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The genetic control of aposematic black pigmentation in hemimetabolous insects: insights from *Oncopeltus fasciatus*

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SUMMARY

Variations in body pigmentation, encompassing both the range of specific colors as well as the spatial arrangement of those colors, are among the most noticeable and lineage-specific insect features. However, the genetic mechanisms responsible for generating this diversity are still limited to several model species that are primarily holometabolous insects. To address this lack of knowledge, we utilize *Oncopeltus fasciatus*, an aposematic hemimetabolous insect, as a new model to study insect pigmentation. First, to determine the genetic regulation of black pigment production in *Oncopeltus*, we perform an RNAi analysis on three core genes involved in the melanin pathway, *tyrosine hydroxylase* (*TH*), *dopa decarboxylase* (*DDC*), and *laccase 2* (*lac2*). The black pigmentation is affected in all instances, showing that the black pigments in this species are derived from the melanin pathway. The results of the *DDC* RNAi are particularly informative because they reveal that it is Dopamine melanin, not DOPA melanin, which is the predominant component of black pigments in *Oncopeltus*. Second, we test whether pigmentation follows a two-step model where the spatial pre-mapping of enzymatic activity is followed by vein-dependent transportation of melanin substances. We confirm the existence of the first step by observing that premature wings develop black pigmentation when exposed to melanin precursors. In addition, we provide evidence for the second step by showing that wing melanin patterning is disrupted when vein transportation is halted. These findings bring novel insights from a hemimetabolous species and establish a framework for subsequent studies on the mechanisms of pigment production and patterning responsible for variations in insect coloration.

INTRODUCTION

Pigmentation is one of the most conspicuous and variable features of insect diversity, playing essential functions in a variety of behavioral, physiological, and reproductive processes (Nijhout 1991). Although highly diverse, variations in insect pigmentation are generated through a common mechanism that encompasses both pigment patterning and pigment production (Wittkopp and Beldade 2009). Because most of the available genetic information was generated from the studies in model species such as *Drosophila*, which not only have a limited color spectrum but also undergo a more derived holometabolous development, it is unclear whether this mechanism is broadly applicable to other insect lineages.

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Pigment patterning is determined by regulatory factors, which directly or indirectly control the spatial distribution of enzymes involved in pigment biosynthesis (Wittkopp and Beldade 2009). In *Drosophila*, wing patterning follows a two-step model, described by True et al. (1999). First, the enzymes are pre-patterned prior to the initialization of wing pigmentation. Then, in newly molted adults, the precursors are transported through the wing veins and gradually diffuse out into the wing tissue, allowing the pre-patterned enzymes to oxidize the precursors forming dark brown or black patterns. At present, the role of vein transport in pigment patterning is still not fully understood due to a limited number of studies that provided conflicting results. As previously reported, the disruption of this transport in *Drosophila* and the swallowtail butterfly *Papilio*, by either genetic or surgical means, resulted in abnormal color patterns in the wing regions associated with the affected veins (True et al. 1999; Koch and Nijhout 2002). However, in the brush-footed butterfly *Heliconius*, mutants with severe vein deficiency do not display significant alteration in wing coloration, suggesting that wing patterning may be vein independent in this species (Reed and Gilbert 2004). Such contradictory findings make it hard to generalize whether the two-step model is also applicable to other insect species.

Most of our insight into pigment production comes from *Drosophila*, in which body and wing pigments are mainly produced via the melanin pathway (Wright 1987; Wittkopp et al. 2002; Wittkopp et al. 2003; True et al. 2005; Riedel et al. 2011). As described by Wright (1987), the process starts with the conversion of tyrosine to DOPA (dihydroxyphenylalanine) by tyrosine hydroxylase (TH)(Fig. 1A). DOPA can either be transformed to produce DOPA melanin (black pigment) or further converted to Dopamine by DOPA decarboxylase (DDC). Dopamine, in turn, can be converted into two other major catecholamine metabolites, NBAD (N- β -alanyldopamine) or NADA (N-acetyldopamine). Finally, Phenol Oxidases (PO) change these products to their relative quinones, which are then converted to Dopamine melanin (brown/black pigment), NBAD sclerotin (yellow), or NADA sclerotin (colorless), respectively. Additional functional data from *Tribolium*, *Apis*, and *Monochamus* (Roseland 1987; Arakane et al. 2005; Niu et al. 2008; Arakane et al. 2009; Elias-Neto et al. 2010; Gorman and Arakane 2010), in combination with recent expression studies suggest that the melanin pathway might also be involved in pigmentation in other holometabolous groups (Futahashi and Fujiwara 2005; Futahashi et al. 2010; Shirataki et al. 2010; Ferguson et al. 2011; Yu et al. 2011). However, much less genetic data has been reported in hemimetabolous insects except a functional report showing that *laccase 2* (*lac2*), a phenol oxidase, is involved in cuticular pigmentation in stinkbug species (Futahashi et al. 2011).

Our understanding of the molecular mechanism of aposematism, which is one of the most successful anti-predator adaptations in insects, remains extremely limited. Model species such as *Drosophila* or *Tribolium*, in which genetic tools are readily available, do not display any striking coloration. In contrast, in traditional aposematic model insects such as *Heliconius*, a functional approach is not yet feasible. To fill in this gap we utilized *Oncopeltus fasciatus* (milkweed bug) as a new model for studying the evolution and divergence of insect pigmentation in a more basal insect group. *Oncopeltus* is ideally suited for such a purpose being a hemimetabolous species in which functional experiments (via

RNAi application) are highly effective. Furthermore, its orange/black aposematic coloration is relatively simple, allowing for an easy and direct molecular analysis of specific warning patterns.

In the first half of this study, we tested the role of the melanin pathway in the black pigmentation in *Oncopeltus*. We performed an RNAi analysis to examine the functions of three essential enzymes in this pathway: *TH*, *DDC*, and *lac2* (Wright 1987; Arakane et al. 2005; Niu et al. 2008; Elias-Neto et al. 2010; Futahashi et al. 2011; Riedel et al. 2011). Our results establish that in *Oncopeltus* black coloration is produced via the melanin pathway, with Dopamine as the predominant contributor to the black pigmentation. Also, we tested if the *Drosophila* two-step wing melanin patterning model is applicable to *Oncopeltus*. Our results show that both the pre-mapping of melanization enzymes as well as the vein-dependent elaboration exist in this species. Overall, these observations suggest that the production of black pigmentation, as well as the two-step wing patterning model may be general mechanisms that are used across all insect lineages.

MATERIALS AND METHODS

Cloning and Sequence Analysis of cDNA Fragments

Mixed stage embryos of *Oncopeltus fasciatus* were used for total RNA extraction. The production of cDNA, RT-PCR, and cloning were performed according to Li and Popadic (2004). Multiple reactions of nested-PCR were utilized to obtain the gene fragments. The *TH* fragment was amplified using three sets of nested degenerate primers, 5'GCIGTIGCIGCIGCICARAARAAY 3', 5'MGIMGIMGIWSI YTIGTIGAYGAY 3' and 5'GCIMGITTYGARACIYTIGTIGT 3' as forward primers, and 5'ARIARICCGCIGCIGGICKIAR 3', 5'RTAIGCRAAIGCDATYTCIGCDAT 3' and 5'YTTTRTCIGCRAAICCGGRTGRTT 3' as reverse primers. *DDC* was amplified using two sets of nested degenerate primers, 5'GGIGTIACICAYTGGCAYWSICC 3' and 5'TTYCAYGCITAYTTYCCIIACIGC 3' as forward primers, and 5'CKRAAICKICKICCIARIGGDAT 3' and 5'YTGCCARTGICKRTARTCIGGIGC 3' as reverse primers. *lac2* was amplified using two sets of primers, 5'TCCACACATTCCCTGGTACA 3' and 5'TCAGGTATGCGAAGGTGACA 3' as forward primers, and 5'AAAGTTAGGCGGTGTTGGTG 3' and 5'GGAGAAGACCCCTTCTGTCC 3' as reverse primers. *Tyr* was amplified using one set of primers, 5'GCTATTATTCACCGGCCTGA 3' as forward primer, and 5'CAATGGCCATGTTACGAGTG 3' as reverse primer. RT-PCR conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 90 seconds; one cycle of 72°C for 7 minutes. As a result, a 693 bp fragment of *TH*, a 819 bp fragment of *DDC*, a 1359 bp fragment of *lac2*, and a 1615 bp fragment of *Tyr* were obtained. Ten clones of each fragment were isolated and sequenced. The resulting nucleotide sequences were then compared to each other and to known sequences in Genebank. Identities of these *Oncopeltus* genes were confirmed by a phylogenetic analysis (Fig. S1).

RNA interference (RNAi)

Double stranded RNA (dsRNA) was prepared and injected into the abdomens of *Oncopeltus* nymphs, as described by Chesebro et al. (2009). Approximately 2 μ l of dsRNA was injected at a concentration of 2–3 μ g/ μ l into each individual. For *DDC* and *tyrosinase*, dsRNA was injected into 4th nymphs. Injections at this stage for *TH* and *lac 2*, however, were all lethal. For this reason, the functions of these two genes were analyzed by injecting 5th nymphs instead. In total, of the 48 nymphs injected with *DDC* RNAi, 33 successfully molted into adults. For *TH* RNAi, 44 nymphs were injected and 32 survived to adult stage. For *lac2* RNAi, 32 nymphs were injected and 19 molted to adults. Of these 19 individuals, only 5 could eclose completely, whereas the other 14 had residuals of exoskeleton remaining. For *tyrosinase* RNAi, 10 nymphs were injected and all molted to adults. All images were chosen to show the most representative phenotypes.

RT-PCR Analysis

To determine the efficiency of our RNAi approach, we performed independent RT-PCR analyses on *TH*, *DDC*, *lac2*, and *Tyr* as controls (shown in Fig. S2). *Oncopeltus* 5th nymphs were injected with 2 μ l of dsRNA of *TH*, *DDC*, *lac2*, or *Tyr* dsRNA at 2.5 μ g/ μ l and allowed to develop for 3–4 days. Next, two RNAi individuals from each injection had their internal organs removed and the rest of the bodies were used for total RNA extraction and cDNA generation, as described by Chesebro et al. (2009). This same procedure was applied to two wild type individuals. Equal amounts of wild type and RNAi 5th nymphs cDNA were used as templates in individual PCR reactions to assess the amount of relative transcripts that were abolished in injected individuals. As positive controls, primers designed to the *Oncopeltus* 18S ribosomal subunit were used in all cases. *TH* transcript was amplified by using forward primer 5' ACGAACGAAAATGCAGAACC 3' and reverse primer 5' GGGACATATCCACCTTGACG 3'. The PCR reaction condition for *TH* was as follows: 94°C for 2 min; 28 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds; one cycle of 72°C for 7 minutes. *DDC* transcript was assessed by using forward primer 5' CACAGAGCTGGAAGTGGTGA 3' and reverse primer 5' GCATAAGCCCTGAATTTGGA 3'. The PCR reaction condition for *DDC* was as follows: 94°C for 2 min; 27 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 25 seconds; one cycle of 72°C for 7 minutes. *lac2* transcript was assessed by using forward primer 5' AAAGTTAGGCGGTGTTGGTG 3' and reverse primer 5' GGAGAAGACCCCTTCTGTCC 3'. The PCR reaction condition for *lac2* was as follows: 94°C for 2 min; 29 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 90 seconds; one cycle of 72°C for 7 minutes. *Tyrosinase* transcript was assessed by using forward primer 5' TTTGCAAATGTCAGGCTCAG 3' and reverse primer 5' CTTGACCTCCGTTGAATGGT 3'. The PCR reaction conditions for *tyrosinase* was as follows: 94°C for 2 min; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 40 seconds; one cycle of 72°C for 7 minutes. In all four instances, there was a significant reduction in transcription levels in RNAi individuals when compared to wild types. (Fig. S2)

***In Vitro* Induction of Wing Pigmentation**

This assay was performed following the approach described by Walter et al. (1996). The wing tissues were dissected from late 5th nymphs and fixed for 30 min with freshly made 4% formaldehyde in PBS (140 mM NaCl, 2mM KCl, 1.4mM KH₂PO₄, 8.1mM Na₂HPO₄, pH 7.5) containing 10% sucrose. This was followed by two washes in PBS+10% sucrose. After these washes, one forewing and hindwing from the same individual were incubated in a fresh solution of PBS+10% sucrose containing either 5mM L-DOPA or 5mM Dopamine (SIGMA). As a control, the other set of wings from the same individual were incubated in the same buffer but without the added precursors.

Vein Cutting

Within one hour after molting, the subcostal and radial-medial veins of forewings and the radial and medial veins of hindwings were cut using scissors. The wings were then left attached to the body for 3–4 hours or 24 hours, after which they were removed for observation.

RESULTS

Functions of tyrosine hydroxylase (*TH*), dopa decarboxylase (*DDC*) and *laccase 2* (*lac2*) in production of black pigmentation in *Oncopeltus*

The body and wings of *Oncopeltus* display alternating black and orange coloration (Fig. 1B1–B2). The head is characterized by a V-shaped orange stripe flanked by black pigmentation on its dorsal surface (Fig. 1B3). The dorsal plates of the thoracic segments are primarily black, with the exception of the lateral edge on the prothorax (T1) and the posterior tip of the mesothorax (T2) that are orange (Fig. 1B4). From the lateral side, the thoracic plates are almost completely black (Fig. 1B5). In contrast, the abdomen is mainly orange with several rectangular black patches on the ventral side (Fig. 1B6). In order to examine whether the melanin pathway is involved and to what degree it is generating the black wing and body pigmentation in *Oncopeltus*, we performed a functional analysis (via RNAi) on the core enzymes in the pathway.

First, we studied the role of *TH*, the enzyme catalyzing the initial step of the entire melanin pathway. Of the 44 injected bugs, 32 survived to adulthood and the most representative phenotype is illustrated in Fig. 2B. The *TH* RNAi adults display a complete absence of black pigmentation (Fig. 1C1). Specifically, the distinct black patterns that are present in WT are now completely lost in both pairs of wings (Fig. 1C2), head (Fig. 1C3), thorax (Fig. 1C4–C5), and abdomen (Fig. 1C6). These results establish that all of the black pigmentation in *Oncopeltus* is generated solely from the melanin pathway.

Since DOPA and Dopamine are believed to be the two direct precursors of black melanin (Wright 1987; Walter et al. 1991), we examined their individual contributions in *Oncopeltus*. As described in Fig. 1A, *DDC* converts DOPA to Dopamine, hence knocking down this enzyme should cause the irreversible loss of Dopamine. Consequently, any black coloration observed in *DDC* RNAi adults is solely produced by DOPA. As shown in Fig. 1D1–D6, *DDC* RNAi individuals display a severe reduction in black coloration, which

encompasses the whole body. This was observed in all of the 33 surviving adults. Such result shows that Dopamine is the main contributor to black pigmentation in *Oncopeltus*, whereas the remaining grayish coloration can be attributed to DOPA.

The above observations confirm that the melanin precursors contribute to black pigments in *Oncopeltus*. However, these precursors still need to be oxidized into quinones, which are in turn incorporated into the cuticle matrix (Andersen 2010). The *lac2* gene, a phenol oxidase, was reported to be critical in completing the cuticular melanization process in several insect species (Arakane et al. 2005; Niu et al. 2008; Tomoyasu et al. 2009; Elias-Neto et al. 2010; Futahashi et al. 2011; Riedel et al. 2011). To determine its function in *Oncopeltus*, we performed *lac2* RNAi experiments (Fig. 1E1–E6). Out of 32 injected individuals, 19 survived into adults. While 14 of them could not molt completely, they could still live for 2–3 days allowing us to remove most of the residuals of exoskeleton to analyze the body and wing pigmentation (Fig. 1E1). These individuals display an absence of black pigmentation across the whole body (Fig. 1E2–E6), confirming that *lac2* is essential for melanization in this species as well. Tyrosinase (TYR), another phenol oxidase present in insects, is also believed to be involved in the melanization process (Andersen 2010). To test the role of this additional phenol oxidase in *Oncopeltus* body melanization, we performed RNAi experiments. Our results showed identical black pigmentation patterns as observed in wild type (Fig. S3), indicating that it is *lac2*, and not TYR, which is the essential phenol oxidase that governs cuticular melanization in this species.

In addition to significantly affecting black pigmentation in RNAi adults, the depletion of *TH*, *DDC*, or *lac2* (confirmed by our RT-PCR control, as shown in Fig. S2) also causes a slight reduction on the overall orange pigmentation (Fig. 1C1–E6). These observations suggest that the melanin pathway also plays a minor role in the orange pigmentation in *Oncopeltus*.

Wing black pigment patterning requires vein transportation in *Oncopeltus*

Previous conflicting reports make it unclear if the wing veins are essential in the wing melanization process (True et al. 1999; Koch and Nijhout 2002; Reed and Gilbert 2004). To determine the role of veins in wing melanization in *Oncopeltus*, we first determined the dynamics of attaining black pigmentation in freshly molted wild type adults. An hour after eclosion has occurred, these appendages still lack black coloration (Fig. 2A). In contrast, the characteristic orange pattern on the forewings is already present at this stage. The beginning of wing darkening starts three to four hours post eclosion (Fig. 2C1), when both pairs of wings begin to turn grey. The darkening of tissue continues to increase and wings obtain an intense black color within 24 hours (Fig. 2C2). From this stage on, the final black pigmentation levels are reached and wing coloration becomes permanent.

Wing pigment patterning was previously shown to be vein-dependent in *Drosophila* (True et al. 1999), but not in *Heliconius* (Reed and Gilbert 2004). To determine which of the two mechanisms is present in *Oncopeltus* we interrupted the vein transportation by performing surgical cuts on freshly eclosed wings (corresponding to stage depicted in Fig. 2A). The subcostal and radial-medial veins of forewings and the radial and medial veins of hindwings were severed (arrowheads in Fig. 2B). The effects of surgery become noticeable within three

hours, with the affected regions localized in the anterior half of each wing (dotted areas in Fig. 2D1). In forewings, there are two significantly lighter grey regions: a smaller one immediately adjacent to the cut and a larger one encompassing the distal half (compare Fig. 2C1 vs. Fig. 2D1). In a similar fashion, there is a complete loss of grey coloration in the distal region of hindwings delineated by the radial and medial veins. By the 24 hours mark, these affected regions in both forewing and hindwing do become darker – but they never reach the levels of black pigmentation in the unaffected, posterior wing regions (compare dotted areas in Fig. 2D2 to regions immediately posterior to them). These observations show that the vein transportation is critical for completing melanin patterning in *Oncopeltus* wings.

Premature *Oncopeltus* wings can develop black pigmentation *in vitro* by supplying melanin precursors

According to True et al. (1999), prior to the eclosion of wings, the enzymes for black pigmentation are pre-localized in immature wings. In order to determine if such a process is also present in *Oncopeltus*, we performed an *in vitro* induction of wing pigmentation. Wings were dissected from late 5th nymphs and incubated in DOPA or Dopamine. As shown in Figs. 3A, 3B1, and 3B2, wings incubated in buffer alone did not develop any black pigmentation, whereas incubation with either precursor (DOPA or Dopamine) resulted in black pigmentation albeit with different levels of intensity. Wings exposed to DOPA (Fig. 3B1) were significantly lighter than the wings incubated with Dopamine (Fig. 3B2), which is consistent with our *DDC* RNAi observation (Fig. 1D1–D6).

The incubation of *TH* RNAi or *DDC* RNAi wings showed similar levels of melanization when compared to wild type wings (Fig 3C1–D2). On the other hand, the wings from *lac2* RNAi 5th nymphs failed in producing black pigmentation under exposure to either of these two melanin precursors (Fig. 3E1–E2). These observations suggest that the “production genes”, such as *lac2* are essential to oxidize the precursors and need to be pre-localized in premature wings before eclosion in *Oncopeltus*. While the “precursor enzymes” such as *TH* and *DDC* may also be pre-localized, the present data does not provide for a definitive answer in that regard. Together with the previously shown vein-dependent elaboration of melanin substances, these experiments suggest the presence of a two-step wing melanin patterning model in *Oncopeltus*.

DISCUSSION

Black pigment is produced via melanin pathway in *Oncopeltus*

Functional studies in *Drosophila* and *Tribolium* (Wright 1987; Roseland 1987; True et al. 1999; Wittkopp, True, and Carroll 2002; Wittkopp, Carroll, and Kopp 2003; True et al. 2005; Arakane et al. 2005; Arakane et al. 2009; Gorman and Arakane 2010), suggest that the core part of the melanin pathway may be conserved among holometabolous insect species. In both species *TH* triggers the transformation of tyrosine to DOPA and *DDC* transforms DOPA into Dopamine, whereas *PO* is required for the conversion of melanin precursors to cuticular melanins. Our RNAi experiments in *Oncopeltus* show that the depletion of *TH*, *DDC*, and *lac2* causes the loss of black pigmentation, indicating the pivotal roles of these

genes in *Oncopeltus* melanization (Fig. 1C1–E6). In addition, our observations that *TH* RNAi and *DDC* RNAi wings developed black melanin through *in vitro* incubation with melanin precursors whereas *lac2* RNAi wings did not, further support the different positions of these three genes in the melanin pathway. That is, *TH* and *DDC* are “precursor enzymes” that produce substrates for subsequent melanization reactions, whereas *lac2* belongs to “production enzymes” that act on the precursors. Taken together, our findings imply that the basic components of melanin production in insects may have a single common origin.

In spite of the overall progress in understanding the melanization across different insects, the identity of the main precursor of black melanin remains an unresolved issue (Gibert et al. 2007). In the original studies of black pigmentation in *Drosophila*, *DDC* mutants were shown to generate only a low intensity of black pigment (gray), leading to the proposal that Dopamine melanin is the main component of black melanin (Wright 1987; Walter et al. 1996). However, the follow-up analyses emphasized that the contribution of DOPA to black melanin should be equally important as Dopamine (Nijhout, 1991; Johnson et al., 2001; Wittkopp, True, and Carroll 2002; Wittkopp et al. 2003). Of these two possibilities, our *DDC* RNAi study follows the original one, which showed that the contribution of DOPA to black pigment is quite limited and it is Dopamine that generates the majority of black pigments in *Oncopeltus*. While the latter results seem to swing the pendulum toward Dopamine as the main precursor, it will be important to confirm the generality of this insight through the sampling of additional hemimetabolous lineages.

The above discussion mainly focuses on the relationship between the melanin pathway and black pigment production. However, this pathway accounts only for a portion of the extraordinary range of colors that are present in insects. In classically studied aposematic species such as *Precis* and *Heliconius*, the warning colorations are thought to be produced independently from the melanin pathway (Nijhout 1997; Reed and Nagy 2005; Ferguson and Jiggins 2009). Our observations in *Oncopeltus*, showing that *TH*, *DDC*, or *lac2* RNAi cause a very subtle reduction in orange coloration (Fig. 1C1–E6), indicate that the melanin pathway plays only a minor role in orange pigmentation. Since the majority of orange color is still present in these RNAi individuals, the bulk of this pigment is generated by other pigmentation pathways. One possible component might be ommochrome, as reported in butterflies (Nijhout 1997; Reed and Nagy 2005; Ferguson and Jiggins 2009) and red dragonflies (Futahashi et al. 2012). Another candidate is carotenoid, as indirectly suggested by studies in the pea aphid, *Acyrtosiphon pisum* (Moran and Jarvik 2010; Novakova and Moran 2012). However, these studies were only able to correlate either of the two proposed candidates to orange/red regions of the insect body without providing direct functional data showing a causal effect. Thus, a follow up step in further developing *Oncopeltus* as a model for aposematic coloration would be to utilize the RNAi approach to determine the origin of residual orange pigments. This would significantly expand our overall understanding of the diversity of insect pigmentation and the genetic mechanisms responsible.

Wing melanin patterning in *Oncopeltus* is processed via a two-step model

Our current understanding of the mechanism regulating insect wing pigment patterning is restricted to *Drosophila*, on which the proposed two-step model is based (True et al. 1999).

The first step involves the early pre-patterning of melanin enzyme activity. The second step is the vein-dependent transportation of melanin substances. In our study, the existence of the first step in *Oncopeltus* is supported by the fact that pre-mature wings can develop black pigmentation when melanin precursors are provided exogenously (Fig. 3). Confirmation of the second step is provided by our observations from the vein-cutting assay, showing that the melanization in areas of the wing downstream of the cut locations is affected by the physical damages to the veins (Fig. 2). When combined with insights from *Drosophila*, our results indicate that the two-step model may be shared between holometabolous and hemimetabolous species.

The previous studies in *Drosophila* and *Heliconius* provided conflicting views on the actual significance of vein transportation in wing melanization (True et al. 1999; Reed and Gilbert 2004). As illustrated in Fig. 2D2, the vein cutting assay clearly confirms that vein transportation is required for melanin patterning in *Oncopeltus* wings. We also observe that on pre-melanized *Oncopeltus* wings, black bristles are already present (Fig. S4). A similar result was reported in *Drosophila* vein mutants (True et al. 1999), suggesting not only that bristle and wing tissue melanization are separated – but also that the former process is generated by a vein-independent mechanism. The presence of such a mechanism in *Heliconius* could provide for an alternative explanation of the situation observed in this species, in which vein deficiency did not seem to affect melanin patterns. Assuming that the black wing coloration in butterflies is comprised solely of black scales (Janssen et al. 2001), which are reported to be modified bristles (Galant et al. 1998), then such observations may result from bristle melanization. Therefore, two different processes might co-exist during the wing melanin patterning in insects: bristle melanization, which is vein transportation independent and wing tissue melanization, which requires vein transportation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

Functions of *TH*, *DDC*, and *lac2* in *Oncopeltus* adults. (A) The summary consensus view of melanin biosynthesis pathway in *Drosophila* (Wright 1987; Wittkopp, Carroll, and Kopp 2003). (B1–B6) Wild type *Oncopeltus* shows alternating black-orange patterning. The distinct black patterns are established in the wings (B2), head (B3), dorsal thorax (B4), lateral thorax (B5), and ventral abdomen (B6). (C1–C6) *TH* RNAi adults show a complete loss of black pigmentation in the whole body. (D1–D6) *DDC* RNAi adults display a significant reduction of black coloration. (E1–E6) *lac2* RNAi adults show that black pigmentation is abolished. The black legs and antennae are the residuals of un-removed exoskeleton. Scale bars: 1mm (B1, B2, C1, C2, D1, D2, E1, E2); 500μm (B3–B7, C3–C7, D3–D7, E3–E7). Abbreviations: FW, forewing; HW, hindwing; T1, prothorax; T2, mesothorax.

Fig 2.

Black patterning of *Oncopeltus* wings is vein dependent. (A) Freshly molted adults have an alternating orange-white forewing and colorless hindwing. (B) The basic outline of veins, with cut sites represented by black arrowheads. The names of the veins are originated from Nivedita (1982). (C1–C2) The appearance of untreated wings 3–4 hr and 24 hr after molting, respectively. (D1–D2) Forewing with subcostal and radial-medial veins and hindwing with radial and medial veins surgically cut have black patterns disrupted in the subregions distal to the cutting sites (dotted areas). Such disruptions cannot be completely rescued even after 24 hrs, indicating that the black patterning process of *Oncopeltus* wings requires vein transportation. Scale bars: 1mm. Abbreviations: FW, forewing; HW, hindwing; Sc, subcostal; R, radial; M, medial; Cu, cubital.

Fig 3.

Pre-mature *Oncopeltus* wings can develop black pigmentation with exogenously supplied melanin precursors. All wings were dissected from late 5th nymphs. (A) Wings incubated in buffer without melanin precursors were used as a background control. (B1) Wings incubated in DOPA develop a low level of black pigment. (B2) Wings incubated in Dopamine develop a much stronger black pigmentation than the ones incubated in DOPA. (C1–D2) Similar results can be observed in *TH* RNAi and *DDC* RNAi wings. (E1, E2) *lac2* RNAi wings failed to generate any black pigment when incubated with DOPA or Dopamine. Scale bars: 500µm. Abbreviations: FW, forewing; HW, hindwing.