

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.

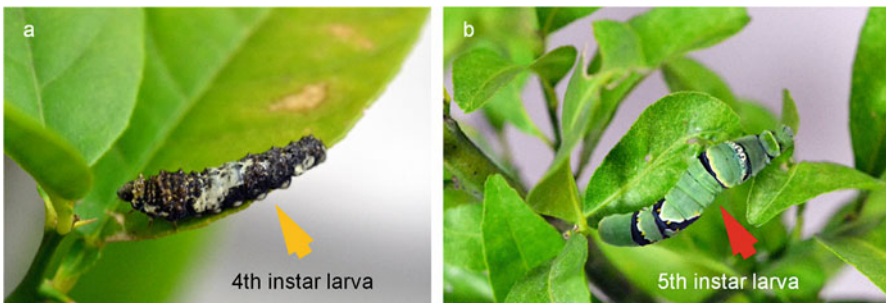


Fig. 15.1 (a) Fourth instar larva with bird-dropping body pattern; (b) Fifth instar larva with green body pattern of *P. xuthus*

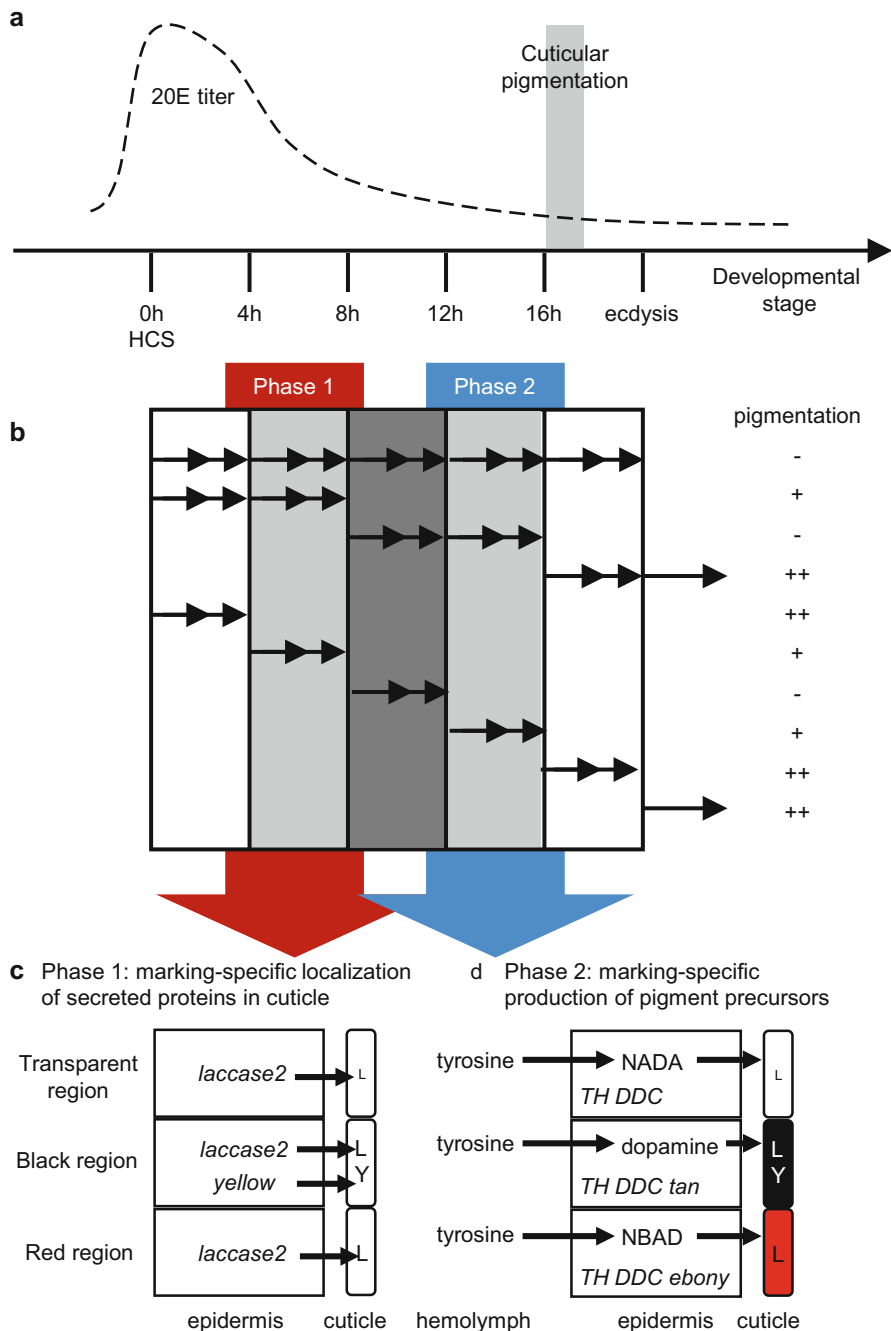


Fig. 15.2 A working model for the two-phase cuticular pigmentation in larvae of *P. xuthus*. (a) 20E titer in hemolymph during the fourth molt; (b) The timing effect of 20E on *black* pigment synthesis. The intervals of 20E applications (*arrows*, every 2 h); (c) phase 1; (d) phase 2. N-b-alanyldopamine (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 3 β -reductase (coded by the *3DE 3 β -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; Ogiwara 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).

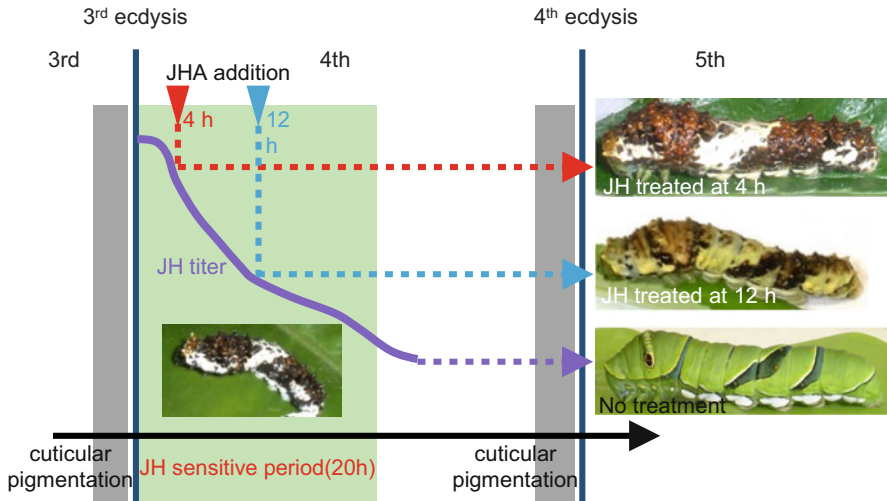


Fig. 15.3 Treatment of juvenile hormone analogs during the JH-sensitive period (Modified from Futahashi and Fujiwara 2008a)

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.

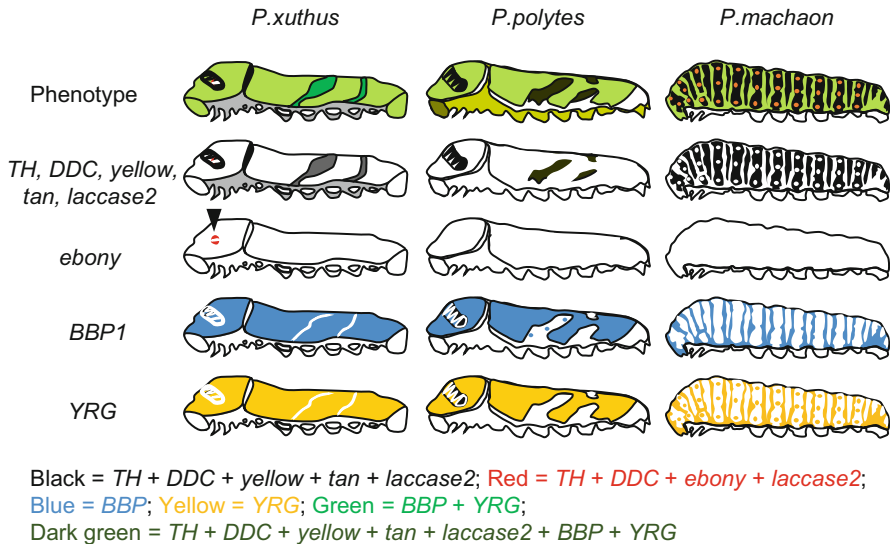


Fig. 15.4 Schema of species-specific body color pattern among three *Papilio* species (Modified from Shirataki et al. 2010)

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 (Px*YRG* and Pp*YRG*) were designed, and the spatial expression pattern was detected in the last instar larvae of the F1 hybrid. Both the Px*YRG* and Pp*YRG* 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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