# The molecular basis of melanism and mimicry in a swallowtail butterfly

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**Melanism in Lepidoptera, either industrial or in mimicry, is one of the most commonly cited examples of natural selection [1,2]. Despite extensive studies of the frequency and maintenance of melanic genes in insect populations [1,2], there has been little work on the underlying molecular mechanisms. Nowhere is butterfly melanism more striking than in the Eastern Tiger Swallowtail (*Papilio glaucus*) of North America [3–5]. In this species, females can be either yellow (wild type) or black (melanic). The melanic form is a Batesian mimic of the distasteful Pipevine Swallowtail (*Battus philenor*), which is also black in overall color. Melanism in *P. glaucus* is controlled by a single Y-linked (female) *black* gene [6]. Melanic females, therefore, always have melanic daughters. Black melanin replaces the background yellow in melanic females. Here, we show that the key enzyme involved is N-β-alanyl-dopamine-synthase (BAS), which shunts dopamine from the melanin pathway into the production of the yellow color pigment papiliochrome and also provides products for cuticle sclerotization. In melanic females, this enzyme is suppressed, leading to abnormal melanization of a formerly yellow**



**sclerotization (maturation) or, in contrast, that the delay in scale maturation precludes expression of BAS at the correct stage. Together, these data show how changes in expression of a single gene product could result in multiple wing color phenotypes. The implications for the genetic control of mimicry in other Lepidoptera are discussed.**

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## Results and discussion

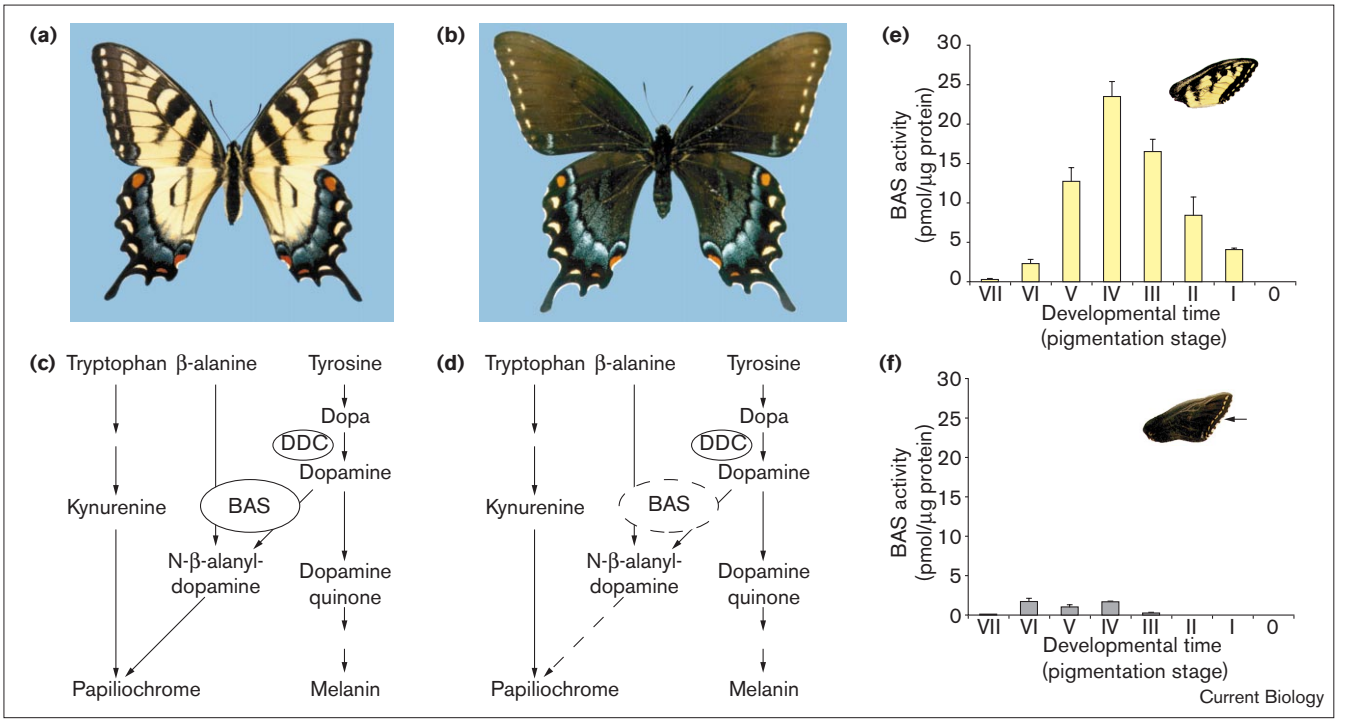
### Sex-linked melanism and the role of dopa decarboxylase in the wild-type pattern

In swallowtail butterflies, females can be either wild type (yellow and black) or melanic (the yellow background is replaced by dark melanin; Figure 1a,b). The yellow and orange colors are formed by papiliochromes [7,8], a pigment group unique to the Papilionidae, and black is formed by melanin. During wing development in the pupa, the yellow and orange colors are deposited first and then the surrounding areas are later melanized black [9]. We have shown previously that both melanin and papiliochrome partly stem from a common biochemical pathway [9]. Thus, dopa decarboxylase (DDC) catalyzes the conversion of dopa to dopamine, which is a precursor for both melanin and papiliochrome synthesis (Figure 1c). As dopamine is required first for the formation of papiliochrome and then later for melanin, the *Ddc* gene is expressed early in presumptive yellow areas and then later only in presumptive black areas [9]. This inversion of *Ddc* expression explains how a given tissue becomes either yellow or black, during the course of wild-type development, when the synthetic pathways for both colored pig-

### BAS effects the switch to the melanic mutant

In *Drosophila*, null mutants of the gene *ebony* show black body phenotypes [10] because of the failure of BAS to catalyze the conversion of dopamine to N-β-alanyl-dopamine (NBAD). Lack of NBAD, which is required for the production of the normally tan-colored cuticle, leads to an excess of dopamine, which results in abnormal cuticle melanization. In *Papilio*, we predicted that BAS would also play a key role in shunting dopamine out of the melanin pathway into the papiliochrome-specific part of pigment biosynthesis (Figure 1c). Therefore, lack of BAS activity throughout most of the wing could be the key switch between the wild-type and melanic patterns (Figure 1d). We developed an assay for BAS activity in wing homogenates of wild-type and melanic forewings by measuring the incorporation of $^{14}$C-β-alanine into NBAD, separating the $^{14}$C-β-alanine label from the synthesized $^{14}$C-NBAD by high performance liquid chromatography (HPLC) [11]. In wild-type wings, BAS activity showed a peak at developmental stage IV (Figure 1e), which corresponds to the peak of papiliochrome synthesis, whereas, in melanic wings, there was almost a complete absence of BAS activity (Figure 1f). In these melanic wings, the low level of BAS activity recorded is

**Figure 1**




Suppression of BAS activity in melanic *P. glaucus*. **(a)** Wild-type female with normal yellow and black pattern. **(b)** Melanic female in which most of the yellow background is replaced with black. Note the distal row of yellow marginal spots that remain. **(c)** Wild-type biochemical pathway for the formation of yellow papiliochrome and black melanin. Note how dopamine is a precursor for both color pigments but that BAS selectively channels dopamine into papiliochrome synthesis. **(d)** Melanic pathway in which BAS activity is suppressed in a tissue-specific fashion (as indicated by the dashed arrows), leading to a failure to produce papiliochrome in the central areas of the wing. The corresponding area is later melanized black. **(e,f)** BAS activities in wild-type versus melanic forewings at different developmental times (stages of wing pigmentation). Note that, in (e) wild-type wings, BAS activity peaked at developmental stage IV, whereas, (f) in melanic wings, BAS activity was almost absent. The residual activity in (f) is derived only from formation of the remaining yellow marginal spots (arrow).

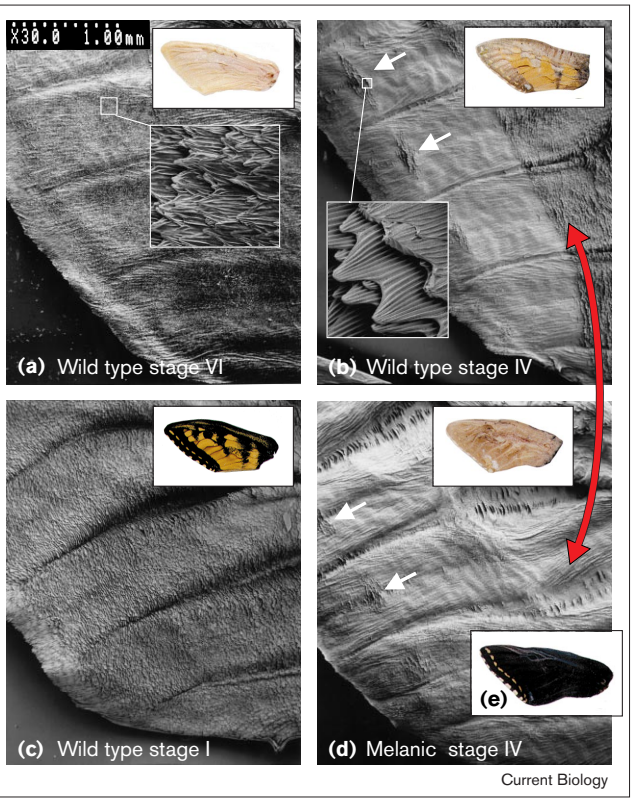
probably associated with the remaining row of distal yellow spots still present in the mutant pattern (Figure 1f, arrow).

The observation that suppression of a single enzyme is involved helps us understand how this phenotype could be under control of a single Y-linked gene. Nevertheless, we note that the changes involved in melanism are not only sex specific but also pattern specific, as not all of the butterfly becomes black. Melanic forms of *P. glaucus* therefore cannot be simple BAS null mutants like some *ebony* strains of *Drosophila*. We therefore speculate that, in *Papilio*, the melanic gene itself (the *black* gene [6]) must be able to suppress BAS activity in both a cell- and sex-specific fashion, perhaps in response to the dosage of a Y-linked factor. This hypothesis of cell-specific suppression was supported by an examination of individual specimens that are 'gynandromorphs', which show a mosaic of male and female wing tissues (see cover illustration). When the female tissues were melanic, a mosaic of melanic (dark brown, female) and wild-type (yellow and black, male) tissues were seen in the same specimen. This supports our belief that control of the melanic gene is both sex specific and cell autonomous.

### Scale maturation is delayed in the melanic mutant

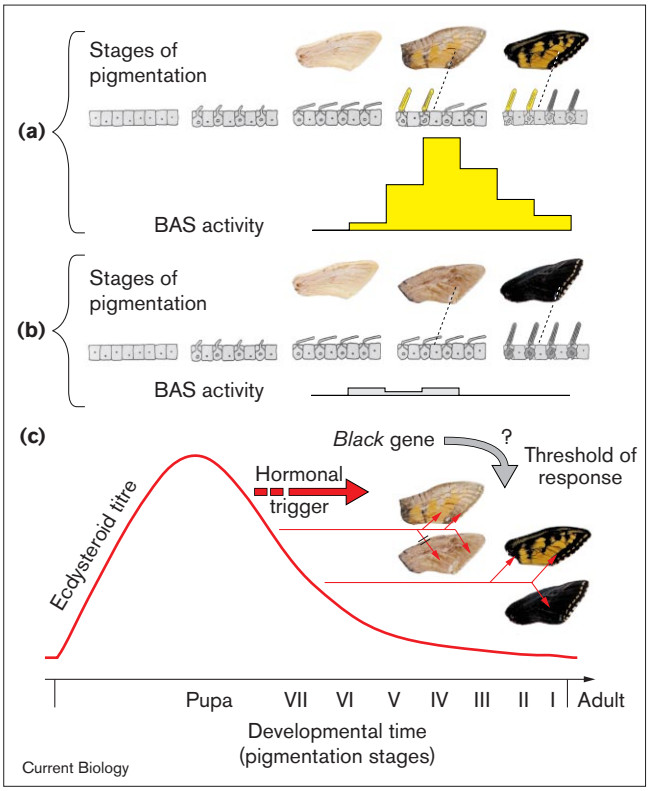
We also investigated changes in the rates of scale maturation in the melanic mutant. Pigment synthesis in butterfly wing scales proceeds in a fixed manner, with colored pigments (in this case, papiliochromes) being laid down first and the remaining areas then being melanized black or brown. By air-drying developing wings excised from the pupa and then sputtering them with gold, the developmental state of wing-scale cells could be readily assessed using scanning electron microscopy. Thus, scales that had finished pigmentation and sclerotization (maturation) remained erect, whereas those that had yet to mature collapsed (Figure 2). Therefore, early in scale maturation, when no pigment was visible, all the scales in the wing collapsed upon air-drying (Figure 2a). In the development of the wild-type color pattern, the presumptive yellow areas (corresponding to the central wing areas and the row of yellow marginal spots) matured first (Figure 2b) then, later, the surrounding black areas matured (Figure 2c). In contrast, in the melanic mutant, the scales in the presumptive yellow area of the central forewing failed to mature early (Figure 2d) and, instead, sclerotized late, at

**Figure 2**




Melanism is correlated with a delay in the maturation of wing scales normally destined to be yellow. **(a)** Scanning electron micrograph (SEM) of a wild-type wing at developmental stage VI. At this early stage, there was no visible coloration on the wing (see inset) and, after air-drying, all the scale cells collapsed (see SEM magnification) as they were soft and unpigmented. **(b)** Wild-type wing at stage IV, which was the peak of yellow (see inset) papiliochrome synthesis (cross reference with Figure 1e). At this later stage, scales destined to be yellow (white arrows indicate yellow spots on the wing margin) were pigmented and remained erect after air-drying (see inset). **(c)** Wild-type wing at stage I, the stage before eclosion. In this final stage, all the scales, both yellow and black (see inset), were pigmented and remained erect. **(d)** Melanic wing at stage IV. Note that, compared with the wild-type wing at the same stage, the presumptive yellow scales of the central wing (red arrow linking panels b and d) had failed to become pigmented and remained soft and collapsed after air-drying, whereas the scales in the yellow marginal spots (white arrows) had become pigmented. **(e)** Melanic wing just before eclosion (stage I) showing final colouration. This figure shows that melanism is correlated with a delay in maturation of scales originally destined to be yellow, which are then abnormally melanized at the same time as those in the black pattern.

**Figure 3**




Diagrams relating the differences in BAS activity between wild-type and melanic *P. glaucus* to the falling ecdysteroid hormone titre that occurs before pupal eclosion. **(a)** Wild-type pattern. Yellow scales mature first, correlating with a peak in BAS enzyme activity and accompanying papiliochrome (yellow) synthesis. The scales in the black pattern are then melanized black. **(b)** Melanic pattern. The presumptive yellow scales fail to mature, show low BAS activity and fail to make papiliochrome. These scales are then abnormally melanized black at the same time as the normal black pattern. **(c)** Model showing how the timing of colour pigment synthesis might be linked to the falling ecdysteroid hormone concentration in the pupa before eclosion. In this model, papiliochrome synthesis is triggered early, when the hormone concentration is high; later, when the hormone concentration is low, melanization occurs. We speculate that the *black* gene interferes with this process by modulating the response of presumptive yellow scales to the falling hormone titre. One mechanism whereby such a change could occur is through differential display of hormone receptors (see text for discussion).

the same stage as those that were melanized black. The net result was that scales in an area originally destined to be yellow matured in the wrong time-frame and were melanized black or dark brown (Figure 2e). This failure of the melanic scales to mature early, and the corresponding lack of papiliochrome synthesis (Figure 3a,b), was documented repeatedly following dissection of several hundred wild-type and melanic female pupae at different developmental stages.

These observations raise two interesting alternatives for the role of BAS in melanism. Firstly, the suppression of BAS activity in the central areas of the wing may lead directly to the delay in scale maturation, as products of BAS activity are also required for scale sclerotization [12]. Alternatively, it may be the delay in scale maturation itself that excludes BAS activity during the appropriate stage of pigment synthesis, leading to the failure of papiliochrome synthesis. In connection with the latter possibility, we note that the timing of pigment synthesis is set against the falling titre of ecdysteroid hormones (Figure 3c) found in the pupa before eclosion [13], perhaps implicating nuclear ecdysteroid hormone receptors as the key triggers for scale

maturation. Therefore, the *black* gene might modulate the hormone concentration at which scale development is initiated by delaying the development of normally yellow scales, which are then atypically melanized black at a later stage in maturation (Figure 3c). Such a change could be effected by a change in display of the associated nuclear ecdysteroid receptors in the presumptive yellow scales.

#### Implications for the evolution of fly and butterfly melanism

To date, studies of butterfly wing-pattern formation have focused on the development of eyespots [14–16] or hindwing–forewing transformation [17,18]. In *Drosophila*, recent genetic studies have shown that the *ebony* gene, which encodes BAS, is important for regulating wing-vein-driven melanic patterns [19]. Although we have shown that BAS is one of the key enzymes suppressed in the melanic phenotype in the swallowtail butterfly, this does not prove that the BAS-encoding gene is the *black* gene itself. We cannot also infer directly that *Drosophila ebony* and *Papilio black* are homologs of one another (despite their apparent similarity in BAS-like function). For example, BAS enzyme activity in *Papilio* could be regulated post-translationally, or the BAS-encoding gene itself could be under sex-linked control from a different regulatory pathway. Understanding the regulation of BAS-like activity in *Papilio* and how it is altered in melanic mutants may therefore benefit from further genetic dissection of genes like *ebony* in *Drosophila*. Nevertheless, our data demonstrate a potential biochemical mechanism whereby a broad color pattern change is associated with regulation of a single key enzyme activity. The challenge now in *P. glaucus* is to determine whether the BAS-encoding gene is the *black* gene itself, or whether BAS activity is regulated by a separate Y-linked factor.

Our results also have implications for two other aspects of Lepidopteran mimicry. Firstly, mimicry in other species often involves switches from colored pigments to black melanin. For example, butterflies of the genus *Heliconius* [20] show shifts from colored pigments (red, orange, white or yellow) to those containing melanin (black). Similar color pathway 'switching' genes may therefore be involved in pattern changes in other butterfly and moth species, although the biochemical nature of the colored pigments themselves may differ between species. Elucidation of these switch genes may help us to study the molecular evolution of mimicry itself. Secondly, our observations that a single genetic factor can affect multiple wing pattern phenotypes (that is, both rates of scale maturation and color pattern itself) may also help to explain why such phenomena in other swallowtail species have been labelled as being under the control of 'super' genes [20]. Super genes have been defined as clusters of two or more genes that are tightly linked that affect several different components of the pattern [20]. The demonstration that BAS activity could regulate scale maturation and, in turn, determine the final color of the scales themselves shows a potential mechanism (regulation of scale maturation) whereby multiple color phenotypes could be effected by control of a single gene. Thus, different delays in scale maturation can produce different arrays of color in different species. This may help explain how multiple color phenotypes could be regulated by a single genetic factor rather than, as previously thought, multiple linked genes (or a super gene) regulating a series of closely related phenotypes.

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