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## From *Aedes* to *Zeugodacus*: A review of dipteran body coloration studies regarding evolutionary developmental biology, pest control, and species discovery

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### Abstract

Over the past two decades, evo-devo (evolution of development) studies have elucidated genetic mechanisms underlying novel dipteran body color patterns. Here we review the most recent developments, which show some departure from the model organism *Drosophila melanogaster*, leading the field into the investigation of more complex color patterns. We also discuss how the robust application of transgenic techniques has facilitated the study of many non-model pest species. Furthermore, we see that subtle pigmentation differences guide the discovery and description of new dipterans. Therefore, we argue that the existence of new field guides and the prevalence of pigmentation studies in non-model flies will enable scientists to adopt uninvestigated species into the lab, allowing them to study novel morphologies.

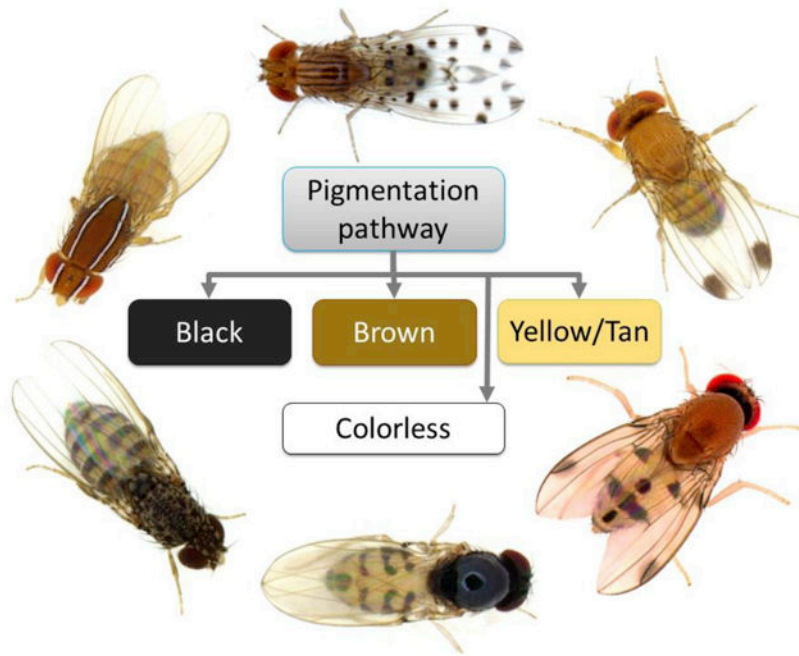
### Graphical abstract

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## Keywords

Diptera; non-model species; color patterns; pigmentation; pigmentation genes; pest control; species discovery; wing patterns; head patterns; thorax patterns; abdominal patterns; Evo-Devo; drosophilids; tephritids; field guides

## Introduction

Diptera, or the “true flies”, is an order of holometabolous insects set apart by their single pair of wings and set of flight-stabilizing halteres [1,2]. With regards to investigating body coloration, the most well-studied dipterans belong to the family Drosophilidae, which includes the “common fruit fly” (Figure 1). Attempts to understand the evolution of color pattern development in *Drosophila* (*D.*) have persisted for decades. In this review, we will discuss the ongoing research in not only “common fruit flies”, but the order Diptera as a whole. One early focus of dipteran evo-devo pertained to simple color patterns, like that of *D. melanogaster*. Today, we investigate body coloration across an array of genera and species. Recently, there appears to be a major trend in the use of the CRISPR/Cas9 system to disrupt pigmentation to develop pest control methods. Furthermore, the critical analysis of color patterns has led to the identification of new species. Here we review the recent literature on dipteran coloration and provide our opinion on how the field should move forward.

## The evolution of dipteran color patterns

Dipterans exhibit a multitude of color patterns; however, the bulk of recent literature pertains to the genus *Drosophila*. Recent studies in these “common fruit flies” examined complex abdominal, wing, and thoracic color patterning. We also note the exhibition of new methods

and tools with the potential to impact the field of evo-devo and a paradigm shift in our understanding of pigment biosynthesis. Finally, we see that a complex color pattern outside of the genus *Drosophila* is thoroughly analyzed, a progression welcome in the field.

### Abdominal color patterns

The myriad of abdominal color patterns seen in *Drosophila* species, ranging from full-body pigmentation to intricate combinations of spots and stripes, have inspired investigators to study how these novel morphologies emerged [3,4]. The evolutionary divergence of coloration between the pale-yellow colored *D. novamexicana* and the brown *D. americana* was shown to have occurred, at least in part, from differences in the *ebony* alleles between the species [5\*]. Sramkoski *et al.* (2020) examined the intraspecific pigmentation of *D. americana*. They noted that populations found in the eastern United States display a darker body coloration than the western populations, and that this progressively lighter body coloration seen in western *D. americana* results in a pigmentation phenotype that resembles *D. novamexicana*. Sramkoski *et al.* (2020) suggested that allelic similarities in *ebony* and *tan* between lightly colored *D. americana* morphs and *D. novamexicana* might underly the pigmentation cline seen in *D. americana*. However, this hypothesis was not supported [6].

The *quinaria* group's 26 species [7] display an impressive array of pattern elements. Dion *et al.* (2020) demonstrated that the co-expression patterns of three key pigmentation genes, *Dopa decarboxylase*, *tan*, and *yellow*, prefigure the spot patterning of three *quinaria* species group members: *D. guttifera*, *D. palustris*, and *D. subpalustris* [8]. *D. guttifera* was further examined by KKB Raja *et al.* (bioRxiv doi: [10.1101/2020.04.09.034900](https://doi.org/10.1101/2020.04.09.034900)), who showed that a *cis*-regulatory element in the *yellow* locus drove the expression of a reporter construct in a pattern that resembled the adult abdominal pigmentation. They also showed correlational *in situ* hybridization data indicating that the toolkit genes *wingless*, *decapentaplegic*, *hedgehog*, *abdominal-A*, and *zerknüllt* may play a role in the formation of the abdominal color pattern. Kalay *et al.* (2019) focused on the *cis*-regulatory capabilities of sequences derived from the 5' intergenic and intronic regions of the *yellow* gene in *D. melanogaster*, *D. pseudoobscura*, and *D. willistoni*. They observed reporter gene expression patterns driven by redundant and cryptic *cis*-regulatory elements, the latter being a sequence with the ability to regulate gene expression alone but not in its naturally occurring position in the genome. They suggested that the observed *cis*-regulatory complexity may underlie the *yellow* gene's wide range of expression patterns within the genus *Drosophila* [9].

Sexually dimorphic pigmentation is widespread across *Drosophila*, a phenomenon that Hughes *et al.* (2020) proposed to have emerged through parallel evolution [10]. Roeske *et al.* (2018) demonstrated that in female *D. melanogaster*, *bab* represses posterior coloration by binding to a *cis*-regulatory element (*body element*) of *yellow* required for the male-specific pigmentation [11]. The development of this male-specific coloration was also shown to require the activity of the gene *rainy head* [12]. The female-specific pigmentation of this fly was shown to be controlled by a gene-regulatory network that is thermally sensitive. *Abdominal B* and (female-specific) *doublesex* initiate the expression of *bab*, a gene with a role in the development of sexually dimorphic body coloration [13], which dials down the activity of three pigmentation genes: *Dopa decarboxylase*, *tan*, and *yellow*. De Castro

*et al.* (2018) demonstrated that higher temperatures yielded increased levels of *bab*, which resulted in reduced pigmentation. They further suggested that *Abdominal B*, a gene whose product has an established role in *Drosophila* pigmentation [14,15], may turn on *tan* expression at lower temperatures [16]. *Abdominal B* was further investigated by Liu *et al.* (2019), who demonstrated that untangling the evolution of a gene-regulatory network is not straightforward. *Abdominal B* facilitates dark body coloration in *D. yakuba*, and modifying its expression was shown to not alter the body pigmentation of the sister species *D. santomea* (whose absence of abdominal pigmentation resulted from an evolutionary loss of *tan* and *yellow* expression [17]) [18]. The morphological diversity of *Drosophila* abdominal coloration continues to be a fruitful resource for investigating the development of novel traits.

### Wing pigmentation

Attempts to understand the development of dipteran wing coloration has been ongoing for more than 20 years [19]. Recent studies of *Drosophila* and *Samoaia* progressed our understanding of how wing color patterns evolved. The gene *wingless* is an established coordinator in the development of wing pigmentation in *D. guttifera* [20,21]. Fukutomi *et al.* (2020) further demonstrated that *wingless* is a key regulator of 151 genes associated with the color pattern on the wings of this polka-dotted fruit fly [22\*]. Massey *et al.* (2020) suggested that the X-chromosomal gene *optomotor-blind* possibly underlies the disparity of pigmentation between two sibling species, *D. elegans* (which has a male-specific wing spot) and *D. gunungcola* (which lacks a wing spot) [23]. The male-specific spot seen in *D. biarmipes* was also the subject of a recent study. CC Galouzis *et al.* (bioRxiv doi: [10.1101/2020.03.23.003103](https://doi.org/10.1101/2020.03.23.003103)) showed that homologous alleles for the X-chromosomal *yellow* gene interact with each other to inactivate the regulatory element (*spot* enhancer) responsible for this trait in females. They further noted that the intron of *yellow* and the protein Mod(mdg4) are required for this function to occur.

The expression of *engrailed* is normally located only in the rear section of the early developing fly wing to dictate posterior wing identity. However, *Engrailed* also plays a role in the development of the wing spot seen in *D. biarmipes* [24]. Dufour *et al.* (2020) suggested that *engrailed* was co-opted into the development of wing pigmentation in *Samoaia leonensis*, a process that occurs after the development of the basic wing morphology. This co-option results in a complex wing color pattern; *engrailed* represses pigmentation in specific areas of an ancestrally black wing, resulting in many irregular light areas spread across the wing surface. Dufour *et al.* (2020) further proposed that *engrailed* gained new temporal and spatial domains of expression with an onset after its vital developmental role in posterior wing identity [25\*\*]. This study provided valuable insights into how toolkit genes can gain novel domains of expression without interrupting their other, essential roles in organismal development.

### Thoracic trident of *D. melanogaster*

One other specific pigmentation feature, the thoracic trident of *D. melanogaster*, inspired recent studies. Gibert *et al.* (2018) suggested that the expression pattern of *stripe* - a gene involved in flight muscle attachment and also the repression of pigmentation -

caused the emergence of this unique color pattern as a consequence of its co-option into a pigmentation gene-regulatory network [26]. Endler *et al.* (2018) showed that single-nucleotide polymorphisms within the *tan* male-specific enhancer and a missense mutation in *CG15370* (a gene upstream of *tan*) have a high impact on trident pigmentation [27]. Based on the discovery that alterations to the regulation of *ebony* impacted the abdominal coloration of *D. melanogaster* [28], Telonis-Scott and Hoffmann (2018) suggested that *ebony* enhancer variation, in part, underlies the diversity in color intensity of the thoracic trident pattern [29].

### Methods and pigment biosynthesis

Besides “common fruit flies” being at the center of experiments that progressed our understanding of the molecular mechanisms underlying the evolution and development of body color patterns, we see techniques relevant to evo-devo designed and refined through the use of both model and non-model *Drosophila* species, such as a method to investigate enhancer-promoter interactions [30], a method to produce transgenics in a much more delicate “common fruit fly”, *D. guttifera* [31\*], and the robust application of the Oxford Nanopore sequencing technology [32]. Also, it is now widely accepted that black pigment is produced by Yellow from dopamine, as opposed to dopa (Figure 2) [8,10,33,34]. These studies show that our evo-devo toolkit and our understanding of pigmentation continue to develop.

### Head pigmentation in a tephritid species

One interesting study, notably outside of the genus *Drosophila*, investigated a complex spot pattern crowning the head of *Bactrocera dorsalis*. Bai *et al.* (2019) demonstrated that the knockout of the *white* gene partially erased this dark pigmentation pattern. These *white* mutants had lower expression levels of *yellow* (*Bd-yellow1* specifically), prompting the authors to speculate that a regulatory relationship between *white* and *yellow* may exist, where White possibly facilitates the transport of secondary messengers that affect transcription factor activity and gene expression [35\*\*]. This study clearly demonstrated the value of incorporating diverse fly species into our efforts to understand both the architecture of gene-regulatory networks and the evolution and development of complex color patterns.

### Pigmentation as a target for pest control

Applying our understanding of the underlying molecular pathways and adapted molecular techniques for model and non-model species has allowed for advancements in translational research. Mosquitoes are an established disease vector, and one method to manage their populations could be through manipulating their pigmentation development. Pigmentation studies in *Aedes albopictus* demonstrated that ovary-specific genes of the *yellow* family have been found to play roles in both melanization timing and proper development of the chorion [36]. CRISPR/Cas9 knockouts of *kynurenine hydroxylase* and *yellow* in *Aedes albopictus* demonstrated the feasibility of genetic manipulation in this mosquito [37], and RNAi knockdown of *Laccase 2* in *Anopheles sinensis* identified a target that could facilitate the control of this mosquito population [38]. Juvenile hormone receptors are integral to insect development. Zhu *et al.* (2019) knocked out the gene *methoprene-tolerant* encoding

one of these receptors in *Aedes aegypti* through the use of CRISPR/Cas9. This knockout led to black third-instar and fourth-instar larvae that eventually died before reaching the pupal stage [39].

In *Cochliomyia hominivorax* and *Lucilia cuprina*, two flesh-eating parasitic flies in livestock, *yellow* plays a key role in the development of dark adult body coloration, and the gene was targeted to prove the viability of the CRISPR/Cas9 system in these insects. Taking it one step further beyond pigmentation, Paulo *et al.* (2019) then knocked out the gene *transformer* in *C. hominivorax*, which disrupted reproductive organ development and demonstrated a possible avenue for pest control [40]. Additionally, the *brown body* locus in *Musca domestica* was shown to be orthologous to the *yellow* gene in *Drosophila* through the use of CRISPR/Cas9 [41].

The Tephritidae, or the “true fruit flies”, are a large and invasive dipteran family at the center of recent studies of genetic manipulation. CRISPR/Cas9 facilitated the first successful manipulation of the gene *scarlet* in *Bactrocera oleae* [42]. In *Anastrepha ludens*, a previously unidentified mutation affecting both pupal and adult pigmentation, *slow larvae*, was identified [43]. Chen *et al.* (2018) and Zhang *et al.* (2019) demonstrated that the pigmentation gene *tyrosine hydroxylase* (a.k.a. *pale*) plays an important role in the pupal pigmentation (tanning) of *Bactrocera dorsalis* [44] and *Zeugodacus tau* [45], respectively. These studies focused primarily on pest control; however, their contributions to refining transgenic techniques in non-model organisms and their data regarding the roles of key pigmentation genes are a boon to the field of dipteran color pattern evo-devo.

## The identification and characterization of new species and traits

A new species, *D. carrolli*, isolated from Brunei has been recognized. Some defining characteristics that separated this species from the closely related *D. rhopaloea* included unique body and wing pigmentation traits [46\*]. Additionally, wing pigmentation supported the identification of five previously unknown species of *Culicoides* [47], and differences in thoracic pigmentation helped to distinguish a new member of the genus *Corethrella* from an established species [48].

While body pigmentation is easily visible to the human eye, wing interference patterns require multispectral digital imaging to be properly visualized. Hawkes *et al.* (2019) suggested that these structural colors in the wings of *D. simulans* play a role in sexual selection [49], and NJ Butterworth *et al.* (bioRxiv doi: [10.1101/2020.02.18.948646](https://doi.org/10.1101/2020.02.18.948646)) observed species-specific and sexually dimorphic wing interference patterns in species of the genus *Chrysomya*.

As a discipline, evo-devo is incomplete without field work. Field guides and references providing high-quality images and accurate descriptions of species across the order Diptera have recently become available [50–52, 53\*, 54\*]. We hope that these guides facilitate the identification and investigation of rarely studied flies.

## Conclusion

The advancement in our understanding of dipteran body color evo-devo, though impressive, is mainly localized to well-characterized *Drosophila* species. We propose two directions for the field: First, we hope to see an even greater incorporation of non-model *Drosophila* species into evo-devo research. The availability of new, comprehensive field guides coupled with broadly applicable methods for genetic manipulation will facilitate the broader exploration of novel morphologies - a scenario that should excite every naturalist. Second, we propose that in-depth studies of body color patterns displayed by the family Tephritidae should be pursued. Recent literature demonstrated that genetic manipulation is practical in this dipteran family and that novel gene-regulatory architectures underlying pigmentation development may exist. We believe that the broader inclusion of non-model *Drosophila* and the “true fruit flies” into a field dominated by well-studied “common fruit flies” is a vital step towards understanding the evo-devo of very complex dipteran body color patterns.

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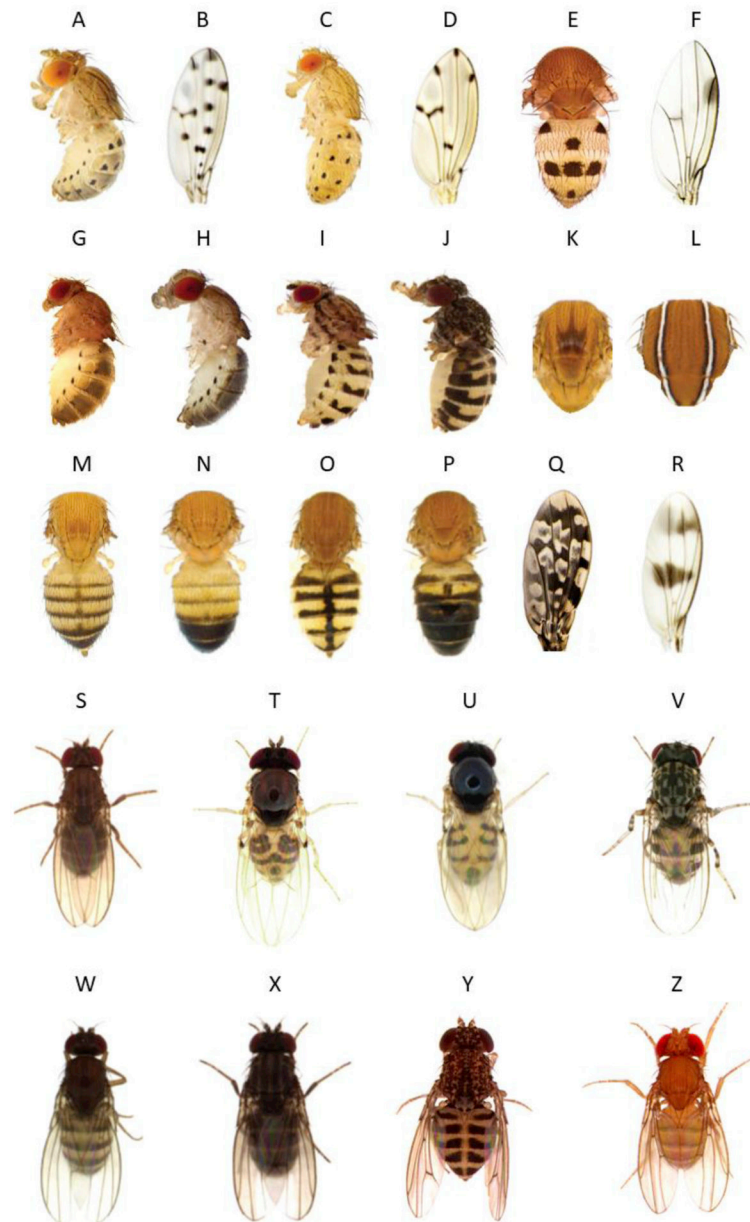
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**Figure 1: Drosophilid body coloration.**

Here, we see just a fraction of the diverse body color patterning exhibited within the order Diptera. Some of these unique morphologies are the subjects of recent investigations detailed within this review. However, many other traits shown here are not mentioned in the literature, and their developments deserve to be investigated. Row 1: (A, B) *D. guttifera*, (C, D) *D. deflecta*, and (E, F) *Leucophenga varia*. Row 2: (G) *D. palustris*, (H) *D. subpalustris*, (I) *D. busckii*, (J) *D. hydei*, (K) the thoracic trident of *D. melanogaster*, and (L) the striped thorax of *Zaprionus indianus*. Row 3: (M) *D. melanogaster* (female), (N) *D. melanogaster* (male), (O) *Hirtodrosophila duncani* (female), (P) *Hirtodrosophila duncani* (male), (Q) *Samoia leonensis*, and (R) *Chymomyza amoena*. Row 4: (S) *D. americana*, (T) *Mycodrosophila claytonae*, (U) *Mycodrosophila dimidiata*, and (V) *Phortica variegata*. Row

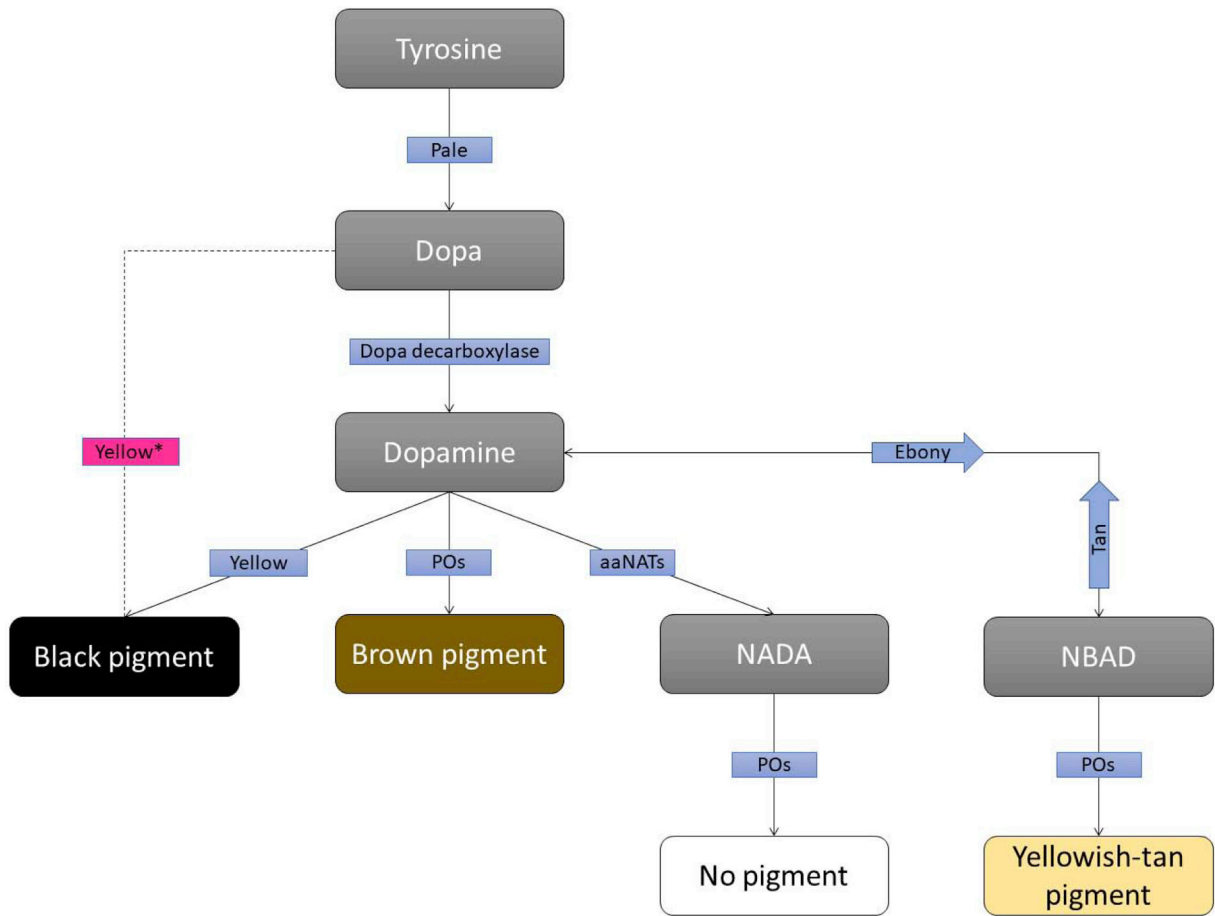
5: (W) *D. macrospina*, (X) *D. borealis*, (Y) *D. peninsularis*, and (Z) *D. ananassae*. Images are from [53\*, 54\*].

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**Figure 2: The pigmentation biosynthesis pathway.**

The previously understood role of Yellow (Yellow\*) is shown by a dashed line. POs are phenol oxidases, aaNATs is arylalkylamine *N*-acetyl transferases, NADA is *N*-acetyl dopamine, and NBAD is *N*- $\beta$ -alanyl dopamine. Figure adapted from [8,10,33,34].