A Pathway Analysis of Melanin Patterning in a Hemimetabolous Insect

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ABSTRACT Diversity in insect pigmentation, encompassing a wide range of colors and spatial patterns, is among the most noticeable features distinguishing species, individuals, and body regions within individuals. In holometabolous species, a significant portion of such diversity can be attributed to the melanin synthesis genes, but this has not been formally assessed in more basal insect lineages. Here we provide a comprehensive analysis of how a set of melanin genes (*ebony*, *black*, *aaNAT*, *yellow*, and *tan*) contributes to the pigmentation pattern in a hemipteran, *Oncopeltus fasciatus*. For all five genes, RNA interference depletion caused alteration of black patterning in a region-specific fashion. Furthermore, the presence of distinct nonblack regions in forewings and hindwings coincides with the expression of *ebony* and *aaNAT* in these appendages. These findings suggest that the region-specific phenotypes arise from regional employment of various combinations of the melanin genes. Based on this insight, we suggest that melanin genes are used in two distinct ways: a "painting" mode, using predominantly melanin-promoting factors in areas that generally lack black coloration, and, alternatively, an "erasing" mode, using mainly melanin-suppressing factors in regions where black is the dominant pigment. Different combinations of these strategies may account for the vast diversity of melanin patterns observed in insects.

KEYWORDS *Oncopeltus fasciatus*; insect pigmentation; melanin patterning; melanin-promoting factors; melanin suppressors

PIGMENT patterns are among the most striking and variable features of insect morphology. An extraordinary diversity in coloration distinguishes species, populations within species, individuals within populations, and different body regions (Wittkopp and Beldade 2009). Most insights into the mechanisms underlying such diversity have come from studies on melanization in *Drosophila* (Wittkopp *et al.* 2003; Wittkopp and Beldade 2009). Melanization is the pigmentation process wherein precursors (catecholamines) are converted into pigment molecules that are incorporated into the cuticle (Wittkopp and Beldade 2009). These studies have helped to identify a network of melanin genes and their roles in body color patterning (Wright 1987; Wittkopp *et al.* 2003; Wittkopp and Beldade 2009). The core part of this proposed pathway is shown in Figur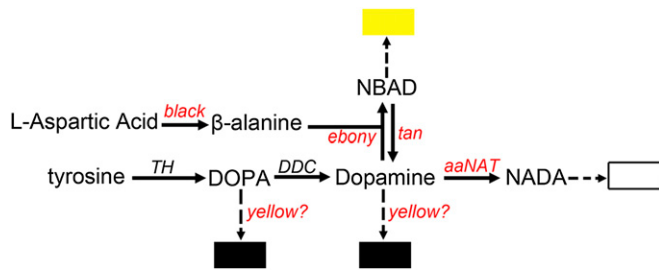e 1. The pathway begins with the conversion of tyrosine to dihydroxyphenylalanine (DOPA). DOPA can then be used in two different manners: to produce DOPA melanin (black) or to be converted to dopamine, another precursor of black melanin. In the conversions from DOPA/dopamine to black melanin, the *yellow* gene is thought to play an essential role in promoting these processes. However, it is still unclear whether *yellow* plays a role in producing DOPA melanin, dopamine melanin, or both (question marks in Figure 1). Alternatively, production of dopamine melanin can be suppressed by converting dopamine to N-β-alanyldopamine (NBAD) or N-acetyldopamine (NADA). The NBAD branch contains three main genes: *ebony*, *black*, and *tan*. Black catalyzes the production of β-alanine, which binds to dopamine by the activity of Ebony, thus forming NBAD, the precursor of yellow sclerotin. It is also possible to convert NBAD back to dopamine by the activity of Tan. The dopamine produced under such circumstance is then converted to dopamine melanin, thus promoting dark coloration (Wittkopp *et al.* 2002b; True *et al.* 2005). In *Heliconius*, Tan also has been recognized as an additional factor that promotes dark melanization (Ferguson *et al.* 2011). The NADA branch relies on the function of arylalkylamine-N-acetyltransferase

Figure 1 A summary of the melanin pathway in insects. [Redrawn from Wright (1987).] Black melanin originates from either DOPA or dopamine, which are converted from tyrosine by TH and DDC. While yellow is recognized as a melanin-promoting factor, it is still unclear whether it converts DOPA melanin, dopamine melanin, or both (hence the question marks next to it). Dopamine can be converted to NBAD sclerotin (yellow pigment), which requires the functions of both *ebony* and *black*. This conversion can be reversed by *tan*, another melanin-promoting factor. Dopamine also can be converted to NADA sclerotin, which is colorless and catalyzed by *aaNAT*. All conversions from precursors (DOPA, dopamine, NBAD, and NADA) to the final pigments require multistep biochemical reactions, as illustrated by dashed lines.

(AANAT), which converts dopamine to NADA, the precursor of colorless sclerotin. These five genes, including three melanin-suppressing factors (*ebony*, *black*, and *aaNAT*) and two melanin-promoting factors (*tan* and *yellow*), are thought to be the main genes in the mechanism responsible for producing different melanin patterns (Wright 1987; Wittkopp *et al.* 2003; Wittkopp and Beldade 2009).

Assays in *Drosophila* have provided functional evidence associating *ebony*, *yellow*, and *tan* with body and wing pigmentation (Walter *et al.* 1996; Wittkopp *et al.* 2002b; Gompel *et al.* 2005; True *et al.* 2005; Jeong *et al.* 2008). In addition, functional studies in *Tribolium* have also reported the essential involvement of *ebony*, *black*, and *yellow* (Arakane *et al.* 2009; Tomoyasu *et al.* 2009; Arakane *et al.* 2010), whereas the pigmented phenotype of *aaNAT* was reported only in *Bombyx* (Zhan *et al.* 2010; Osanai-Futahashi *et al.* 2012). A systematic functional profile of these melanin genes is lacking in any of the more basal hemimetabolous insect lineages. In this mode of development, the embryo hatches as a miniature adult that undergoes a succession of molts, with melanization occurring at each stage. Thus, as a complement to the studies already performed in holometabolous insects, it is important to begin evaluating the roles of melanization genes in the pigmentation of hemimetabolous species. This also establishes a foundation for understanding the evolution of the melanin pathway in insects.

Accordingly, we performed the first systematic functional analysis of *ebony*, *black*, *tan*, *yellow*, and *aaNAT* in a hemimetabolous species, the milkweed bug *O. fasciatus*. This insect is well suited for the study of pigmentation because it features striking aposematic black/orange warning colorations and color patterns that are distinct in different body regions. The black coloration has been confirmed recently to be produced via the melanin pathway, whereas most of the orange coloration comes from another source (Liu *et al.* 2014). The RNA interference (RNAi) approach in *Oncopeltus* is also highly efficient and generates phenotypes displaying

systemic responses (Liu and Kaufman 2004a, b, 2005; Angelini and Kaufman 2005). This allows for the functional analysis of pigmentation genes at a whole-body scale, as was highlighted by a previous study (Liu *et al.* 2014). The depletion of master synthesis genes (*tyrosine hydroxylase* and *dopa decarboxylase*, which initialize the melanin pathway) (Figure 1) caused a great reduction in black coloration throughout the whole body (Liu *et al.* 2014). In the present study, RNAi knockdown of the five melanin genes caused different regional pigment phenotypes. More specifically, the phenotypes observed in the abdomen and hindwings of RNAi adults were different from those in the head, thorax, and forewings. These observations are unexpected because RNAi depletion of *ebony*, *black*, or *aaNAT* in holometabolous species (*Tribolium* and *Bombyx*) caused alterations in color patterns over the entire body (Tomoyasu *et al.* 2009; Arakane *et al.* 2010; Zhan *et al.* 2010; Osanai-Futahashi *et al.* 2012). Results presented here from *Oncopeltus* suggest that different melanin genes are used to form black patterns in distinct body regions. This finding provides novel insights regarding how pigment diversity is created in different body regions by mechanisms that could be general to other insects with complex color patterns, including other aposematic species whose color patterns play critical roles in interspecies communication.

Materials and Methods

Cloning and sequence analysis of complementary DNA (cDNA) fragments

Extraction of total RNA from embryos of *O. fasciatus*, as well as the follow-up cDNA generation, nested RT-PCR, and cloning were carried out according to Liu *et al.* (2014) and Li and Popadić (2004). cDNA fragments of *ebony* and *aaNAT* were obtained from *Periplaneta americana* embryos (another hemimetabolous species used here for comparison and confirmation) following an established protocol from Chesebro *et al.* (2009). Multiple reactions of nested PCR were performed to amplify the gene fragments. The primers used for PCR cloning, as well as the lengths of the cDNA fragments obtained, are listed in Supplemental Material, Table S1. All these cDNA fragments cover most of the coding region of each gene. The orthologies of these *Oncopeltus* genes were confirmed by phylogenetic analysis (Figure S1 and Figure S2). In the case of *Oncopeltus Ubx*, a previously described clone was used (Medved *et al.* 2015).

RNA interference (RNAi)

For all genes in this assay, double-stranded RNA (dsRNA) was generated using the entire cDNA fragment to ensure the specificity of RNAi depletion. The prepared dsRNA was then injected into the abdomens of *Oncopeltus* nymphs following established protocols (Chesebro *et al.* 2009; Liu *et al.* 2014). Approximately 2 µl of dsRNA was injected at a concentration of 2–3 µg/µl into each individual. Injections were performed around the beginning of the last nymphal stage (fifth nymphs). *Ubx* RNAi was performed following an established protocol (Medved *et al.*, 2015). For *ebony* RNAi, 20 nymphs

were injected, and 11 successfully molted into adults. For *tan* RNAi, 45 nymphs were injected, and 37 survived to adult stage. For *black* RNAi, 32 nymphs were injected, and 20 molted to adults. For *yellow* RNAi, 45 nymphs were injected, and 38 molted to adults. For *aaNAT* RNAi, 25 nymphs were injected, and 17 molted to adults. All images were chosen to show the representative phenotypes. For *Ubx* RNAi, 17 nymphs were injected, all of which were dissected at the fifth nymphal stage for *in situ* hybridization.

The RNAi analysis in *Periplaneta* was carried out following Chesebro *et al.* (2009). dsRNA was prepared and injected into the ventral abdomens of late nymphs of *Periplaneta*. Approximately 3 μl of dsRNA was injected at a concentration of 3–4 μg/μl into each nymph. Of the 10 nymphs injected with *ebony*, six successfully molted into adults. For *aaNAT* RNAi, of the 10 nymphs injected, seven successfully molted into adults. All images were chosen to show the representative phenotypes.

RT-PCR analysis

To determine the efficiency of our RNAi approach, we performed independent RT-PCR analyses on *ebony*, *black*, *tan*, *yellow*, *aaNAT*, and *Ubx* as controls (shown in Figure S3). Total RNA was extracted from the whole bodies (with internal organs removed). Two individuals were used for RNA extraction in each group. The procedures of total RNA extraction and cDNA synthesis were carried out following Liu *et al.* (2014). Primers used for RT-PCR are listed in Table S1. Complementary RT-PCR analyses on *ebony* and *aaNAT* were performed in *P. americana* (Figure S4).

In addition, to determine whether the RNAi depletion was consistent throughout the body, we performed RT-PCR analyses on isolated body regions of *black* RNAi individuals that showed the greatest contrast in pigmented phenotypes between the abdomen and anterior body regions (Figure S5). Two wild-type or *black* RNAi fifth nymphs (day 8 of development) were used. The epidermis of head, thorax, forewing, hindwing, and abdomen were dissected and separated. Each regional sample was used for total RNA extraction and cDNA synthesis following Liu *et al.* (2014).

Image processing

Microscopic images were obtained using a SZX16 Microscope (Olympus) and DP72 Camera (Olympus). To minimize the possible variation in captured images, the background and all the microscope and camera settings were standardized: the images under a particular magnification were taken under the same light conditions, aperture, exposure time, and white balance.

In addition, to quantify the changes in black intensity observed in *yellow* and *tan* RNAi adults, we measured the mean grayscales (at a scale of 0–255) of the black subregions using ImageJ in separate microscopic images of the head, thorax, forewing, hindwing, and ventral abdomen. The measurements were carried from 10 individuals of wild-type, *yellow* RNAi, or *tan* RNAi *Oncopeltus* adults. The grayscale

values were converted to brightness level as grayscale value/255 × 100. The converted brightness levels from these three groups were then compared to each other using group *t*-tests (the level of significance was 0.05). As a control, the same measurements and analyses were also applied to the orange subregions in the dorsal abdomens.

In situ hybridization

The digoxigenin-labeled antisense RNA probes of *ebony* and *aaNAT* were synthesized as described in Li and Popadić (2004). For each gene, both sense and antisense probes were used. The formation of wing tissue is completed at day 5 of the fifth nymphal stage, after which the cuticle starts forming, which, in turn, restricts the use of postembryonic *in situ* hybridization analysis. Before day 5, the wing tissue is not completely formed and cannot be fixed intact. After day 6, the cuticle prevents the riboprobes from penetrating into the wing tissue. Therefore, in this assay, the wings were dissected from wild-type and *Ubx* RNAi fifth nymphs that were exactly 5–6 days old. In each group, 16–22 wings were analyzed, and the images shown represent the consensus expression patterns. The *in situ* hybridization was modified from Tomoyasu *et al.* (2009). Details will be provided on request.
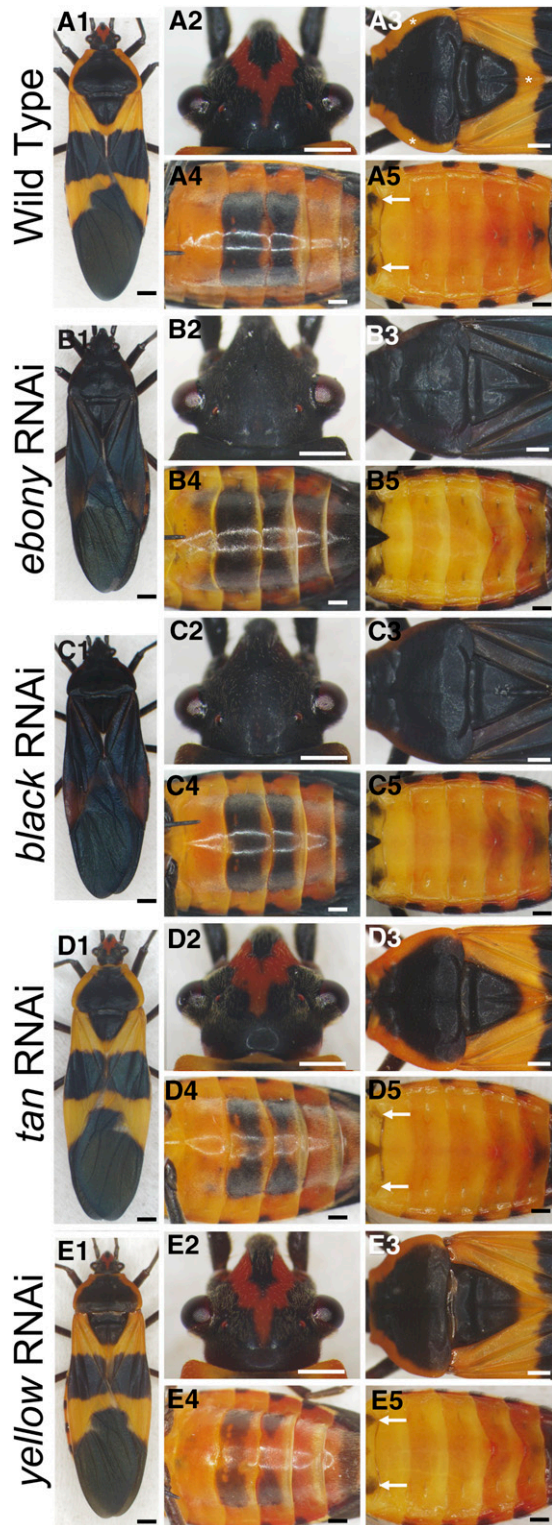
Data availability

The authors state that all data necessary for confirming the conclusions presented in this article are represented fully within the article. The accession numbers of the *O. fasciatus* sequences from GenBank are as follows: KX023894 (*ebony*), KX023895 (*black*), KX023896 (*yellow*), KX023897 (*tan*), and KX023898 (*aaNAT*).

Results

Functions of melanin genes on black patterns of body pigmentation in Oncopeltus

The *Oncopeltus* body has warning coloration consisting of alternating black and orange subregions and patches (Figure 2A1). The dorsal head features a V-shaped orange stripe flanked by black pigmentation covering the rest of the head (Figure 2A2). Dorsal plates of the thoracic segments are primarily black, with the exception of orange at the lateral edges on the prothorax (T1) and posterior tip of the mesothorax (T2; asterisks, Figure 2A3). In contrast, most of the abdomen is orange, with the exception of two rectangular black patches on the ventral side of A3–A4 segments (Figure 2A4) and two small spots on the dorsal A1 segment (white arrows, Figure 2A5).

To examine the role of the melanin pathway in *Oncopeltus* color patterning, we cloned five putative core melanin genes: *ebony*, *black*, *tan*, *yellow*, and *aaNAT*. Phylogenetic analyses confirmed that the clones obtained are indeed *Drosophila* orthologs (Figure S1 and Figure S2). To test the efficiency of RNAi, we performed RT-PCR analyses for each gene, which showed that in all instances the original transcription level was significantly reduced throughout the body, including the epidermis (Figure S3). These levels of depletion are

consistent with previous RNAi analyses in *Oncopeltus* showing changes in coloration encompassing the entire body (Liu *et al.* 2014). These findings, combined with an additional RT-PCR result showing a significant reduction in the level of *black* transcripts in all body regions (Figure S5), support the conclusion that the observed phenotypes resulted from a systemic response.

Studies in *Drosophila* and *Tribolium* have shown that *ebony* plays the central role in suppressing black pigmentation (Figure 1). On loss of function of *ebony*, a global darkening of body pigmentation was observed in both species (Wittkopp *et al.* 2002b; Takahashi *et al.* 2007; Tomoyasu *et al.* 2009). Based on these observations, we expected *ebony* RNAi *Oncopeltus* adults to exhibit black coloration throughout the body. Among 11 *ebony* RNAi adults, all but one exhibited a significant expansion of black melanization in the anterior body regions. As illustrated in Figure 2, B1–B3, all nonblack anterior subregions, including the orange V-shaped stripe on the head, the lateral edges on T1, and the posterior tip on T2, became black. However, in these 10 individuals, the expansion of black pigmentation was only moderate in posterior body regions. The black rectangle on the third abdominal segment (A3) expanded anteriorly into the A2 segment, whereas the A4 rectangle expanded posteriorly into A5 and A6 segments (Figure 2B4). The remainder of the ventral abdomen and the non-black-pigmented dorsal abdomen were generally unaltered (Figure 2B5). Therefore, the different outcomes observed between anterior and posterior body regions suggested that while *ebony* was critical for nonblack patches in the head and thorax, it played only a minor role in the majority of nonblack subregions in the abdomen.

Ebony is the core enzyme in the conversion of dopamine to NBAD, catalyzing the binding of β-alanine to dopamine (Figure 1). Therefore, the synthesis of β-alanine, which is catalyzed by the product of the *black* gene, is also critical for the NBAD branch (Wright 1987; Arakane *et al.* 2009). Among 20 *black* RNAi adults, 18 showed an expansion of black coloration in the head and thorax that covered the original orange pigmentation (Figure 2, C1–C3). In the ventral abdomen, the effects were highly consistent among these 18 individuals, showing little of the melanin expansion that was observed in *ebony* knockdowns (compare Figure 2C4 with Figure 2B4). The dorsal abdomen and most of the nonblack

Figure 2 Functions of *ebony*, *black*, *tan*, and *yellow* in different body regions of *Oncopeltus* adults. (A1–A5) Wild-type *Oncopeltus* shows alternating black-orange patterning. Black pigmentation is present in the head (A2), thorax (A3), ventral abdomen (A4), and dorsal abdomen (A5). (B1–B5) The *ebony* RNAi adult phenotypes showed the expansion of black pigment in the head (B2) and thorax (B3), whereas such an expansion was only moderate in the ventral abdomen (B4) and barely noticeable in dorsal abdomen (B5). (C1–C5) The *black* RNAi adults showed a pheno-

type similar to the *ebony* RNAi individuals described earlier. (D1–D5) The *tan* RNAi adult phenotypes showed a differential defect of black pigment across the body. The defect of black was subtle in the head (D2) and thorax (D3), while it was quite significant in ventral abdomen (D4). Also, on the dorsal A1 segment, the two black patches were reduced (arrows, D5). (E1–E5) The *yellow* RNAi adults showed varying levels of reduction in black pigment between body regions. Reduction was moderate in the head (E2) and thorax (E3) whereas quite significant in ventral abdomen (E4). The two black patches on the dorsal A1 segment were also significantly reduced (arrows, E5). Scale bars, 1 mm (A1, B1, C1, D1, and E1) and 500 μm (A2–A5, B2–B5, C2–C5, D2–D5, and E2–E5).

portions of the ventral abdomen also remained unchanged (Figure 2, C4 and C5). These observations show that *black* was required for the nonblack patches in the anterior body regions but not for the abdomen. Combined with *ebony* RNAi insights, these results establish that suppression of melanin by the NBAD branch is used differently between the anterior and posterior body regions of *Oncopeltus*.

While these results focus on the mechanism that creates nonblack subregions, it is equally important to understand the complementary process—the generation of black areas across the insect body. Studies in *Drosophila* have shown that other than tyrosine hydroxylase (TH) and dopa decarboxylase (DDC), the enzymes required for the production of DOPA and dopamine, additional promoting enzymes are required for melanization (Wright 1987; Wittkopp *et al.* 2002b; True *et al.* 2005; Jeong *et al.* 2008). One of the essential enzymes is Tan, which counteracts Ebony in the NBAD branch (Figure 1), thus promoting melanin production (True *et al.* 2005; Jeong *et al.* 2008). Functional analyses on *tan* have been reported only in *Drosophila*, which showed that its loss causes a global reduction of melanin patterns (True *et al.* 2005; Jeong *et al.* 2008). Of 37 *tan* RNAi *Oncopeltus* adults, 34 exhibited a consistent phenotype (Figure 2D1). Black patterns in the head and thorax were generally unaltered (Figure 2, D2 and D3). In contrast, the melanin patterns showed noticeable changes in the abdomen (Figure 2, D4 and D5). In particular, the two black spots on the dorsal A1 segment were significantly reduced (arrows in Figure 2D5). The same trend also was observed in the ventral A3 and A4 segments, exhibiting a reduction in the middle portion of each black rectangle (Figure 2D4). In three individuals, the degree of reduction was more pronounced, with either the left or right half of the A4 rectangle disappearing completely. It is worth noting that the intensity of black coloration within the remaining A3 and A4 rectangles was not significantly different from wild type (Figure S6, $P > 0.05$). These observations suggest that *tan* is required for proper patterning of black pigmentation in the *Oncopeltus* abdomen but not for its intensity. Such a role for *tan* in abdominal melanin patterning is consistent with previous studies of *Drosophila* species (True *et al.* 2005; Jeong *et al.* 2008).

As shown earlier, the depletions of *ebony*, *black*, and *tan* did not affect the majority of the nonblack subregions in the abdomen. These observations suggest that the NBAD branch of the melanin pathway is not involved in the generation of these subregions. Another possible candidate is *arylalkyl-amine-N-acetyltransferase* (*aaNAT*), which is the core gene in the NADA branch of the melanin pathway (Figure 1). Studies in *Drosophila* have shown that *aaNAT* is responsible for converting dopamine to NADA as a way of depleting melanin and creating colorless sclerotin (Wright 1987; Hintermann *et al.* 1995; Brodbeck *et al.* 1998). Consistent with this, the depletion of *aaNAT* causes an increase in melanization across the body in *Bombyx* (Zhan *et al.*, 2010). In order to determine whether this mechanism also can explain the nonblack subregions in the abdomen of *Oncopeltus*, we depleted *aaNAT* in fifth instar nymphs. The consequent adults, however, showed no effect in color patterns in the head, thorax, or abdomen (Figure S7). This observation indicates that the NADA branch of the melanin pathway is not required for black patterns in these body regions.

In addition to the preceding four genes within the NBAD and NADA branches, we also tested the function of *yellow*, another important gene that promotes melanin production (Wright 1987; Wittkopp *et al.* 2002a, b; Jeong *et al.* 2008; Tomoyasu *et al.* 2009; Arakane *et al.* 2010). Although this gene is not within the NBAD branch, its depletion in *Drosophila* causes a severe reduction in melanization across the whole body (Wittkopp *et al.* 2002b; Jeong *et al.* 2008). Among the 38 *yellow* RNAi adults, 32 displayed a significant reduction in the intensity of black pigments (Figure S6, $P < 0.05$) in ventral A3 and A4 rectangles (Figure 2, E4 and E5). In addition, in 30 of 32 individuals, a reduction in the middle portions of the A3 and A4 rectangles also was observed (Figure 2E4). These pigmented phenotypes in the abdomen are similar to those reported in *Drosophila melanogaster* (Wittkopp *et al.* 2002b; Jeong *et al.* 2008). While abdomens showed an increase in average brightness of 51%, the reduction in black intensity in the head and thorax was more moderate (Figure 2, E1 and E3), with an increase in average brightness of 28 and 15%, respectively (Figure S6). These findings indicate that *yellow* was involved in regulating both the extent of melanin patterns and their intensity in the abdomen but had much less effect in the thorax and abdomen. Based on *yellow* and *tan* RNAi results, we speculate that these melanin-promoting factors may be critical for the melanin patterns in the posterior body regions.

Distinct black patterns between the forewing and hindwing are generated by different branches of the melanin pathway in Oncopeltus

The forewing and hindwing in *Oncopeltus* also have distinct melanin patterns. The forewing has an alternating black and orange pattern (Figure 3A1), whereas the hindwing is colorless at the proximal end and black throughout the distal region (Figure 3A2). To determine whether the NBAD branch regulates the melanin patterns in both pairs of wings, we examined the wings of *ebony* and *black* RNAi adults. In both instances, black pigmentation greatly expanded into the orange subregions on the forewing (Figure 3, B1 and C1). However, the depletion of either gene generated no noticeable changes in color patterns in the hindwing (Figure 3, B2 and C2). These observations indicate that the NBAD branch was required for repressing melanization in the nonmelanized areas on the forewing, whereas similar nonmelanized regions on the hindwing used a different mechanism.

Candidate genes responsible for suppressing melanization in the proximal hindwing include the NADA branch of the melanin pathway (Figure 1). Because *aaNAT*, the core gene within this branch, is reported to be responsible for converting dopamine to colorless NADA sclerotin in *Drosophila* (Wright 1987; Hintermann *et al.* 1995; Brodbeck *et al.* 1998),
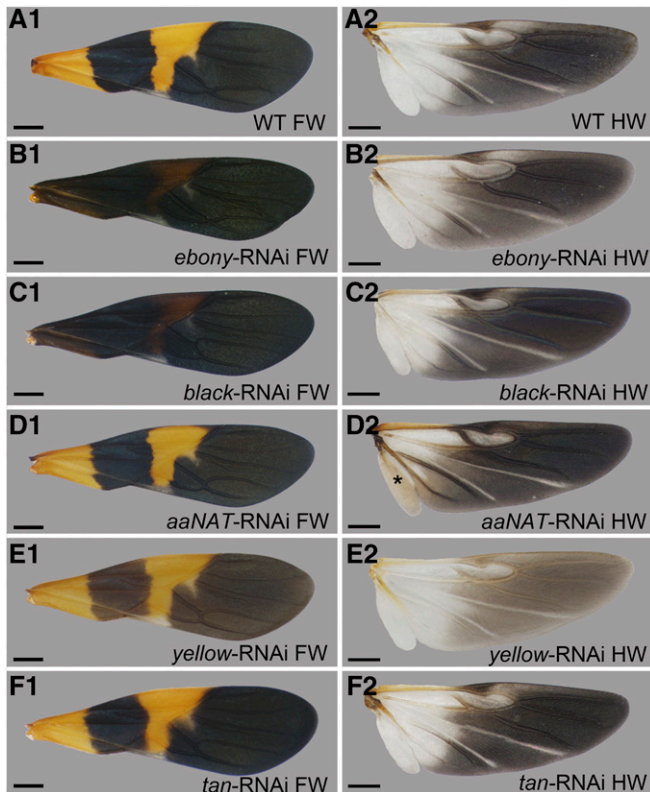
Figure 3 Functions of *ebony*, *black*, *aaNAT*, *yellow*, and *tan* in the forewing and hindwing of *Oncopeltus* adults. (A1–A2) Pigmentation patterns of wild-type wings. (B1–B2) The *ebony* RNAi adults showed an expansion of black pigmentation into the orange subregion of the forwing (B1), while the hindwing remained unaffected (B2). (C1–C2) The *black* RNAi adult wings showed a similar phenotype to the *ebony* RNAi adults, as described earlier. (D1–D2) The *aaNAT* RNAi adult phenotypes show no effect on the forewing (D1), while melanization expanded into the anal region of the hindwing (asterisk in D2). (E1–E2) The *yellow* RNAi adult wing phenotype showed a reduction of black pigment on the forewing (E1) and the hindwing (E2). (F1–F2) The *tan* RNAi adult phenotype showed no noticeable pigmentation effect in the forewing and hindwing. FW, forewing; HW, hindwing. Scale bars, 1 mm.

it is possible that such a mechanism also can generate the colorless pattern in the hindwing of *Oncopeltus*. To test this hypothesis, we observed the hindwing coloration in *aaNAT* RNAi adults. In all 17 resulting RNAi individuals, the anal lobe region of the hindwing became melanized (Figure 3D2), whereas the black pigmentation of the forewing was not affected (Figure 3D1). These observations indicate that *aaNAT* was involved in suppression of melanin formation in the colorless anal lobe region of the hindwing. However, there was no indication that this role was required for proper pigmentation of the forewing. In summary, the *Oncopeltus* forewing and hindwing seem to use distinct mechanisms to generate nonblack subregions: the NBAD branch is applied to suppress melanization in the orange areas of the forewing, whereas the hindwing employs the NADA branch to generate the colorless anal lobe.

In addition to the melanin-suppressing factors *ebony*, *black*, and *aaNAT*, we tested the function of the melanin-promoting factors *yellow* and *tan* in the black subregions of *Oncopeltus*

wings. In *yellow* RNAi adults, there was a significant reduction in black intensity in both pairs of wings (Figure 3, E1 and E2, and Figure S6, $P < 0.05$ in forewing and $P \ll 0.05$ in hindwing). This effect was much greater in the hindwing (average brightness increased by 89%) than the forewing (19%) (Figure S6). In contrast, the depletion of *tan* did not generate any noticeable effect on the black patterns of either the forewing or hindwing (Figure 3, F1 and F2) nor significant reduction in their black intensity (Figure S6, $P > 0.05$), indicating that this gene was not essential for wing melanin patterns. These observations suggest that the roles in wing melanization are distinct between different melanin-promoting factors: *yellow* is required for the proper intensity of black melanin in the wings, especially the hindwing, whereas *tan* may not be involved at all in wing pigmentation in *Oncopeltus*.

Differential involvement of pigmentation genes between forewing and hindwing correlates with their expression patterns

Previous studies in the wings of *Drosophila* and *Heliconius* have shown strong correlations between melanin patterns and the expression patterns of melanin genes (Wittkopp *et al.* 2002b; Gompel *et al.* 2005; Ferguson *et al.* 2011; Hines *et al.* 2012). In particular, such correlations have been seen in the *D. melanogaster* abdomen (Rebeiz *et al.* 2009; Camino *et al.* 2015) and forewing (Gompel *et al.* 2005) and occur in diverse *Drosophila* species with divergent patterns of melanic pigmentation (Werner *et al.* 2010; Arnoult *et al.* 2013; Ordway *et al.* 2014; Camino *et al.* 2015), indicating that these correlations are functionally meaningful. Thus, we hypothesized that the difference in mechanisms regulating nonblack patterns between forewing (NBAD branch) and hindwing (NADA branch) comprise differential expressions of the relevant core genes. To test this hypothesis, we used *in situ* hybridization to detect in developing wing pads of *Oncopeltus* the expression patterns of *ebony* and *aaNAT*, the two essential genes in the NBAD and NADA branches. Note that because formation of the cuticle precludes the expression analysis later in development (see *Materials and Methods*), we can only study early stages during which initial patterns are beginning to be laid out and do not fully correspond to the final patterns. As shown in Figure 4A1, *ebony* expression was observed in three distinct patches on the forewing: two are located on the anterior and posterior edges of the proximal portion and the other in the middle of the anterior edge. These locations correspond with the anterior and posterior margins of orange subregions on the forewing (Figure 3A1). The expression of *ebony* on the hindwing, however, was not detectable (Figure 4A1), consistent with the fact that *ebony* RNAi displayed no phenotype. However, *aaNAT* expression was not observed in the forewing but was present in the anal lobe region of the hindwing (Figure 4B1). This expression pattern of *aaNAT* was strongly correlated with its RNAi phenotype, where the colorless anal lobe region of the hindwing became melanized (Figure 3A2). Hence, the expression
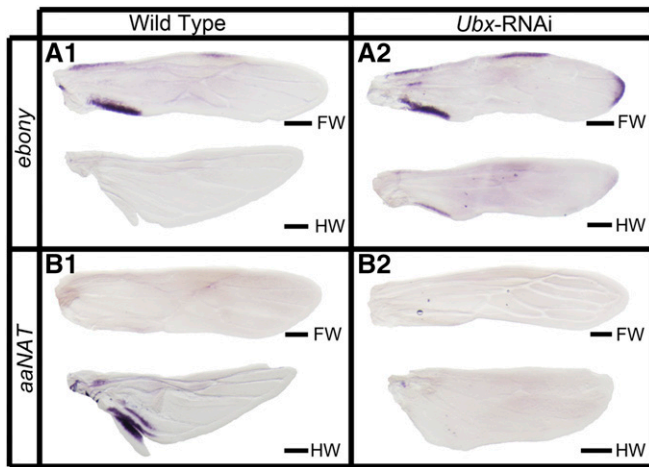
Figure 4 *In situ* hybridization of *ebony* and *aaNAT* in developing wild-type and *Ubx* RNAi *Oncopeltus* wings. (A1) In wild-type wings, *ebony* is expressed exclusively in the forewing. (A2) In *Ubx* RNAi wings, the hindwing exhibits expression of *ebony*. (B1) In wild-type wings, *aaNAT* is expressed only in the hindwing. (B2) In *Ubx* RNAi wings, *aaNAT* is no longer expressed in the hindwing. FW, forewing; HW, hindwing. Scale bars, 200 μm.

patterns of *ebony* and *aaNAT* correlated with their functions in generating the nonblack patches on the forewing and hindwing.

These differences in RNAi phenotypes between forewing and hindwing pigmentation may be explained by the differential activations of *ebony* and *aaNAT*. If so, this regional regulation of melanin genes would require specific selector genes (Wittkopp *et al.* 2003; Wittkopp and Beldade 2009). In the butterfly *Junonia coenia*, the Hox gene *Ultrabithorax* (*Ubx*) plays such a role by differential regulation of hindwing and forewing color patterns (Weatherbee *et al.* 1999). Recently, *Ubx* was shown to also control the identity of hindwing in *Oncopeltus* (Medved *et al.* 2015), allowing us to test the generality of the selector gene's role in regional regulation. Therefore, on depletion of *Ubx*, we would expect *ebony* to be expressed in the developing hindwing, whereas *aaNAT* should be absent. As shown in Figure 4A2, the hindwing expresses *ebony* at the proximal margins, which resembles the patterns observed in the wild-type forewing. However, the expression of *aaNAT* is lost in the hindwing, even in a moderate phenotype showing an intermediate shape transformation (Figure 4B2). These findings indicate that *Ubx* governs the differential activation of melanin genes between the wings. This further suggests that the different pigmentation roles of melanin genes across different *Oncopeltus* body regions, as shown by the present RNAi analyses, may be due to the functions of other region-specific regulatory genes.

The expression patterns of *black*, *yellow*, and *tan*, however, could not be detected using *in situ* hybridization. Note that the wing tissue is amenable to this procedure only during the middle part of the fifth nymphal stage (see *Materials and Methods*) when these three genes may not be active. To test this possibility, we examined the expression of *black*, *yellow*,

and *tan* during the entire fifth nymphal stage (Figure S8). As predicted, expression of these genes was noticeable only during the latter half of the stage (Figure S8). These observations suggest that during the development of *Oncopeltus* wings, enzymes such as Ebony and AANAT, which use dopamine as direct substrates (Figure 1), perform their patterning roles at early-middle fifth nymphal stage. In contrast, Black, Yellow, and Tan are turned on at a later stage.

Functions of melanin-suppressing factors in *Periplaneta*

The preceding sections have shown that in *Oncopeltus*, melanin-suppressing factors (*ebony* and *aaNAT*) play critical roles in the appearance of light patches of color in body regions that are mostly dark in coloration (*e.g.*, the head, thorax, and wings). To determine whether such a mechanism is applicable to nonaposematic hemimetabolous insects, we examined the American cockroach, *P. americana* (Figure 5A1), whose uniform brown body coloration is also generated by the melanin pathway (Lemonds 2015). In the middle of the dorsal T1 plate, however, there are paired oval regions created by clear sclerotin (Figure 5A2). The *Periplaneta* forewing is dark brown (Figure 5A3), whereas the hindwing features a brown anterior distal half and clear sclerotin in the posterior proximal half (Figure 5, A3 and A4). To determine how the melanin-suppressing factors can generate the color patterns in *Periplaneta*, we performed RNAi depletion of *ebony* and *aaNAT* using previously reported gene fragments (Bembenek *et al.* 2005; Blenau and Baumann 2005).

Adults from *ebony* RNAi treatments showed a significant increase in overall melanization, with black pigment covering most of their bodies (Figure 5B1). However, the clear sclerotin on the dorsal T1 plate remained (Figure 5B2). The wings also showed a substantial increase in pigmentation (Figure 5, B3 and B4), especially the forewing, which became black in color (Figure 5B3). These observations indicate that in *Periplaneta*, *ebony* plays an essential role in maintaining the proper intensity of melanization in areas that are already melanized, but it has no effect on clear sclerotin regions. In other words, *ebony* can regulate the darkness levels of the already-established melanin pattern, but it cannot alter the pattern. This finding is similar to that of a previous report in *Tribolium*, where the colorless patch on the hindwing was not altered by *ebony* RNAi (Tomoyasu *et al.* 2009).

The *aaNAT* RNAi adults retained wild-type brown pigmentation (Figure 5C1). However, the clear sclerotin areas in the T1 plate became melanized (Figure 5C2), with the overall coloration in the forewing and hindwing remaining similar to wild type (Figure 5, C3 and C4). These results reveal that *aaNAT* is responsible for creating some (T1 plate), but not all, colorless regions (posterior half of the hindwing). Alternatively, it is possible that these latter regions of clear sclerotin are produced by putative *aaNAT* paralogs (Mehere *et al.* 2011; Barbera *et al.* 2013; Long *et al.* 2015). These findings suggest that in both *Oncopeltus* and *Periplaneta*, some aspects of melanin patterning may be
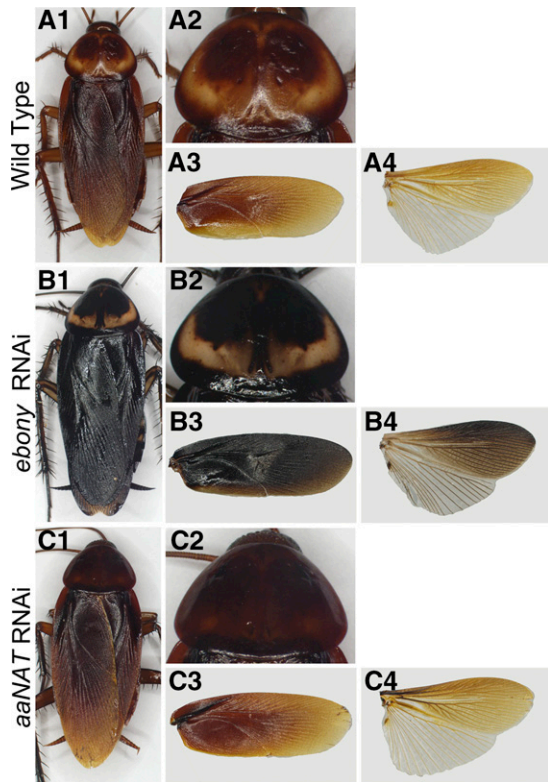
Figure 5 The functions of *ebony* and *aaNAT* in *P. americana* adults. (A1–A4) Pigmentation patterns of wild-type adult *Periplaneta*. (A1) Most of the body is covered by dark brown pigments. (A2) The T1 plate has a clear sclerotin patch in the shape of a horseshoe and two brown oval patches in the middle. (A3) The forewing displays a uniform brown coloration. (A4) The hindwing has a distinct pigmentation pattern with a brown pigment on the entire anterior region but is unpigmented in the posterior region, except for the veins, which are also brown in color. (B1–B4) The *ebony* RNAi adults established a significant increase in melanization. (B1) The increase in melanization resulted in a black appearance. (B2) Most of the T1 plate became black pigmented, with the exception of clear patches. (B3) The forewing showed a similar increase in melanization throughout the wing. (B4) The hindwing appeared darker compared to wild type, especially the anterior region. (C1–C4) The *aaNAT* RNAi phenotypes showed similar brown coloration to those observed in wild type, except for the T1 plate (C2), which lost the clear patches and is completely brown. FW, forewing; HW, hindwing.

conserved (*e.g.*, creating clear sclerotin by using AANAT in hindwing in *Oncopeltus* and in T1 in *Periplaneta*).

Discussion

Pigmentation functions of melanin genes in Oncopeltus

In this study, we performed a comprehensive functional analysis of the putative core melanin genes using RNAi. Although there has been limited success in RNAi silencing by direct body cavity injection in *Drosophila*, this approach has proven to be highly effective in other insects (Li *et al.* 2015). This is especially the case in *Oncopeltus*, which is characterized by a strong systemic gene depletion following RNAi treatment (Liu and Kaufman, 2004a, b, 2005; Angelini and Kaufman 2005). The recent RNAi analysis of the essential enzymes for

the production of black melanin in this species also showed a systemic reduction in coloration encompassing the entire body (Liu *et al.* 2014). In this study, the whole-body RNAi response is documented by RT-PCR results showing similar reduction in the amount of transcript across different body regions in *black* RNAi individuals (Figure S5). Hence, the observed region-specific effects of RNAi against melanin genes are likely true phenotypes resulting from systemic responses rather than low-penetrance phenotypes. These results suggest distinct regional utilization of melanin genes.

The NBAD branch of the melanin pathway has been reported to be essential for melanin patterning in *Drosophila* (Wright 1987; Wittkopp *et al.* 2002b). In this pathway, both *black* and *ebony* can suppress melanin formation via the production of β-alanine and NBAD, respectively (Figure 1). In *Oncopeltus*, RNAi knockdown of these two genes caused the nonblack subregions to become black in the head (Figure 2, B2 and C2), thorax (Figure 2, B3 and C3), and forewing (Figure 3, B1 and C1), whereas most of the nonblack patches in the hindwing (Figure 3, B2 and C2) and abdomen (Figure 2, B4 and B5 and C4 and C5) were not affected. These findings suggest that the melanin-suppressing role of *ebony* and *black* is crucial in the head, thorax, and forewing but not necessary for proper coloration in the hindwing and abdomen. This is in contrast with findings in *Drosophila* and *Tribolium*, where loss of function of either *ebony* or *black* results in an overall darkening of body pigmentation (Wright 1987; Wittkopp *et al.* 2002b; Arakane *et al.* 2009; Tomoyasu *et al.* 2009).

Another gene of the NBAD branch, *tan*, counteracts the function of *ebony* and promotes black pigmentation by converting NBAD back to dopamine (Wright 1987; True *et al.* 2005; Jeong *et al.* 2008). *tan* RNAi phenotypes in *Oncopeltus* indicate that it is essential for the black patterns present in the abdomen (Figure 2, D4 and D5) but is not a significant player in forming the melanin patterning in the head (Figure 2D2), thorax (Figure 2D3), and wings (Figure 3, F1 and F2). Again, these results are different from those in *Drosophila*, where *tan* mutants display a reduction in melanization spanning the entire body (True *et al.* 2005; Jeong *et al.* 2008).

Despite the fact that the reactions catalyzed by *tan* and *ebony* appear as a circuit from the biochemical perspective (Figure 1), in *Oncopeltus*, they are employed differently in distinct body regions. It is possible that in hemimetabolous insects, only half of this circuit is essential for melanization in one specific body region, whereas the other half might be active but not required for pigmentation. Instead, they might be involved in other biological processes such as behavior (Wittkopp and Beldade 2009). Subsequently, in more derived groups such as dipterans, the entire *tan-ebony* circuit (both reactions) became fully involved in melanization (True *et al.* 2005). Extending future studies of *tan* to additional hemimetabolous species may show how the NBAD branch of the melanin pathway has changed during insect evolution.

Finally, the NADA branch also can suppress melanin production by transforming dopamine to NADA, thus creating colorless tissue (Wright 1987). The key gene in this pathway

is *aaNAT* (Wright 1987; Hintermann *et al.* 1995; Brodbeck *et al.* 1998). Here we observed that the role of *aaNAT* is restricted to the anal lobe region of the hindwing (Figure 3D2), which correlates with its expression profile (Figure 4B1). This observation is in contrast to the depletion of *aaNAT* in the silkworm *Bombyx*, which causes a global darkening of the adult body (Zhan *et al.* 2010; Osanai-Futahashi *et al.* 2012). Thus, *Oncopeltus* appears to preferentially activate *aaNAT* expression in wings to create the colorless tissue in the anal lobe region. It is worth noting that *aaNAT* may not be the only gene used in this region because the anterior portion of the proximal half of the hindwing remains colorless when *aaNAT* is depleted by RNAi (Figure 3D2). Future studies will need to assess whether other members of the AANAT family are involved in generating colorless patches in this region (Mehere *et al.* 2011; Long *et al.* 2015).

The pigmented phenotypes of *yellow* have been reported in both *Drosophila* and *Tribolium*, with distinct results. Loss of *yellow* causes global reduction of melanin in *Drosophila* (Wittkopp *et al.* 2002b), whereas only the hindwing is affected in *Tribolium* (Tomoyasu *et al.* 2009; Arakane *et al.* 2010). In *Oncopeltus*, *yellow* RNAi adults displayed a mix of features previously observed in *Drosophila* and *Tribolium*: black coloration in the abdomen and hindwing is greatly reduced, but other body regions were only moderately affected (Figure S6). Therefore, the restriction of *yellow* function appears to have changed during insect evolution. At present, we cannot rule out the possibility that in regions where *yellow* RNAi was observed to have a moderate effect, other *yellow* family members might play additional roles in melanization (Han *et al.* 2002). However, currently the functional roles of most of the *yellow* family genes are yet to be determined. Even for the *yellow* gene itself, despite the fact that it is known to be required for melanization in *Drosophila* and *Tribolium* (Wright 1987; Wittkopp *et al.* 2002a, b; Jeong *et al.* 2008; Tomoyasu *et al.* 2009; Arakane *et al.* 2010; Bray *et al.* 2014), its enzymatic activity has not yet been established. In terms of the position of *yellow* in the melanin pathway, two different hypotheses have been proposed (Figure 1): it may be essential for the production of either DOPA melanin (Wright 1987; Walter *et al.* 1996) or dopamine melanin (Wittkopp *et al.* 2002b, 2003). Our present results and another recent report (Liu *et al.* 2014) show that black pigment in the hindwing is dopamine melanin, suggesting that *yellow* may be involved only in dopamine melanin production.

Overall, our results show that the pigmentation functions of the five genes under study are regionalized in *Oncopeltus*. This is in contrast with previous studies in *Drosophila*, *Tribolium*, and *Bombyx*, in which depletion of *ebony*, *black*, *tan*, *aaNAT*, or *yellow* resulted in alteration of melanin patches throughout the body (Wright 1987; Walter *et al.* 1996; Wittkopp *et al.* 2002b; True *et al.* 2005; Gibert *et al.* 2007; Jeong *et al.* 2008; Arakane *et al.* 2009; Tomoyasu *et al.* 2009; Zhan *et al.* 2010; Osanai-Futahashi *et al.* 2012). Hence, the insight from *Oncopeltus* is that the entire melanin pathway can be split into different sections that are used in different body regions.
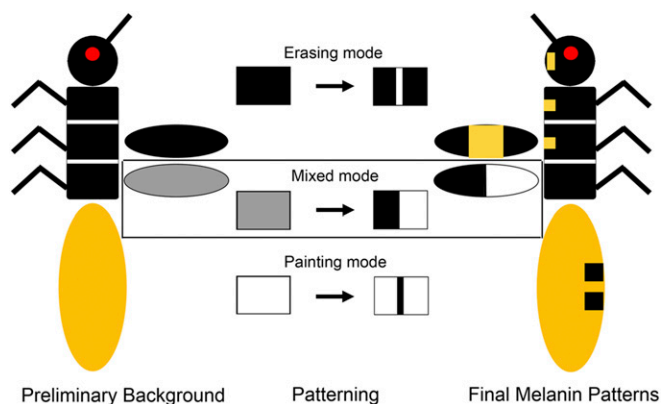
Figure 6 Three proposed modes of insect melanin patterning, as illustrated by pigmentation in *Oncopeltus*. The final melanin patterns (right) are generated from the preliminary background (left) via different modes of melanin patterning. In the head, thorax, and forewing, where the background is fully black, the nonmelanin patches are generated by "erasing" the background. In the abdomen, where the background is nonblack, the melanin patches are "painted" onto the background. A mixture of these two modes may be used in the hindwing, in which black is intensified in the melanin subregions, whereas melanin is suppressed in the nonblack subregions.

In this study, the regional patterns of melanin genes are observed from analyses at a whole-body scale. To obtain a deeper comprehension of how complex melanin patterns are generated will require analyzing pigmentation genes at a finer morphologic scale (*e.g.*, a subregion of a segment). Classical studies in *Drosophila* have shown that selector genes involved in a general anteroposterior axis determination, such as *hedgehog* (*hh*), *engrailed* (*en*), and *optomotor-blind* (*omb*), also regulate melanin patterning in the abdominal segments (Kopp and Duncan 1997; Kopp *et al.* 1997). It is tempting to speculate that similar mechanisms may account for the presence of centrally positioned black rectangles on A3 and A4 segments in *Oncopeltus*. Also, other axis-determination mechanisms, especially mediolateral, may also be involved in generating the black spots located on the lateral edges of each abdominal segment (Figure 2A5). Furthermore, it is interesting to note that *hh* and *en* regulate melanin genes in a cell-autonomous manner in *Drosophila* (Kopp *et al.* 1997). In contrast, melanin genes themselves can function nonautonomously, semiautonomously, or autonomously in different body regions (Hanna 1953; Hotta and Benzer 1970; Borycz *et al.* 2002; Wittkopp *et al.* 2002b; True *et al.* 2005). At present, we do not know whether such region-specific cell autonomy of melanin genes also may account for production of the regional color patterns observed in *Oncopeltus*. Extending future studies in this direction will be required to gain a better understanding of the regulatory mechanisms that generate species-specific melanin patterns in insects in general.

Region-specific employment of melanin genes

To better understand the regional utilization of melanin genes, we provide a summary of the general principles that

appear to guide melanin patterning (Figure 6). In this summary, we refer to a putative case as the "preliminary background," in which only the basic enzymes required for melanin production, such as TH and DDC, would be active. In other words, none of the melanin-promoting or melanin-suppressing factors function in such a background. Under the first scenario, the "painting" mode would be used in a body region that lacks melanin patterns, such as the *Oncopeltus* abdomen, which is predominantly orange. The preliminary background in this case would be nonmelanin because most of the abdomen may not be capable of producing black pigments. The dark melanin is "painted" onto specific areas of this background, which requires melanin-promoting factors (*tan* and *yellow*) to ensure the proper boundary and intensity of black patterns. Under a second scenario, the "erasing" mode would apply to a body region where dark melanin is the predominant pigment (as observed in the head, thorax, and forewing in *Oncopeltus*, as well as the body and wings of *Periplaneta*). In this instance, the preliminary background would be fully melanized, and the melanin suppressors such as *ebony*, *black*, and *aaNAT* are used for "erasing" melanin production in a specific area or for lowering the overall melanin intensity. In addition to situations where one of these two modes is used exclusively, there are also regions in which the melanin-promoting factors and suppressors can both play active roles, such as the hindwing in *Oncopeltus*, where melanin and nonmelanin subregions are equally distributed. Under this scenario, the preliminary background is defined as a region undergoing melanization but lacking a proper level of intensity. Finalizing the melanin patterns requires both the melanin-suppressing factor (*aaNAT*), which generates the nonmelanin patches, and the melanin-promoting factor (*yellow*), which intensifies the dark color within melanin patches.

In terms of their generality, these three scenarios can account for most of the functional results in the previously studied species. The painting mode may be evidenced in the dark-colored pterostigma on the hindwing of *Tribolium*. In those studies, this specific black pattern becomes lighter in *yellow* RNAi individuals, whereas *ebony* RNAi has no effect on the flanking nonblack subregions (Tomoyasu *et al.* 2009; Arakane *et al.* 2010). The erasing mode may be evidenced in the butterfly *Papilio*, where the default fully melanized forewing was observed in *ebony* mutant adults (Koch *et al.* 2000). Another example is the depletion of *ebony* or *black* in *Tribolium*, which results in the general blackening of the whole body (Arakane *et al.* 2009; Tomoyasu *et al.* 2009). In addition to the present results in *Oncopeltus*, the mixed mode has been fully confirmed only in *D. melanogaster*, where functional analyses revealed that *ebony* and *yellow* contribute equally to both wing and body pigmentation (Wittkopp *et al.* 2002b; Gompel *et al.* 2005). In general, our summary of the principles of melanin patterning can serve as a practical framework explaining the diversity in melanin coloration observed in previously reported insects. A broader taxonomic sampling in basal groups, from which we can infer the ancestral melanin patterning, will be required to determine whether this framework can be applied to a wider range of insect species.

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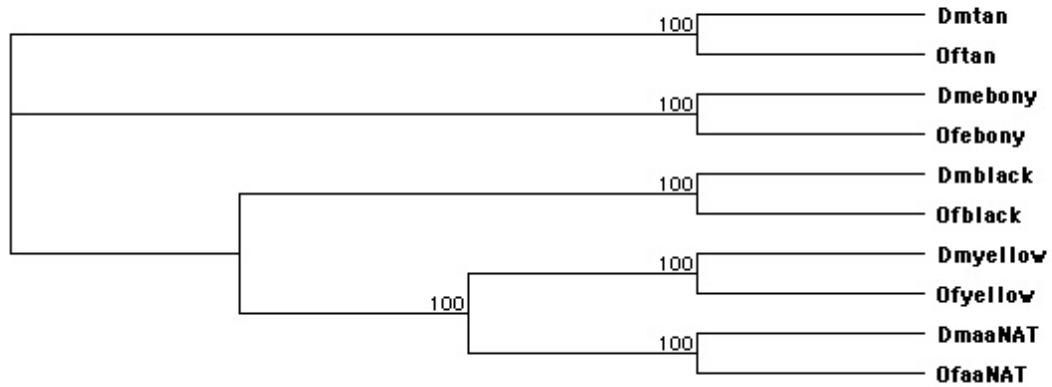*Communicating editor: D. M. Parichy*

GENETICS

A Pathway Analysis of Melanin Patterning in a Hemimetabolous Insect

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Method:    Neighbor Joining; Bootstrap (500 reps); tie breaking = Systematic
Distance:  Poisson-correction
           Gaps distributed proportionally
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Fig. S1. Phylogenetic analysis of *Drosophila melanogaster* and *Oncopeltus facsiatus* melanin pathway genes. Phylogeny was inferred using the Neighbor-Joining method based on amino acid sequences. Branch support values are bootstrap percentages from 500 replicates.
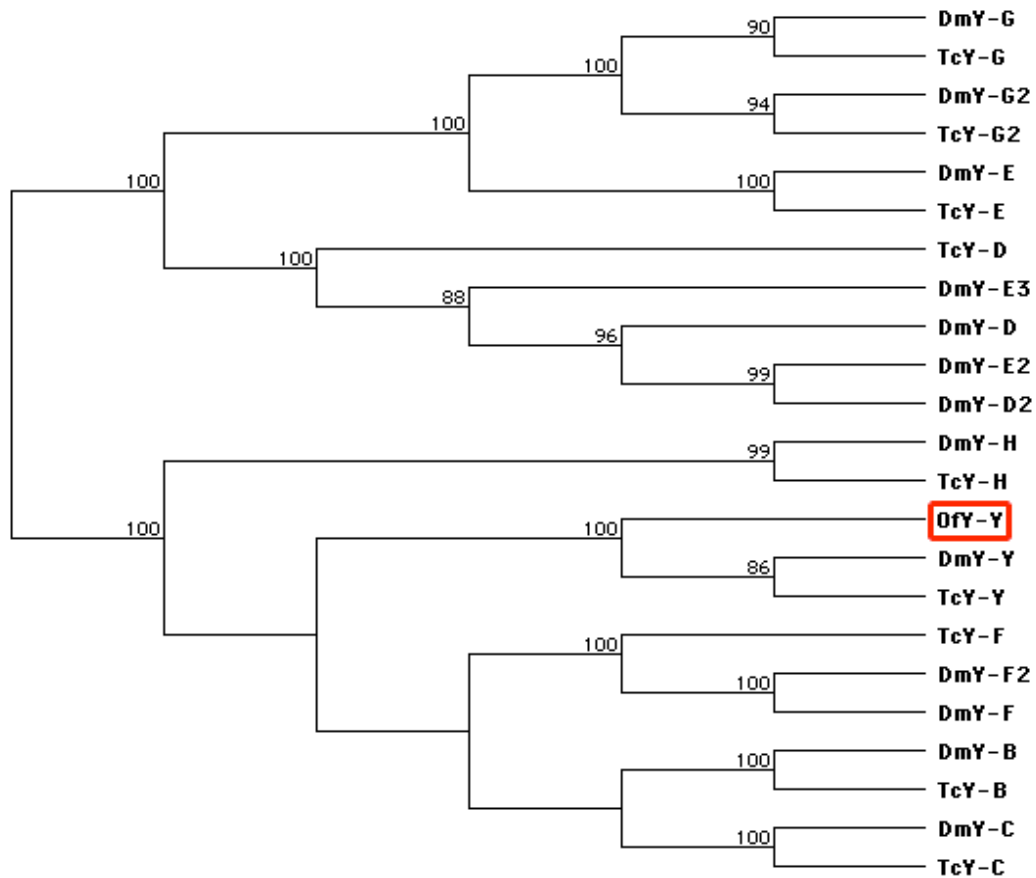
Fig. S2. Phylogenetic analysis of *yellow* gene family. *Oncopeltus* Yellow (OfY-Y) is orthologous to Yellow protein in *Drosophila melanogaster* (DmY-Y) and *Tribolium castaneum* (TcY-Y). Phylogeny was inferred using the Neighbor-Joining method based upon amino acid sequences. Branch support values are bootstrap percentages from 500 replicates.
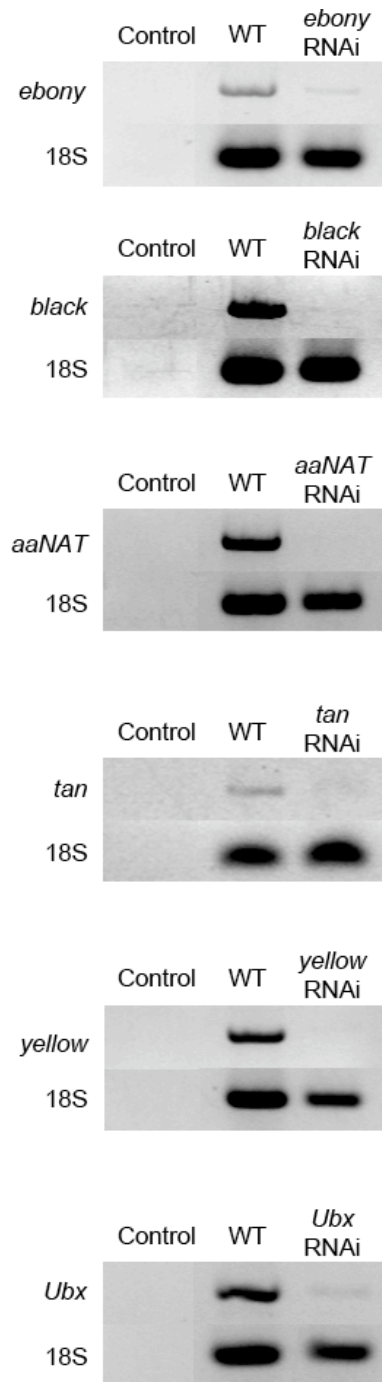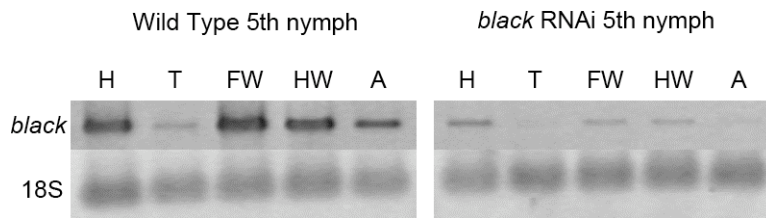
Fig. S3. RT-PCR analysis of *ebony*, *black*, *aaNAT, tan, yellow,* and *Ubx* mRNA in *Oncopeltus* 5th nymphs. Only trace levels of individual gene transcripts were detected in RNAi individuals when compared to wild types.
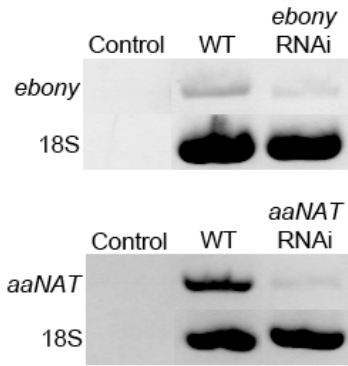
Fig. S4. RT-PCR analysis of *ebony* and *aaNAT* mRNA in *Periplaneta americana*. There was a significant reduction in the amount of targeted RNA transcript.

Fig. S5. RT-PCR analysis of *black* mRNA in isolated body regions of *Oncopeltus* 5th nymphs at Day8 of development. There is a significant reduction in black transcript in every body region in *black* RNAi individuals compared to wild type 5th nymphs. Abbreviations: H, head; T, thorax; FW, forewing; HW, hindwing; A, abdomen.
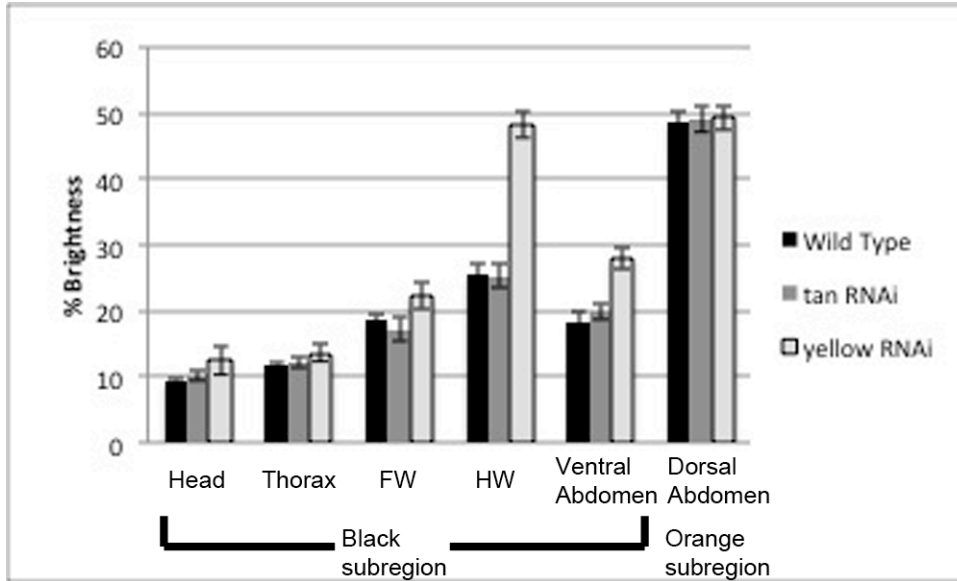
Fig. S6. The comparison of black intensity in isolated body regions of wild type, *tan* RNAi and *yellow* RNAi adults. The levels of black in the melanized subregions in the head, thorax, forewing, hindwing, and ventral abdomen are shown in average percent brightness. The error bars are showing the 95% confidence intervals. The depletion of *tan* did not show significant changes in the black intensity in all body regions (P > 0.05). The black intensity in *yellow* RNAi is significantly different from that in wild type in all body regions (P < 0.05). The amount of reduction in black is most significant in the hindwing and ventral abdomen, whereas it is moderate in the head, thorax, and forewing. In both *tan* RNAi and *yellow* RNAi adults, the brightness of the orange subregions of the dorsal abdomen are not significantly different from those in the wild type (P > 0.05). Abbreviations: FW, forewing; HW, hindwing.
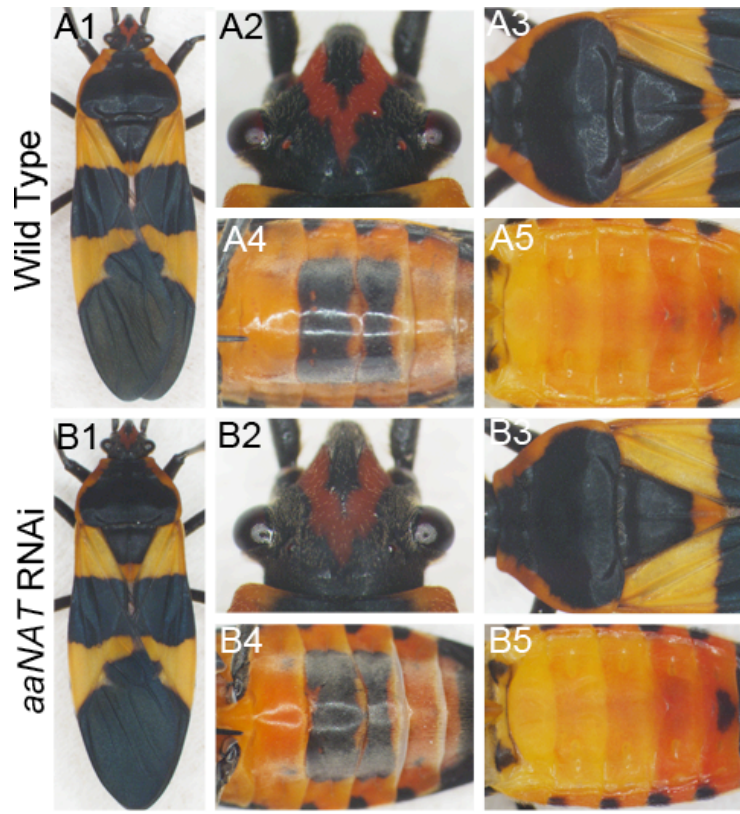
Fig. S7. Phenotype of *aaNAT* RNAi in black coloration of *Oncopeltus* body. (A1-A5) Wild type fully-melanized *Oncopeltus* adults establish black pigmentation in the head (A2), thorax (A3), ventral abdomen (A4), and dorsal abdomen (A5). (B1-B5) *aaNAT* RNAi adults showed similar black coloration as wild type, which can be observed in the head (B2), thorax (B3), ventral abdomen (B4) and dorsal abdomen (B5).
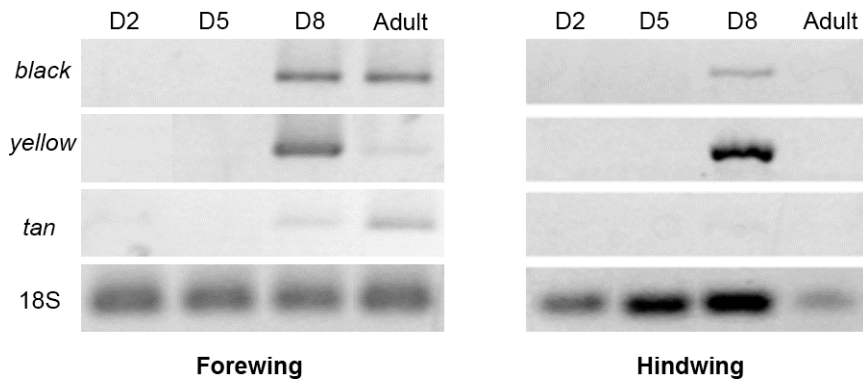
Fig. S8. RT-PCR analyses of *black*, *yellow*, and *tan* mRNA in the developing wings of *Oncopeltus* throughout the entire 5th nymphal stage. The chosen time points are: Day2 (initialization of adult melanin cycle), Day5 (formation of wing tissue), Day8 (localization of melanin enzymes), and freshly molted adults (initialization of melanin process). The expression levels of the targeted mRNA are dynamic among these four time points. At Day2 and Day5, none of these three genes were detectable in the wings. Starting by Day8, the expression of *black, yellow* and *tan* showed up with different patterns. *black* was expressed in both Day8 and adult forewings. However, its expression in the hindwing was only observed at Day8. The expression of *yellow* was observed in both wings at Day8, which were significantly reduced in the adults. *tan* expression was very low on day 8 in both wings. The expression in the forewing later reached its peak in adult, whereas the hindwing expression was not detected at adult stage.

	Primers for PCR cloning	Primers for RT-PCR	Length of fragment	Corresponding AA residues of *Dm* orthologs
Of-ebony	Forward: 5' ACGACCGCAACTCTCAAACT 3' 5' TCACCAGCTCTTCTCGTTGA 3' Reverse: 5' TGCTGAGTTAGAGGGCTGGT 3' 5' TGCCAGTAAAGCCTCTGGAT 3'	Forward: 5' CTTCTCCCGACCTCATTCTG 3' Reverse: 5' GGTCCACAGGGCATCTAAAA 3'	2333 bp	135~822 (out of 879aa)
Of-black	Forward: 5' GGTGCAGCGTCTTCTCCTAC 3' 5' GGCCGGTACTGACACTCTGT 3' Reverse: 5' AGAAGTTTGGGTGTGCCTTG 3' 5' TTGTTCCTTCACGCATCATC 3'	Forward: 5' GGCCGGTACTGACACTCTGT 3' Reverse: 5' TTGTTCCTTCACGCATCATC 3'	1160 bp	147~532 (out of 575aa)
Of-yellow	Forward: 5' ATTCGCCTGGAAGTATGTGG 3' 5' GGACAAAGGTGTCCCGAGTA 3' Reverse: 5' GAGAATTCTGGCAGCTGAGG 3' 5' ATCGGCAACCTGTGGTAGAG 3'	Forward: 5' AGTCCAGAAACACCCCTCCT 3' Reverse: 5' ATCGGCAACCTGTGGTAGAG 3'	1062 bp	80~408 (out of 541aa)
Of-tan	Forward: 5' CGTCCGTTCCTTACTTCTCG 3' Reverse: 5' CCTGGCATTGCAGTTGAATA 3'	Forward: 5' GGACATTCTACCAGCCCAAA 3' Reverse: 5' CAATCGATCTCCAGCCTGAT 3'	1338 bp	6~386 (out of 387aa)
Of-aaNAT	Forward: 5' TGGGCGAAAGAGAGAATGAC 3' 5' CCAGGAAGACGTGGAAAGAG 3' Reverse: 5' GTGCAGACTTTGCAGTGTGG 3' 5' TCGGTCTTCCATCATTGTCA 3'	Forward: 5' CCAGGAAGACGTGGAAAGAG 3' Reverse: 5' CAAGGCTGTAGATGCTGTGG 3'	554 bp	30~206 (out of 240aa)
Pa-ebony	Forward: 5' TCAAGACGTGGGTATGCAGT 3' 5' GAATGGCAAGACAGACCGAC 3' Reverse: 5' CATCTGCATCTGGGTGAACG 3' 5' TGCTGTGTTAGGGGATTGGT 3'	Forward: 5' GAATGGCAAGACAGACCGAC 3' Reverse: 5' TGCTGTGTTAGGGGATTGGT 3'	880 bp	538~833 (out of 879aa)
Pa-aaNAT	Forward: 5' TGCGAAGCAACGAGTGTAAC 3' 5' CACCGATGGAGGTCTACGTT 3' Reverse: 5' AGGAACCAGTGGCAAATCAC 3' 5' AGGCTGTCCATTCTTGCAGT 3'	Forward: 5' CACCGATGGAGGTCTACGTT 3' Reverse: 5' AGGCTGTCCATTCTTGCAGT 3'	646 bp	6~221 (out of 240aa)

Table S1. Primers for RT-PCR and the lengths of the obtained cDNA fragments of relevant genes. Abbreviations: *Of, Oncopeltus fasciatus; Pa, Periplaneta americana; Dm, Drosophila melanogaster*