Total		20	20	24	25	20	18	18	33	23	16	15	15

Fig. 17.6 Numbers and expression patterns of each type of opsin gene of 12 dragonfly species. Phylogenetic relationship of the dragonflies (Futahashi 2014) is shown on the *top*: *S.fre.*, *Sympetrum frequens; O.alb.*, *Orthetrum albistylum; S.uchi.*, *Somatochlora uchidai; M.amp.*, *Macromia amphigena; A.sie.*, *Anotogaster sieboldii; T.pry.*, *Tanypteryx pryeri; A.mel.*, *Asiagomphus melaenops; A.par.*, *Anax parthenope; E.sup.*, *Epiophlebia superstes; I.asi.*, *Ischnura asiatica; M.cos.*, *Mnais costalis; I.per.*, *Indolestes peregrinus*. SW and LW opsin genes are categorized into three (**a**–**c**) and six (**A**–**F**) groups, respectively. In expression pattern, major tissues and stages expressing each group of opsin genes are shown in parentheses, wherein D, V, O, and L indicate dorsal region of adult eyes, ventral region of adult eyes, adult head region containing ocelli, and larval whole head, respectively (Figure modified from Futahashi et al. 2015)

(Calopterygidae), and *Indolestes peregrinus* (Lestidae) (Fig. 17.6) (Futahashi et al. 2015). The former six species belong to true dragonflies (suborder Anisoptera), while the latter three species belong to damselflies (suborder Zygoptera). *E. superstes* belongs to ancient dragonflies (suborder Anisozygoptera, sometimes including into Anisoptera) (Ozono et al. 2012; Futahashi 2014). Among dragonfly families, the total number of opsin genes varied widely from 15 to 33 (Fig. 17.6).

One of the significant advantages of RNA-seq analyses is that the gene expression information of different developmental stages in multiple species could be efficiently obtained. Based on molecular phylogeny and expression pattern, SW and LW opsin genes were categorized into three (a, b, and c) and six (A, B, C, D, E, and

F) groups, respectively (Fig. 17.6). Nonvisual opsin genes were scarcely expressed in the larval and adult visual organs of all examined species (Futahashi et al. 2015). Stage- and region-specific expressions of opsin genes were widely conserved across dragonfly species as follows:

1. The group-a SW, group-B LW, and group-C LW opsin genes were predominantly expressed in larvae.
2. The group-b SW and group-F LW opsin genes were mainly expressed in the ventral region of adult compound eyes.
3. The group-c SW and group-E LW opsin genes were primarily expressed in the dorsal region of adult compound eyes.
4. The group-D LW opsin genes were specifically expressed in the adult ocelli (Fig. 17.6).

The dorsoventrally differentiated expression patterns were obscure in the three damselfly species (Fig. 17.6). It should be noted that compensational expression patterns associated with losses of some visual opsin genes were observed (e.g., loss of the ocellus-specific group-D LW opsin gene entailed ocellus-associated expression of the group-C or group-E genes (Fig. 17.6)). Given that the group -C, -D, -E, and -F genes were located in tandem on the genome of *L. fulva*, the rearrangement among these genes may have occurred in the course of evolution, resulting in lineage-specific expression pattern changes of these genes. Thus, dragonflies may utilize different sets of opsin genes depending on types of light environment, which can be achieved by an extraordinary increase in the number of opsin genes.

The repertoire of opsin genes differed among dragonfly species, suggesting that the opsin genes may have evolved according to the habitat or behavior of each species. For example, the absence of the SW opsin genes at larval stage coincided with their sand- or pit-dwelling behaviors in *A. sieboldii*, *T. pryeri*, and *A. melaenops*, whereas the multitude of SW and/or LW opsin gene numbers in the dorsal region of adult compound eyes are correlated with twilight flying activity for predation in *S. uchidai*, *M. amphigena*, *A. parthenope*, and *E. superstes* (Fig. 17.6; Futahashi et al. 2015). Plausibly, although speculative, the large variation of opsin genes is associated with the evolution of diverse color pattern in dragonflies.

The variety and beauty of color pattern are also prominent in Lepidopteran and Coleopteran insects, although they have only a few opsin genes (Fig. 17.5a). The small numbers of opsin genes in these insects may be attributed to nocturnal lifestyle of their ancestors like mammals (Briscoe and Chittka 2001; Feuda et al. 2016). By contrast, almost all dragonfly species are diurnal, and they diverged from other insects over 350 million years ago (Fig. 17.5a, Misof et al. 2014).

17.6 Molecular Mechanisms Underlying Color Changes in Red Dragonflies

Dragonflies display a wide variety of coloration such as red, yellow, blue, and green. Most of animal colors are derived from structural colors and/or pigment colors. The mechanisms of structural coloration of several dragonfly species have been recently investigated, wherein multilayer structures are generally involved in iridescent coloration (Vukusic et al. 2004; Hariyama et al. 2005; Schultz and Fincke 2009; Stavenga et al. 2012; Nixon et al. 2013, 2015; Guillermo-Ferreira et al. 2015). Non-iridescent blue color is also structural, attributed to coherent light scattering from the quasi-ordered nanostructures within pigment cells (Prum et al. 2004). By contrast, information on pigments in dragonflies is still limited.

We analyzed the red epidermal pigments from three species of red dragonfly, namely, the autumn darter *Sympetrum frequens*, the summer darter *Sympetrum darwinianum*, and the scarlet skimmer *Crocothemis servilia* (Futahashi et al. 2012). Two ommochrome pigments, xanthommatin (vivid red color in reduced form) and decarboxylated xanthommatin (dull red color in reduced form) were consistently identified in all these species (Fig. 17.7a), in which the ratio of xanthommatin is higher in vivid red color species. Previous studies have shown that the color of ommochrome pigments changes reversibly by redox reactions in vitro (Linzen 1974). By injecting a reductant (vitamin C) solution, we confirmed that the yellowish body color of both immature males and mature females changed into red as observed in mature males (Fig. 17.7b). Redox conditions of the extracted ommochrome pigments were measured electrochemically, and the relative abundance of the oxidized and reduced forms of pigments were evaluated. In all three species, only the mature males exhibited very high proportions of the reduced ommochrome pigments (Fig. 17.7c), indicating that sex-specific color change in mature red dragonflies is primarily attributed to redox states of the ommochrome pigments (Futahashi et al. 2012).

Pigment-based color changes in animals are mainly attributed to the following three mechanisms: synthesis and degradation of pigments, changes in localization of pigments, and accumulation of pigments from food (Stevens and Merilaita 2011). Red dragonflies adopt a previously unknown mechanism, namely, a body color change by redox reaction of the pigments. Male-specific color change of dragonflies has been considered as an ecologically important trait for reproductive success. Considering that mature males exhibit territorial behavior under the scorching sun and the reduced pigments show antioxidant abilities (Futahashi et al. 2012), male-specific red pigments may have additional role in preventing oxidative stress from UV radiation.

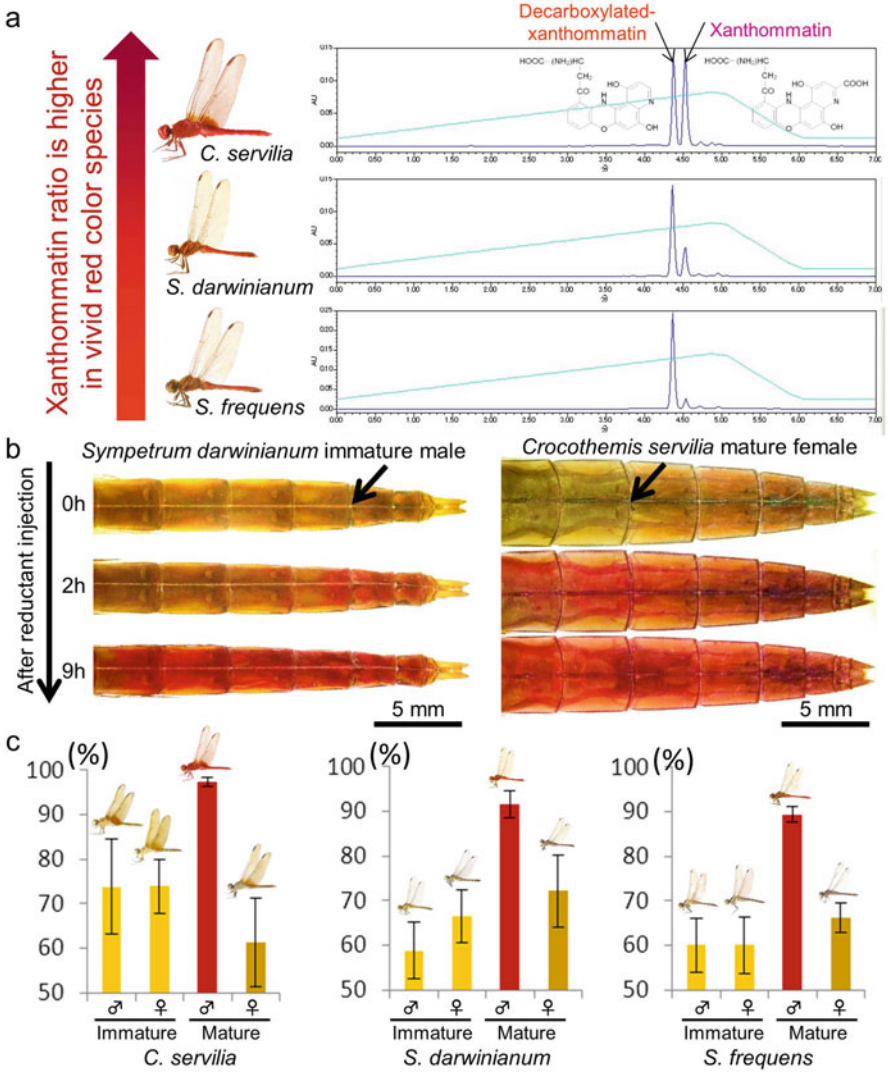


Fig. 17.7 Redox-dependent color change of the ommochrome pigments in red dragonflies. (a) Chromatograms of ommochrome pigments from males of three red dragonflies. Blue lines denote the acetonitrile gradient. (b) Reductant-induced yellow/red color change. Arrows indicate the injection sites. (c) Reduced form ratios of the extracted ommochrome pigments. Means and standard deviation are shown (n = 10 ~ 12) (Figure modified from Futahashi et al. 2012)

17.7 Conclusion and Perspective

The well-developed sense of sight and the great variety of color pattern in dragonflies have been already pointed out a century ago (Tillyard 1917). Recent progress on molecular mechanisms of color vision and color formation unveiled the outstanding diversity of visual opsin genes and the unique mechanisms of body color changes in dragonflies. Meanwhile, only limited information is available on genes potentially involved in color formation (Chauhan et al. 2014, 2016). Rapid spread of next-generation sequencing technology makes it easier than ever to analyze non-model organisms, although careful evaluation for de novo assembly is still important as described above, especially without genomic information. Molecular bases underlying color pattern formation and its evolution in dragonflies are just as fascinating and challenging as in butterflies. Recently, effective RNAi- and genome-editing methods have been developed for gene functional analyses in butterflies (Ando and Fujiwara 2013; Nishikawa et al. 2015; Li et al. 2015; Perry et al. 2016; Zhang and Reed 2016; Beldade and Peralta 2017). Applying these methods to dragonflies will be an important step toward future studies in this field (Okude et al. 2017).

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Errata to: Diversity and Evolution of Butterfly Wing Patterns

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In Chapter 3: Camouflage Variations on a Theme of the Nymphalid Ground Plan

Takao K. Suzuki

The original version of this chapter was inadvertently published without figure 3.4. The figure is inserted in the current version.

In Chapter 11: Chemical Ecology of Poisonous Butterflies: Model or Mimic? A Paradox of Sexual Dimorphisms in Müllerian Mimicry

Ritsuo Nishida

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In Chapter 17: Molecular Mechanisms Underlying Color Vision and Color Formation in Dragonflies

Ryo Futahashi

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