# 博士論文

**Analysis of gene network for color pattern formation and the mechanism of the prepattern determination in *Drosophila guttifera***

（ミズタマショウジョウバエを用いた模様形成遺伝子ネットワーク

とプレパターン決定機構の解析）

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# 要旨

様々な分類群の動物において、多様なパターンの模様が進化してきた。多様なパターンの模様がどのように生じてきたのかを明らかにするためには、模様の形成メカニズムを理解する必要がある。そのメカニズムの一部として、モルフォゲン（発生の際に拡散によって体のパターンを形成する因子; Wolpert 1969）の拡散によって模様のパターンが決定されていると想定されてきた。また、模様形成にはモルフォゲンだけでなく、色素形成遺伝子も関与する。そのため、模様形成メカニズムの解明には、パターンを決定する機構と模様形成に関与する遺伝子同士の制御関係を理解する必要がある。本研究で私は、ミズタマショウジョウバエ（*Drosophila guttifera*）の翅のメラニンによる模様を、モルフォゲンによる模様形成メカニズムを研究する上でのモデルとして用いた。先行研究では、モルフォゲンをコードする *wingless* 遺伝子（*Wnt1* ホモログ）が模様形成をコントロールすると示されていた（Werner et al. 2010）。第 1 章では、ミズタマショウジョウバエの翅の模様形成プロセスの解析について記述した。私はミズタマショウジョウバエの蛹のステージを決定し、翅脈を通じて運ばれるメラニンの前駆体や細胞外に分泌されて模様形成部位に残った因子が形成に関与していると示唆した。第 2 章では、模様形成部位において *wingless* の下流で制御される遺伝子群の解析について記述した。結果として、神経発生に関与する遺伝子群、Wnt シグナルや Dpp シグナルに関与する遺伝子群、メラニン合成に関与する遺伝子群などが模様形成部位で *wingless* の下流に組み込まれていることがわかった。第 3 章では、翅の模様のプレパターン（将来模様ができる範囲）に Wingless タンパク質の拡散が必要かについて解析したことを記述した。実験はキイロショウジョウバエ（*Drosophila melanogaster*）を用いて行った。ミズタマショウジョウバエの *yellow* 遺伝子（メラニン合成に必要な遺伝子）のエンハンサーによって翅に EGFP が発現するキイロショウジョウバエではミズタマショウジョウバエの模様の一部が EGFP の発現で再現される。このハエと、Wingless を人工的に拡散しないように改変したタンパク質をコードする NRT-Wg 遺伝子を持つキイロショウジョウバエを交配し、翅の模様のプレパターン決定に Wingless の拡散が必要だと示唆した。第 1 章から第 3 章までの結果に基づき、General Discussion では、本研究が模様の進化を説明する発生メカニズムの理解にどのように貢献するのかについて記述した。

## Abstract

Various color patterns have evolved in a wide range of animal taxa. Understanding developmental mechanisms of color pattern formation would explain how various animal color patterns have been generated. As a mechanism of color pattern formation, it has been estimated that diffusion of morphogens (molecules which determines the pattern of animal body plans by diffusion in ontogeny; Wolpert 1969) determines the patterns. In color pattern formation, not only morphogens but products of pigmentation genes are involved. Therefore, elucidation of mechanisms of color pattern formation requires understanding mechanisms of the pattern determination and regulatory relationships of genes involved in color pattern formation. In this study, I used a polka-dotted melanin pigmentation pattern on wings of *Drosophila guttifera* as a model to study how morphogen controls color pattern formation. A previous research showed *wingless* gene, which encodes one of morphogens, controls formation of the wing pigmentation pattern (Werner et al. 2010). In Chapter 1, I described the analysis of the process of pigmentation pattern formation in *D. guttifera*. I determined the pupal stages of *D. guttifera* and suggested that melanin precursors transported through wing veins and extracellular factors left in the cuticle of pigmentation areas are involved. In Chapter 2, I described the analysis of genes regulated downstream of *wingless* in pigmentation areas on wings. As a result, it was shown from transcriptome analysis that genes for neural development, genes involved in Wnt and Dpp signaling, and genes for melanin synthesis were regulated downstream of *wingless* in pigmentation areas. In Chapter 3, I described an analysis of the necessity of Wingless diffusion in determination of the prepattern (the area where pigmentation pattern forms in future) of wing pigmentation. The experiment was conducted on *Drosophila melanogaster*. Transgenic *D. melanogaster* in which expression of EGFP on wings was driven by a *yellow* enhancer of *D. guttifera* was crossed with another transgenic *D. melanogaster* whose *wingless* gene was replaced with *NRT-Wg* gene which encodes artificially made Wingless protein that does not diffuse. From the obtained result, Wingless diffusion was suggested to be necessary for the prepattern formation of wing pigmentation in *D. guttifera*. Based on the results in Chapter 1 to 3, I described how my study contributes to understanding of developmental mechanisms underlying evolution of color patterns in General Discussion.

## General Introduction

Various color patterns have evolved in a large number of animal taxa. Some of those play roles in mimicry and aposematism, and others have a physiological function such as thermoregulation (Cott 1940; Ruxton et al. 2004). What kind of developmental mechanisms underlie the generation of various color patterns? Construction of mathematical models and transplantation of tissues led to the presumption that, in the case of color patterns on mammal furs and butterfly wings, the patterns were determined by diffusion of intercellular signal transduction molecules such as morphogens (molecules which determines the pattern of animal body plans by diffusion in ontogeny; Wolpert 1969) (Turing 1952; Murray 1981; Nijhout 1980; Kondo and Shirota 2009). Thereafter, gene overexpression and knock-out experiments on fruit flies and lepidopterans showed that diffusible intercellular signal transduction molecules have function in color pattern formation (Werner et al. 2010; Yamaguchi et al. 2013; Mazo-Vargas et al. 2017). However, it is unclear whether diffusion is essential to determine the patterns in such organisms. In color pattern formation, not only intercellular signal transduction molecules but products of pigmentation genes are involved (Arnoult et al. 2013; Zhang et al. 2017). Understanding of developmental mechanisms of color pattern formation requires revealing pattern determination and regulatory relationships among genes involved in the formation.

*Drosophila guttifera* (Insecta, Diptera) has a polka-dotted pattern of melanin pigmentation on wings. *wingless* gene (a gene coding one of morphogens, a homologue of vertebrate *Wnt-1*) is known to control formation of the pigmentation pattern. *D. guttifera* can be a model organism for studying how morphogen controls color pattern formation (Martin and Courtier-Orgogozo 2017), but knowledge about the developmental mechanism is limited. Diffusion of Wingless protein from its source was assumed to determine the prepattern (the area where pigmentation pattern forms in future) in the pupal period. Ectopic expression of *wingless* on wings induced ectopic pigmentation, accompanied by upregulating the expression of *yellow*, which is necessary for melanin synthesis (Gompel et al. 2005; Werner et al. 2010).

When I started the research, there was a lack of fundamental information about the process of pigmentation pattern formation in *D. guttifera*. It was unclear when the expression of *yellow* starts, and when wing pigmentation starts and ends. Therefore, I analyzed the process of formation of wing pigmentation. I also observed the movement

of Yellow expressing cells and tested the necessity of wing veins for formation of wing pigmentation. As a result, melanin precursors transported through wing veins were suggested to be converted to melanin by extracellular factors which were secreted from epithelial cells and left in the cuticle (Chapter 1).

To understand the mechanism of formation of the wing pigmentation pattern, it is necessary to know which genes are regulated downstream of *wingless*. In *D. guttifera* and *Drosophila melanogaster* (common fruit fly), *wingless* is expressed on wings. However, there is no wing pigmentation pattern in *D. melanogaster*. Genes regulated downstream of *wingless* in *D. guttifera* were assumed to be responsible for formation of wing pigmentation (Werner et al. 2010). Therefore, I comprehensively detected genes differentially expressed in pigmentation areas on wings by transcriptome analysis. For those genes, I also tested whether their expression was regulated by *wingless*. As a result, genes for neural development, genes involved in Wnt and Dpp signaling, and genes for melanin synthesis were shown to be regulated downstream of *wingless* in pigmentation areas (Chapter 2).

Furthermore, we have to reconsider how Wingless protein determines the prepattern in *D. guttifera*. In *D. melanogaster*, when Wingless protein was artificially tethered to the cell membrane, the body plan was normal (Alexandre et al. 2014). Therefore, some reserchers argued that diffusion of Wingless protein might be unnecessary for determining the pattern of body plans. I estimated the necessity of Wingless diffusion for determining the prepattern of wing pigmentation of *D. guttifera*, from an experiment on *D. melanogaster*. When *eGFP* gene connected with a *yellow* enhancer of *D. guttifera* is introduced to *D. melanogaster*, a part of the wing pigmentation pattern of *D. guttifera* is reconstructed as the expression of EGFP on wings of *D. melanogaster* (Werner et al. 2010). I made individuals of *D. melanogaster* whose Wingless was artificially tethered to the cell membrane and which had *eGFP* gene connected with a *yellow* enhancer of *D. guttifera*. As a result, the expression area of EGFP disappeared or became dramatically narrow. It was suggested that diffusion of Wingless might be necessary for determining the prepattern in *D. guttifera* (Chapter 3).

Based on the above, in the section of General Discussion, I will discuss how my study of the wing pigmentation pattern of *D. guttifera* contributes to understanding developmental mechanisms underlying evolution of color patterns.

# Chapter 1

## Pupal development and pigmentation process of a polka-dotted fruit fly, *Drosophila guttifera* (Insecta, Diptera)

**Abstract**

An adult fly of *Drosophila guttifera* (Insecta: Diptera: Drosophilidae) has melanin pigmentation patterns on its body and wings. Though *D. guttifera* has been used for research into color pattern formation, how its pupal development proceeds and when the pigmentation starts have not been well studied. In this study, I defined the pupal stages of *D. guttifera* and measured the pigment content of wing spots from the pupal period to the period after eclosion. Using a transgenic line which carries *eGFP* connected with an enhancer of *yellow*, a gene necessary for melanin synthesis, I analyzed the timing at which the *yellow* enhancer starts to drive *eGFP*. I also analyzed the distribution of Yellow-producing cells, as indicated by the expression of *eGFP* during pupal and young adult periods. The results suggested that Yellow-producing cells were removed from wings within 3 h after eclosion, and wing pigmentation continued without epithelial cells. Furthermore, the results of vein cutting experiments showed that the transport of melanin precursors through veins was necessary for wing pigmentation. These results showed the importance of melanin precursors transported through veins and of extracellular factors which were secreted from epithelial cells and left in the cuticle.

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**Introduction**

Color patterns of animals have evolved in various taxa. Adaptive functions of animal color patterns such as mimicry and influence on mate choice have been studied (Cott 1940; Ruxton et al. 2004). One of the major substances that constitute animal color patterns is melanin (Riley 1997). Melanin is a core component of dark pigment that plays roles in both vertebrates and invertebrates, such as the stripes of zebras or the eyespots of butterflies (Nijhout 1985; Sugumaran 2002; Mills and Patterson 2009; Wittkopp and Beldade 2009). Although the mechanisms of color pattern formation are largely different between vertebrates and insects, the key step is how melanin is synthesized and distributed (Wittkopp et al. 2003; Kopp 2009; Kronforst et al. 2012; Tadokoro et al. 2016).

The pathway of melanin synthesis in *Drosophila melanogaster* is relatively well understood based on the contributions of genetic and biochemical studies (Wright 1987; Wittkopp et al. 2003; Gibert et al. 2007; Kopp 2009, Massey and Wittkopp 2016). In the first step, Pale (tyrosine hydroxylase; TH) converts tyrosine to dopa (dihydroxyphenylalanine), and then Ddc (dopa decarboxylase) converts dopa to dopamine. Phenol oxidases and Yellow-family proteins convert dopamine to melanin (Fig. 1-1, Hirsh and Davidson 1981; Biessman 1985; Neckameyer and White 1993; Han et al. 2002; Riedel et al. 2011). Based on evidence showing that dopa, dopamine, and Yellow-family proteins are secreted from cells, the latter part of melanin synthesis is believed to proceed in the cuticle layer, which is outside of cells (Kramer and Hopkins 1987; Hopkins and Kramer 1992; Walter et al. 1996; Wittkopp et al. 2002).

Wing pigmentations have been studied in several *Drosophila* species, which have various wing pigmentation patterns. Adult males of *Drosophila biarmipes* have a pigmentation pattern on the anterior distal part of the wings. In this species, *yellow* is expressed where pigmentation occurs, whereas *ebony* is expressed where pigmentation does not occur (Wittkopp et al. 2002; Gompel et al. 2005). Engrailed represses and Distal-less promotes the expression of *yellow* (Gompel et al. 2005; Arnoult et al. 2013). *Drosophila guttifera* has a polka-dotted pigmentation pattern on its wings. In this species, the expression of *yellow* foreshadows the pigmentation pattern and the expression of *ebony* occurs where there is no expression of *yellow* (Gompel et al. 2005). Ectopic expression of *wingless* in the wings of this species induces ectopic pigmentation (Werner et al. 2010). The unique expression pattern of *wingless* is due to the evolution of *cis*-regulatory elements of *wingless* (Koshikawa et al. 2015; Koshikawa 2015).

Based on experiments in various fruit fly species, True et al. (1999) argued that precursors of melanin are transported through veins, and wing pigmentation starts after eclosion. They observed that it takes 1 day to complete wing pigmentation. In *D. melanogaster*, which has no wing pigmentation pattern, it is observed that all the cells except those in veins are retrieved and disappear into the thorax by 2 or 3 h after eclosion (Johnson and Milner 1987; Kimura et al. 2004; Tögel et al. 2008). I term this phenomenon "wing cell clearance". The question then arises, if wing cell clearance occurs in a species which has a pigmentation pattern on the wings, does this pigmentation continue without cells in the blade after wing cell clearance?

In this study, I addressed this question using *D. guttifera*, which has a black

melanin pigmentation pattern on the wings (Fig. 1-2), is easy to breed and suitable for transgenic experiments, and hence is one of the potential model organisms for the study of color pattern formation (Werner et al. 2010; Koshikawa et al. 2015; Koshikawa et al. 2017). I observed and established the sequence of developmental events during the pupal period. Then, I quantified the optical density of wing spots from the pupal period to the period after eclosion and analyzed the timing of wing cell clearance in the wings. Although wing pigmentation started during the pupal period, I found that the majority of the pigmentation was developed after eclosion without the involvement of epithelial cells during that time.

**Materials and methods**

**Flies**

A wild-type strain of *D. guttifera* (stock no. 15130-1971.10) was obtained from the *Drosophila* Species Stock Center at the University of California, San Diego. I also used a transgenic line of *D. guttifera* that carries *nuclear eGFP* connected with a *yellow* enhancer (*vein spot* CRE-*nuclear eGFP*, gut 1c+R GFP #12; Werner et al. 2010). Flies were reared with standard cornmeal/sugar/yeast/agar food at 25 °C and exposed to a 12: 12 h day/night cycle. For measuring the duration of pupal stages, the wild-type strain was kept under the condition of constant light.

**Staging of pupae**

Considering the temporal differences of developmental events between *D. guttifera* and *D. melanogaster*, I defined the pupal stages of *D. guttifera* on the basis of the definition of pupal stages in *D. melanogaster* determined by Bainbridge and Bownes (1981). In the experiment to determine these stages, larvae were collected from vials and put in 1.5 ml microtubes with food (10 larvae/tube). When larvae became pupae, they were moved onto moist tissue paper in a plastic Petri dish. In order to take photographs, puparia were removed with forceps. I used an M125 microscope (Leica Microsystems, Wetzlar, Germany) and a digital camera DFC 290 HD (Leica Microsystems) to take photographs.

**Measurement of the duration of pupal stages**

To measure the duration of pupal stages, Dr. Koshikawa, Dr. Matsumoto, and I

continued to observe pupae without removing puparia. To observe all the stages during 4 days of development, I prepared pupae that were collected 1–2, 2–3, and 3–4 days after pupal formation and newly formed pupae collected once every 30 min. These samples (total 80 pupae) were observed once every 30 min, and the stage of each individual was recorded over four straight days (96 h) by a rotating shift of three persons. I then calculated the average of counts, which showed how many times each stage was recorded. I estimated the duration of each stage (in hours) by multiplying the average count of each stage by 0.5 (h).

**Quantifying the optical density of wing spots**

From the pupal period to the adult period, I calculated optical density (OD) in order to evaluate the darkness of wing spots with ImageJ software (https://imagej.nih.gov/ij/). For relative quantification, I used the right wings of the wild-type strain. When I used pupae, puparia were removed with forceps and the right wings were dissected from flies in phosphate buffered saline (PBS, pH 7.4), and then the wings were extended in water. When I used adult flies, I collected newly eclosed flies once every 10 min from vials. When flies reached the stages of interest, they were anesthetized with $CO_2$ and the right wings were dissected. Dissected wings were mounted with 10 µl of PBS (pH 7.4) and photographed using an SZX16 stereomicroscope (Olympus, Tokyo, Japan) and a DSE-330-A digital camera system (Olympus). Using the photographs of wings, I measured the OD of wing spots. The details of these measurements are described in Fig. 1-3. Briefly, “mean gray values” of wing spots around a campaniform sensillum (Lees 1942), at a tip of a vein, and around a posterior crossvein were converted to OD using the Rodbard function (DeLean et al. 1978). I also measured the OD of a control area that had no pigmentation. Then, I calculated ΔOD by subtracting OD of the control area from OD of wing spots.

**Observation of wing cell clearance**

I observed wing cell clearance, i.e., the migration of wing epithelial cells. For this experiment, I used a transgenic line (*vein spot* CRE-*nuclear eGFP*). I collected newly eclosed flies once every 10 min from vials. I anesthetized the flies with $CO_2$, dissected the right wings from flies, mounted, and photographed them. An SZX16 stereomicroscope (Olympus) and a DSE-330-A digital camera system (Olympus) were

used to take photographs.

**Vein cutting experiments**

I cut the veins of the wild-type *D. guttifera* to clarify the role of veins in wing pigmentation. I collected newly eclosed flies once every 10 min from vials and recorded how many hours had passed since the eclosion. Before I cut veins, flies were anesthetized with $CO_2$. The first longitudinal vein (costa) and the third longitudinal vein were cut with a surgical knife. After the surgery, flies were moved to vials with food (one fly per vial). One day (24 h) after eclosion, flies were anesthetized with $CO_2$ and the right wings of flies were dissected. Wings were mounted with 10 μl of PBS (pH 7.4) and photographed with an SXZ16 stereomicroscope (Olympus) and a DSE-330- A digital camera system (Olympus).

**Results**

**Staging of pupae**

Bainbridge and Bownes (1981) defined pupal stages in *D. melanogaster* on the basis of the description of changes that occur in the color or position of the eyes, body trunk, bristles, Malpighian tubules, and "yellow body" (mass of shed cells within the midgut; Robertson 1936). Werner et al. (2010) applied the system of Bainbridge and Bownes (1981) for some of the *D. guttifera* pupal stages, but their determinations were limited to the period from P5(ii) to P7. I determined the pupal stages of *D. guttifera* (P1-P15) mostly based on the description of Bainbridge and Bownes (1981), and Dr. Koshikawa, Dr. Matsumoto, and I measured the duration of each stage (Table 1-1). In this section, I describe the definitions and changes observed during these stages.

P1

Definition: The puparium is white (Fig. 1-4a).

Change: The pupa stops crawling. The color of the puparium starts to become light brown.

P2

Definition: The color of the puparium is light brown (Fig. 1-4b).

Change: A bubble emerges on a lateral side of the pupa.

P3

Definition: A bubble is observed (Fig. 1-4c).

Change: The bubble enlarges in the lateral side. The pupa becomes buoyant in PBS (pH

7.4).

P4(i)

Definition: The bubble is larger than that in P3 (Fig. 1-4d). The pupa is buoyant in PBS (pH 7.4).

Change: The bubble moves to the posterior side. The pupa is pushed to the anterior side of the puparium. The color of the puparium darkens to medium brown. The lateral trunk tracheae become obscured.

P4(ii)

Definition: The bubble moves to the anterior part.

Change: The pupa withdraws to the posterior end (Fig. 1-4e, f). The imaginal head sac is everted.

P5–6

Definition: Malpighian tubules migrate (Fig. 1-5a).

Change: The extension of wings and legs becomes completed. During this stage, Malpighian tubules migrate from the thorax to the abdomen. A tissue called yellow body emerges between the thorax and the abdomen (Robertson 1936). A translucent patch becomes visible in the middle of the eye.

P7

Definition: The yellow body can be observed in the dorsal side (Fig. 1-5b).

Change: Wings become folded. The color of eyes becomes light yellow.

P8

Definition: The eyes are yellow (Fig. 1-5c).

Change: The eyes become completely yellow (Fig. 1-5d). Then, the eyes become amber.

P9

Definition: The eyes are amber (Fig. 1-5e).

Change: The eyes become red.

P10

Definition: The eyes are red (Fig. 1-5f).

Change: Orbital and ocellar bristles, vibrissae and thoracic macrochaetae become black.

P11

Definition: Orbital and ocellar bristles, vibrissae, thoracic macrochaetae, and tarsal bristles are black and visible (Fig. 1-6a).

Change: At first, wings are white (Fig. 1-6b), and then they start to become gray from the

tips of the wings.

P12(i)

Definition: The tips of wings are gray (Fig. 1-6c).

Change: All parts of the wings become gray. Abdominal bristles become black.

P12(ii)

Definition: All parts of the wings are gray (Fig. 1-6d).

Change: The wings become black.

P13

Definition: The wings are completely black (Fig. 1-6e).

Change: The head and legs become dark.

P14

Definition: The head and the legs are completely darkened (Fig. 1-6f, g).

Change: A green patch, called "meconium", emerges on the dorsal abdomen in a patchy pattern (Robertson 1936; Bainbridge and Bownes 1981).

P15(i)

Definition: The meconium can be observed on the dorsal abdomen (Fig. 1-6h).

Change: Tarsal claws become black. The fly becomes able to walk when the puparium is removed with forceps. The fly expands the ptilinum and opens the lid (operculum).

P15(ii)

Definition: The fly is eclosing (Fig. 1-6i).

Change: Eclosion becomes complete.

**Expression of *yellow* started in stage P7 and wing pigmentation started in stage P12(i)**

In early P7, EGFP was not expressed in the wings when wings were not folded yet (Fig. 1-7a, b), but EGFP was expressed when the wings were folded in late P7 (Fig. 1-7c, d), suggesting that there is some mechanism of synchronization between the onset of wing folding and *yellow* expression. To clarify when wing pigmentation starts, I analyzed ΔOD, namely, the difference between the ODs of wing spots and the OD of the control area (Fig. 1-8). I determined ΔOD of wings at P10, P11, and P12(i) and examined the statistical significance of differences among these values by one-way ANOVA. Tukey's method of multiple comparison showed that there was a significant difference between the campaniform sensillum ΔODs of P10 and P12(i) and also between the

campaniform sensillum ΔODs of P11 and P12(i). No significant difference was detected between the campaniform sensillum ΔODs of P10 and P11 (Fig. 1-8a). The same tendency was observed in the data for spots at the tip of the vein and around a crossvein (Fig. 1-8b, c). In P11, no wing pigmentation was observed in most individuals (Fig. 1-8d), although pigment was observed around the crossvein in some rare cases. In contrast, in P12(i), the polka-dotted pigmentation pattern of wings was observed in all the individuals I observed (Fig. 1-8e). To determine the timing of the completion of pigmentation, ΔOD was compared at various times from P10 to 7 days after eclosion (Fig. 1-9). Campaniform sensillum ΔOD increased in the pupal period and the period after eclosion. No significant difference was observed between campaniform sensillum ΔOD at 1 and 7 days after eclosion, but the differences between ΔODs at all the other times compared were significant (Fig. 1-9a, Tukey's test). The same tendency was observed in the data for spots at the tip of the vein and around a crossvein (Fig. 1-9b, c). These data showed that wing pigmentation started in P12(i) and was completed within 1 day after eclosion.

**Wing pigmentation continued after wing cell clearance**

It is known that almost all the wing cells except those in veins are retrieved and move to the thorax in *D. melanogaster* (Kimura et al. 2004). Apoptosis-related genes are required for this process, and nuclear breakdown precedes the cell detachment (Link et al. 2007). I analyzed melanin pigmentation during and after wing cell clearance in *D. guttifera.* In the transgenic line, *vein spot* CRE-*nuclear eGFP*, cells located where black pigmentation develops are labeled with EGFP. These cells are thought to produce Yellow protein, which is necessary for melanin synthesis. Immediately after wing expansion, cells labeled with EGFP were located in the polka-dotted pattern (Fig. 1-10a, d). In contrast, cells labeled with EGFP were scattered at 1.5 h after eclosion (Fig. 1-10b, e). Almost all the labeled cells had disappeared at 3 h after eclosion (Fig. 1-10c). These observations showed that cells involved in melanin synthesis (labeled with EGFP) were retrieved and disappeared from the wings of *D. guttifera*. However, ΔOD continued to increase from 3 h after eclosion (Fig. 1-9), and it was observed qualitatively that wing spots became darker from 3 h after eclosion (Fig. 1-10f, g). These findings showed that wing pigmentation continued without cells involved in melanin synthesis.

**Provision of melanin precursors after wing cell clearance**

My finding that wing pigmentation continues without epithelial cells raises the question: how are precursors of melanin provided to the pigment? In insects, dopa and dopamine, precursors of melanin, are generally found in hemolymph or cuticle (Hopkins and Kramer 1992; Kramer and Hopkins 1987). Dissected wings of *Drosophila rajasekari* (synonym of *D. biarmipes*) can form their specific pigmentation pattern when they are incubated with dopa or dopamine (True et al. 1999). From the observations of a vein formation mutant of *Drosophila grimshawi* (synonym of *Idiomyia grimshawi*) and a vein cutting experiment on *D. rajasekari*, it is thought that dopa or dopamine is transported through the veins (True et al. 1999). I therefore conducted vein cutting experiments on *D. guttifera* (Fig. 1-11). When the third longitudinal vein was cut, spots on both the proximal and distal sides relative to the injured site became lighter colored than those in intact controls. The spot at the tip of the third longitudinal vein did not become lighter, but its shape was defective (Fig. 1-11a). When the first longitudinal vein (costa) was cut, two spots around campaniform sensilla on the third longitudinal vein and spots at the tip of the third longitudinal vein and fourth longitudinal vein became lighter (Fig. 1-11b). Calculation of the ΔOD of the spots which became lighter showed that the density of those spots was the same as their density 3 h after eclosion (Fig. 1-11e, f). When the third longitudinal vein was cut, the ΔOD of the spot at the tip of the third longitudinal vein was the same as that in intact controls (Fig. 1-11f, not significant by Tukey's method). These data suggest that precursors of melanin are transported through veins in *D. guttifera*. From these data, I inferred the direction of precursor flow (Fig. 1-11d). This inferred direction is consistent with previous observations of hemolymph flow in *D. melanogaster* and *Drosophila funebris* (Perttunen 1955).

**Discussion**

**Interspecific difference of pupal development between *D. guttifera* and *D. melanogaster***

Developmental phenomena known to occur in the pupae of *D. melanogaster* (Robertson 1936; Bainbridge and Bownes 1981) were also observed in almost the same order in *D. guttifera*, indicating that the process of pupal development of *D. guttifera* is very similar to that of *D. melanogaster*. The differences between *D. guttifera* and *D. melanogaster* were as follows.

1. In P3, a large bubble is located laterally in *D. guttifera*, but is located dorsally in *D. melanogaster*. This reflects a difference of internal structure and is not due to the orientation of pupae.
2. In *D. guttifera*, the emergence of a yellow body occurs both before or after Malpighian tubules become green.
3. In *D. guttifera*, in P7, the expression of *yellow* starts in a polka-dotted pattern (it was observed here as EGFP fluorescence driven by the *vein spot* CRE).
4. In *D. guttifera*, head and thoracic bristles darken at the same time (P11), whereas in *D. melanogaster*, head bristles darken faster (P10) than thoracic bristles (P11(i)).
5. In *D. guttifera*, tarsal bristles darken in P11, whereas those in *D. melanogaster* darken in P13.
6. *D. guttifera* has no sex comb (Kopp 2011).
7. Tarsal claws darken in P15(i) in *D. guttifera*, but in P13 in *D. melanogaster*.
8. At 25 °C, the pupal period of *D. guttifera* is 20 h longer than that of *D. melanogaster*.

I did not separate P5(i), P5(ii), and P6, but instead defined P5–6, because the emergence of the yellow body occurs before or after Malpighian tubules become green depending on the individual. Also, I did not separate P11(i) and P11(ii), but instead defined P11, because the head and thoracic bristles darkened at the same time.

**The process of wing pigmentation**

Based on the observation of wing spots and the measurement of ΔOD, I noted some common points and some points of difference between the process of wing pigmentation of *D. guttifera* and other species used in previous research. In *D. grimshawi* and *Drosophila disjuncta* (synonym of *Idiomyia disjuncta*), wing pigmentation does not occur during the pupal period, and pupae only have cell-hair prepatterns. Wing pigmentation starts after eclosion in these species (True et al. 1999), whereas it starts during the pupal period in *D. guttifera*. One of the common points concerning timing is that wing pigmentation is completed about 1 day after eclosion.

The mechanism enabling development of wing pigmentation without epithelial cells could be predicted to be as follows. Yellow and other proteins necessary for wing pigmentation are secreted from cells before wing cell clearance, and then they are located in the cuticle layers where the pigmentation pattern forms and convert dopa and/or dopamine transported through veins to dark-colored melanin. Because ectopic expression

of *yellow* does not induce ectopic pigmentation in wings of *D. melanogaster* (Gompel et al. 2005; Riedel et al. 2011), it is believed that proteins other than Yellow must be involved in wing pigmentation. Moreover, recombinant *D. melanogaster* Yellow protein expressed and purified from *Spodoptera frugiperda* (Sf9) insect cells does not have the activity of dopachrome conversion enzyme (Han et al. 2002), suggesting the contribution of other protein(s) which have this activity. Elucidating which enzyme(s) are involved will be required for understanding the mechanism of *Drosophila* wing pigmentation.

In *D. melanogaster*, it has been suggested that enzymes which convert dopamine to melanin are secreted from epithelial (epidermal) cells. When the cuticle and epithelial cells of the abdomen are incubated with dopamine after fixation with formaldehyde, pigmentation of the abdomen can be induced (Walter et al. 1996). The same phenomenon is observed in the larva of a moth, *Manduca sexta* (Hiruma et al. 1985). The present study is the first to show that synthesis of melanin continues in the absence of epithelial cells *in vivo*. I have thus provided the first direct evidence obtained using living insects to support the idea that the cuticular layer contains proteins necessary for pigmentation. True et al. (1999) argued the importance of two distinct mechanisms in wing pigmentation of *Drosophila*, i.e., prepatterns and melanin formation, and I further confirmed the relevance of this argument.

**The possibility that wing cell clearance and subsequent pigmentation are a general mechanism**

In *D. melanogaster*, wing cell clearance is powered by organs called wing hearts located in the dorsal sides of the thorax (Tögel et al. 2008). Without wing hearts, wing cell clearance does not occur and wings do not mature, resulting in inability to fly (Tögel et al. 2008). Although their anatomical structures are diverse, many pterygote species have wing hearts, and they have a common function, namely, sucking hemolymph from posterior veins (Pass 2000). Also, it is known at least in some insects, and potentially in many, that there are no cells in the membranous part of their mature wings (Grimaldi and Engel 2005). Many insect species, such as mayflies, dragonflies, grasshoppers, and bees, are known to have dark pigmentation patterns on their transparent wings. It will be necessary to verify whether there is wide occurrence in such species of the pigmentation mechanisms shown in this study, namely, that the cells are retrieved after eclosion and proteins deposited on the cuticle keep acting to cause wing pigmentation thereafter

without epithelial cells.

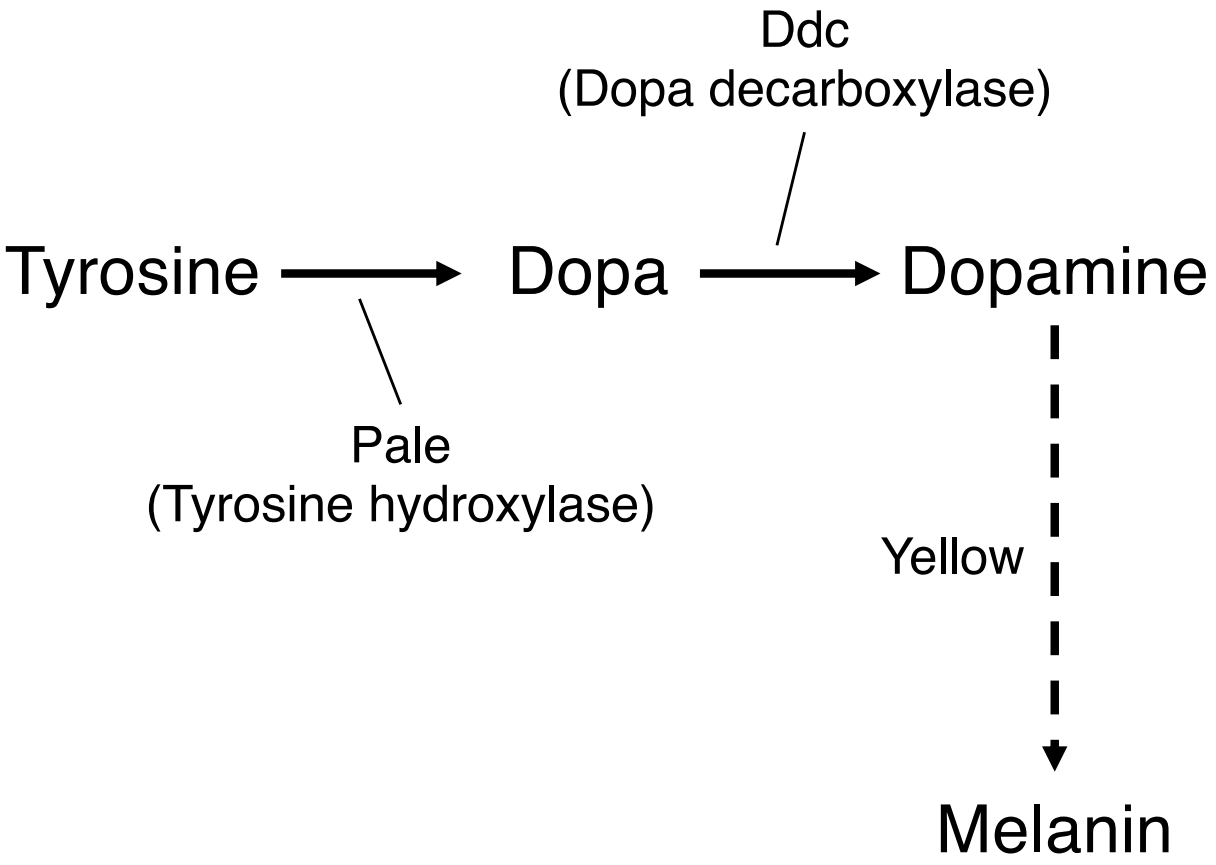


Fig. 1-1
The pathway of melanin synthesis. This figure was made based on Walter et al. 1996 and Wittkopp 2002. Tyrosine is converted to dopa by Pale and dopa is converted to dopamine by Ddc. When dopa is converted to dopamine, multiple chemical reactions are thought to occur. Yellow is considered to involved in conversion of dopa to dopamine (broken arrow).

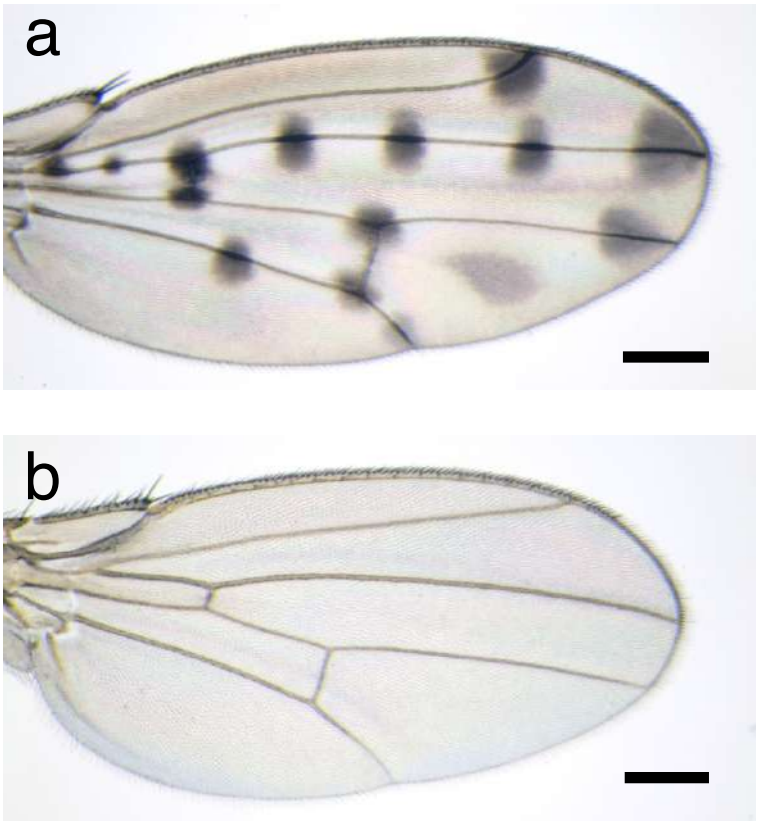


Fig. 1-2
Wings of *Drosophila guttifera* and *Drosophila melanogaster*. **a** A wing of *D. guttifera*. This species has a polka-dotted pigmentation pattern on its wings. **b** A wing of *D. melanogaster*. This species does not have a pigmentation pattern on its wings. Scale bars indicate 250 μm.

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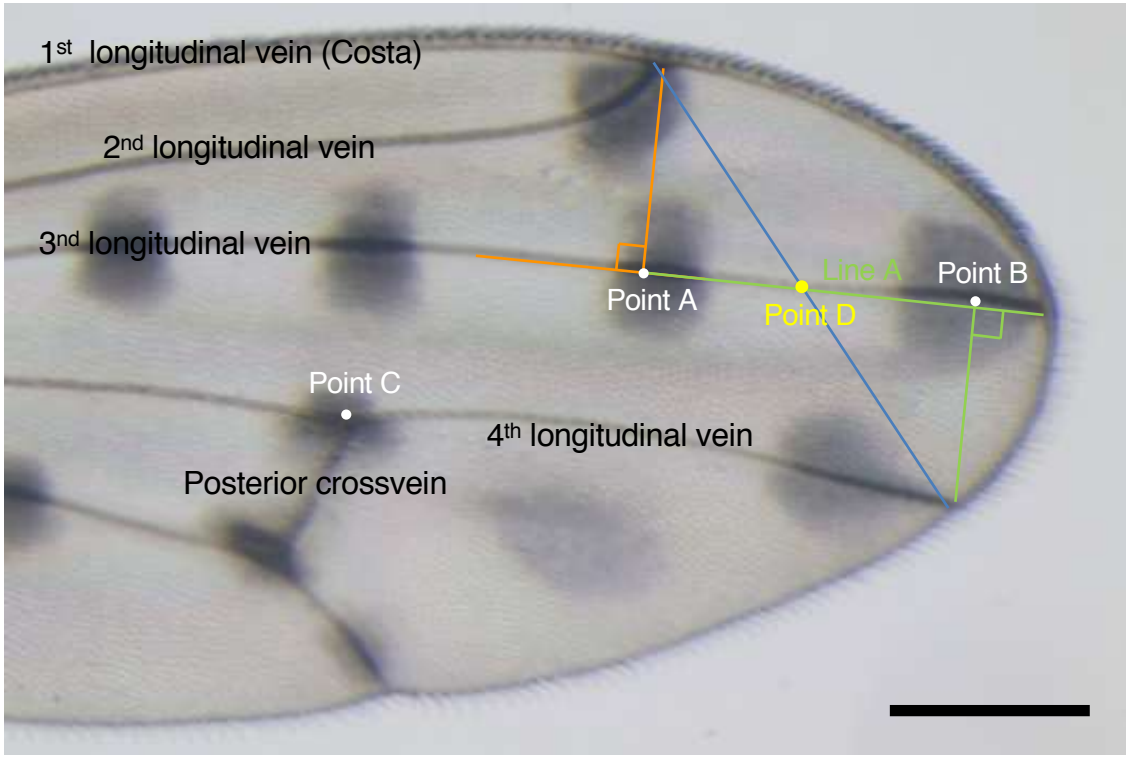


Fig. 1-3
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Fig. 1-3
Quantification of the density of wing spots. First, "mean grey values" of wing spots were calculated using Image J. To calculate O.D. from mean grey value, I used a stepped density filter OD 0.04-3.0 (Edmund Optics, Barrington, NJ, USA). I took photographs of nine parts (from the lightest part to the ninth-lightest part) of the filter and calculated mean grey values. The area selected for calculation was a circle (width 100, height 100) around the center of each image. Then, we calculated O.D. from mean grey values using the Rodbard function (DeLean et al. 1987). We calculated O.D. of wing spots around a campaniform sensillum, at a tip of a vein, and around a posterior crossvein. The center of the circle (width 100, height 100) for calculating O.D. of a wing spot was determined using the following criteria. In the case of the spot around a campaniform sensillum (Lees 1942), a mechanosensor on the vein, the center (Point A) was determined as the foot of a perpendicular line from the end point of the second longitudinal vein to the posterior line of the third longitudinal vein. In the case of the spot at the tip of the vein, two steps were required to determine the center. First, a line was drawn with Image J, connecting Point A and the end point of the posterior line of the third longitudinal vein (Line A). Second, the center (Point B) was determined as the intersection point of the posterior line of the third longitudinal vein and the perpendicular from the end point of the anterior line of the fourth longitudinal vein to Line A. In the case of a spot around a posterior crossvein, the center (Point C) was determined as the most posterior point of the anterior line of the fourth longitudinal vein in the intersection area of the posterior crossvein and the fourth longitudinal vein. I also calculated the O.D. of a control area that had no pigmentation. Two steps were required to determine the center of the circle (width 100, height 100) for calculating the O.D. of the control area. First, a line was drawn with Image J, connecting the end point of the anterior line of the second longitudinal vein and the end point of the posterior line of the fourth longitudinal vein. The crossing point of this line and the posterior line of the third longitudinal vein was used as the center (Point D). Then, by subtracting O.D. of the control area from O.D. of wing spots, I calculated ΔO.D. The scale bar indicates 250 μm.

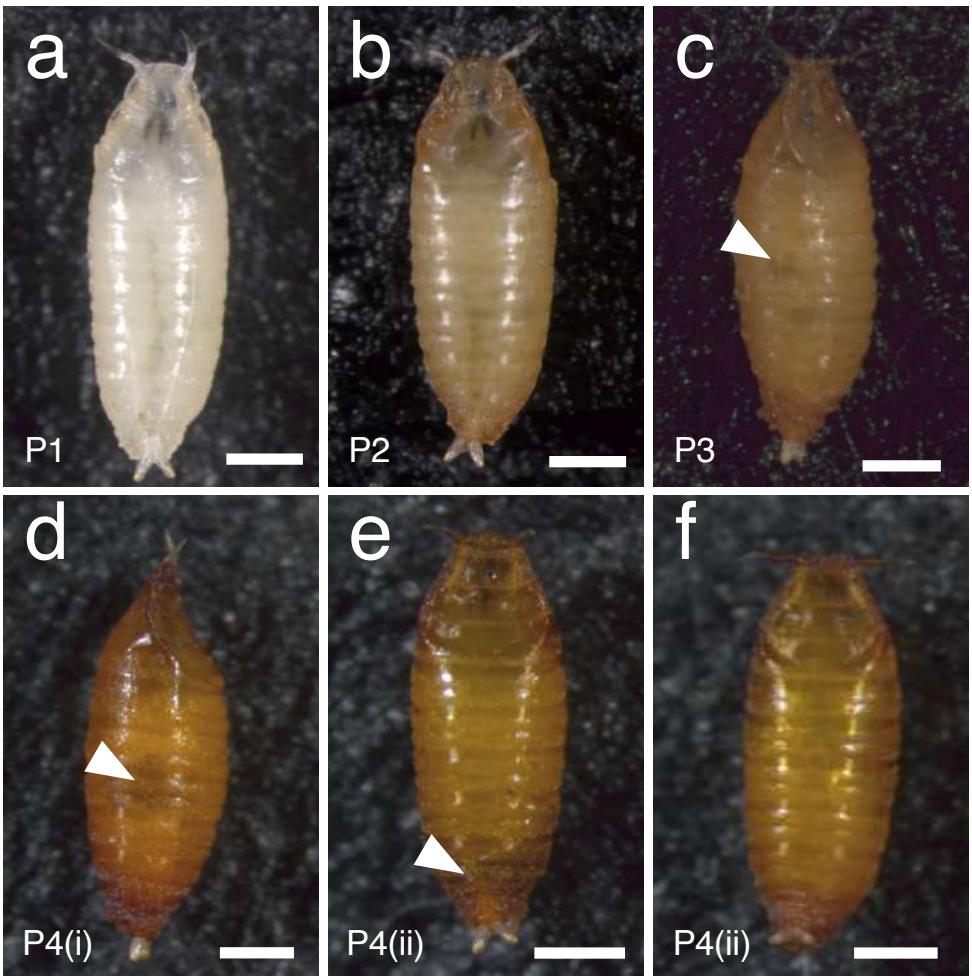


Fig. 1-4
Pupal stages of P1-P4. **a** P1. The color of the puparium is white. **b** P2. The color of the puparium is light brown. **c** P3. A bubble can be observed (white arrowhead). **d** P4(i). The bubble has become larger (white arrowhead). **e** Early P4(ii). The bubble is at the posterior end (white arrowhead). **f** Late P4(ii). The bubble is pushed away when the pupa withdraws. Scale bars indicate 500 μm.

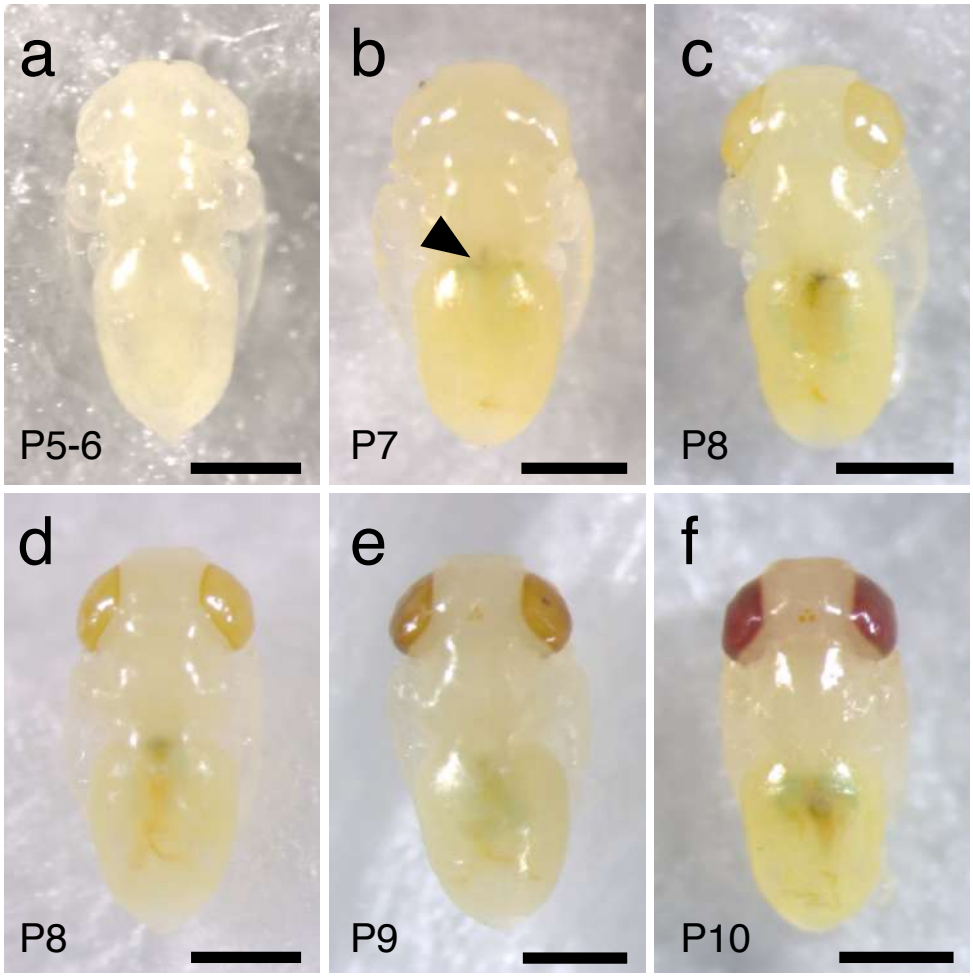


Fig. 1-5
Pupal stages of P5-P10. Puparium is removed in all the images. **a** P5-6. A yellow body cannot be observed in the dorsal side. **b** P7. In the dorsal side, a yellow body can be observed between the thorax and the abdomen (arrowhead). **c** Early P8. The color of eyes is light yellow. **d** Late P8. The color of eyes is completely yellow. **e** P9. The color of eyes is amber. **f** P10. The color of eyes is completely red. Scale bars indicate 500 μm.

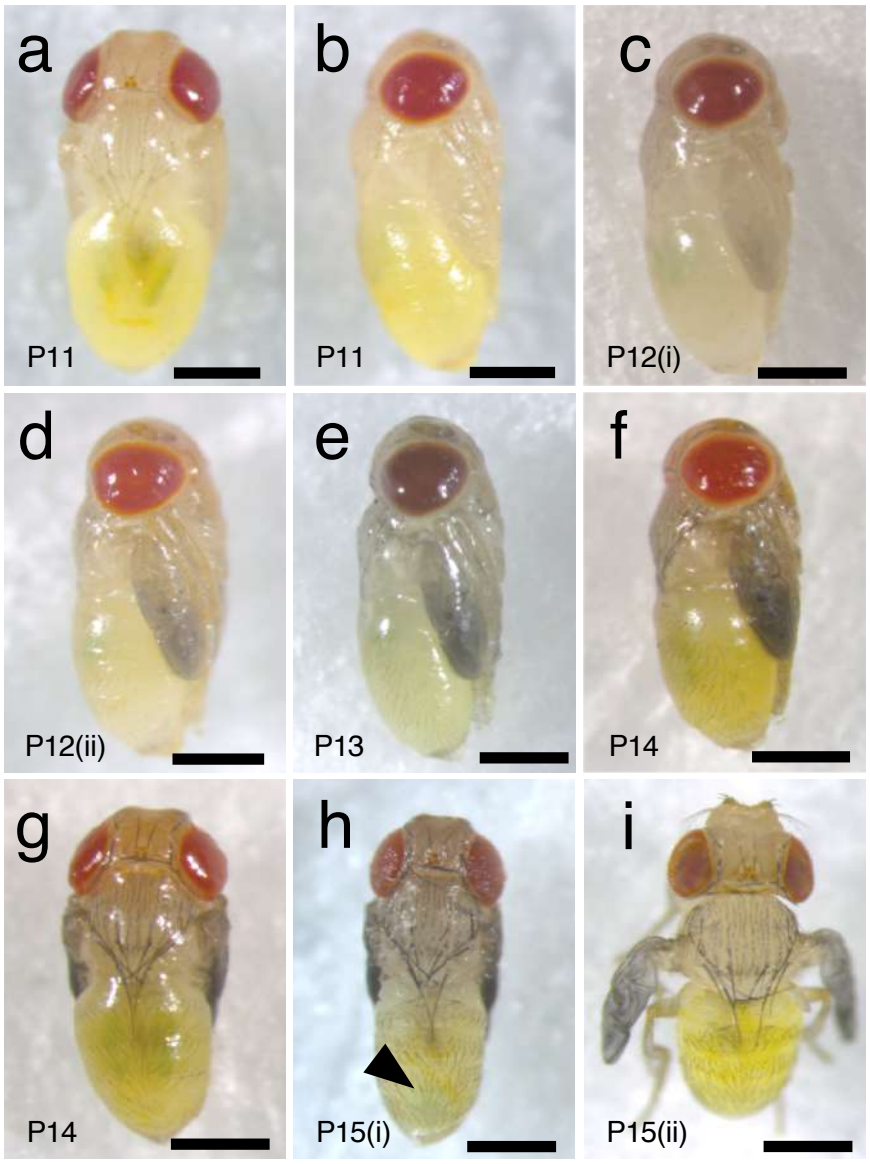


Fig. 1-6
Pupal stages of P11-P15. Puparium is removed in all the images. **a** P11. Head and thoracic bristles in the dorsal side darken. **b** P11 (lateral side). The wing is white. **c** P12(i). The tip of the wing is gray. **d** P12(ii). The entire wing is gray. **e** P13. The wing is completely black. **f** P14 (lateral side). Legs have darkened. **g** P14. The head has darkened. A meconium cannot be observed in the dorsal side of the abdomen. **h** P15(i). A meconium can be observed (arrowhead) in the dorsal side of the abdomen. **i** P15(ii). The fly is enclosing. Scale bars indicate 500 μm.

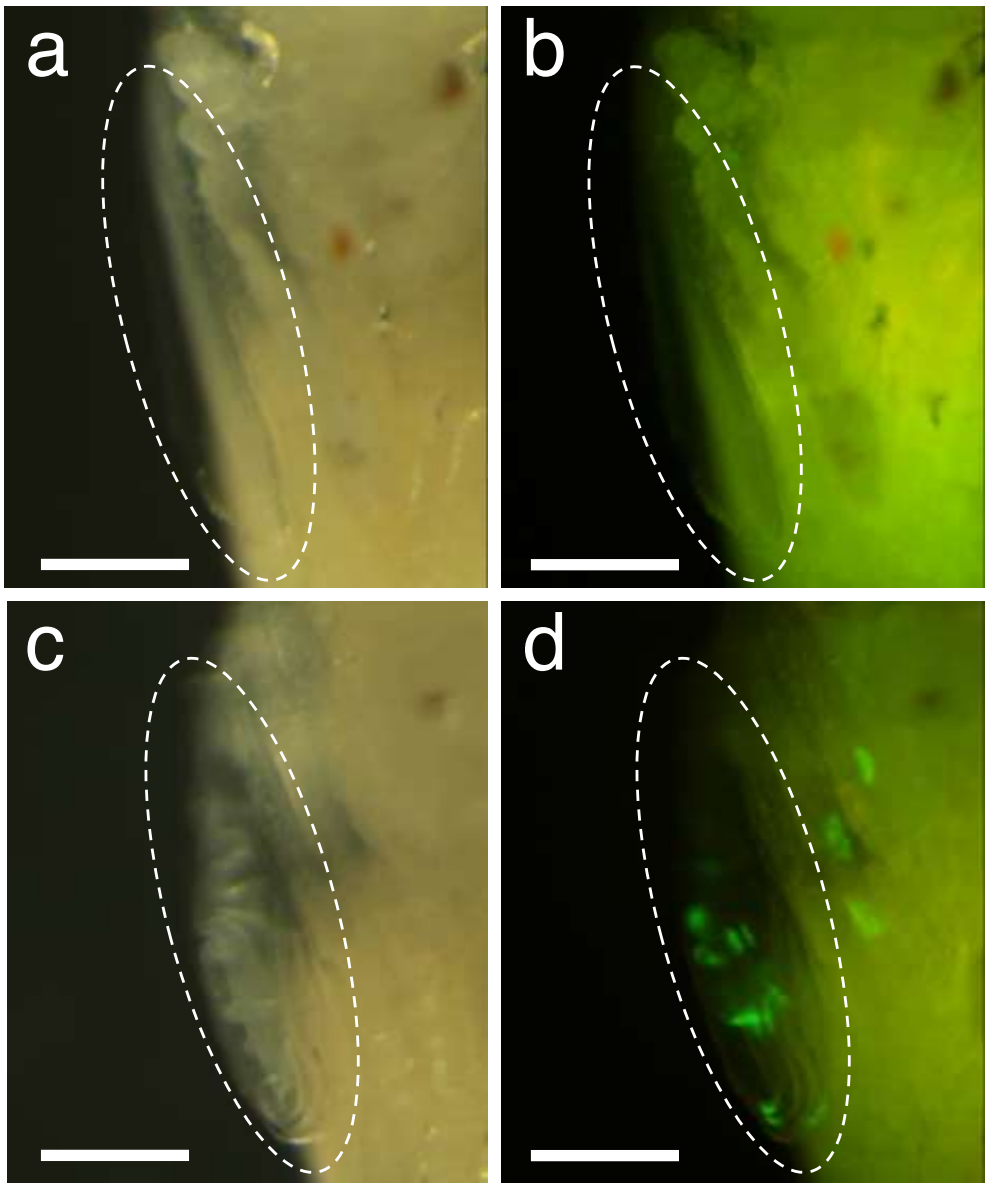


Fig. 1-7
The wings of pupae in P7 (ventral view). Flies in the images carry *nuclear eGFP* connected with a *yellow* enhancer. **a** The wing has not been folded (bright field). **b** The expression of EGFP has not started when the wing is not folded (green channel). **c** The wing is folded (bright field). **d** The expression of EGFP can be seen when the wing is folded (green channel). The same fly was used for images **a** and **b** and for images **c** and **d**. Dashed lines indicate positions of pupal wings. Scale bars indicate 250 μm.

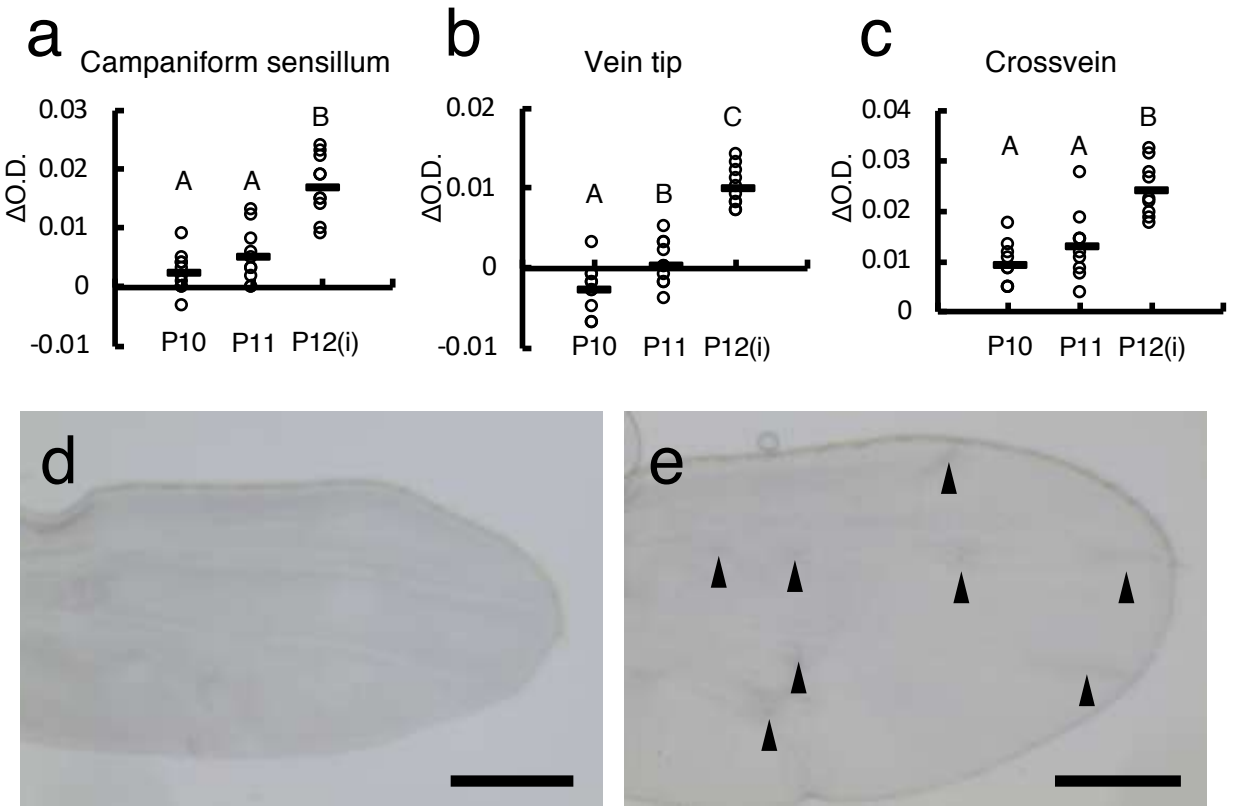


Fig. 1-8

The onset of pigmentation development in pupal stages. **a** Optical density of pigmentation (ΔOD) around a campaniform sensillum of the third longitudinal vein in pupal stages P10, P11, and P12(i). There were significant differences among these stages ($p$ = 1.40E−7, one-way ANOVA). Different letters indicate significant differences ($p$ < 0.05, Tukey's method). **b** ΔOD of wing spots at the tip of the third longitudinal vein. There were significant differences among stages ($p$ = 2.29E−10, one-way ANOVA). Different letters indicate significant differences ($p$ < 0.05, Tukey's method). **c** ΔOD of wing spots around the posterior crossvein. There were significant differences among stages ($p$ = 2.39E−5, one-way ANOVA). Different letters indicate significant differences ($p$ < 0.05, Tukey's method). **d** A wing of pupal stage P11. Wing pigmentation is not observed. **e** A wing of pupal stage P12(i). Weak pigmentation is observed in a polka-dotted pattern (arrowheads).

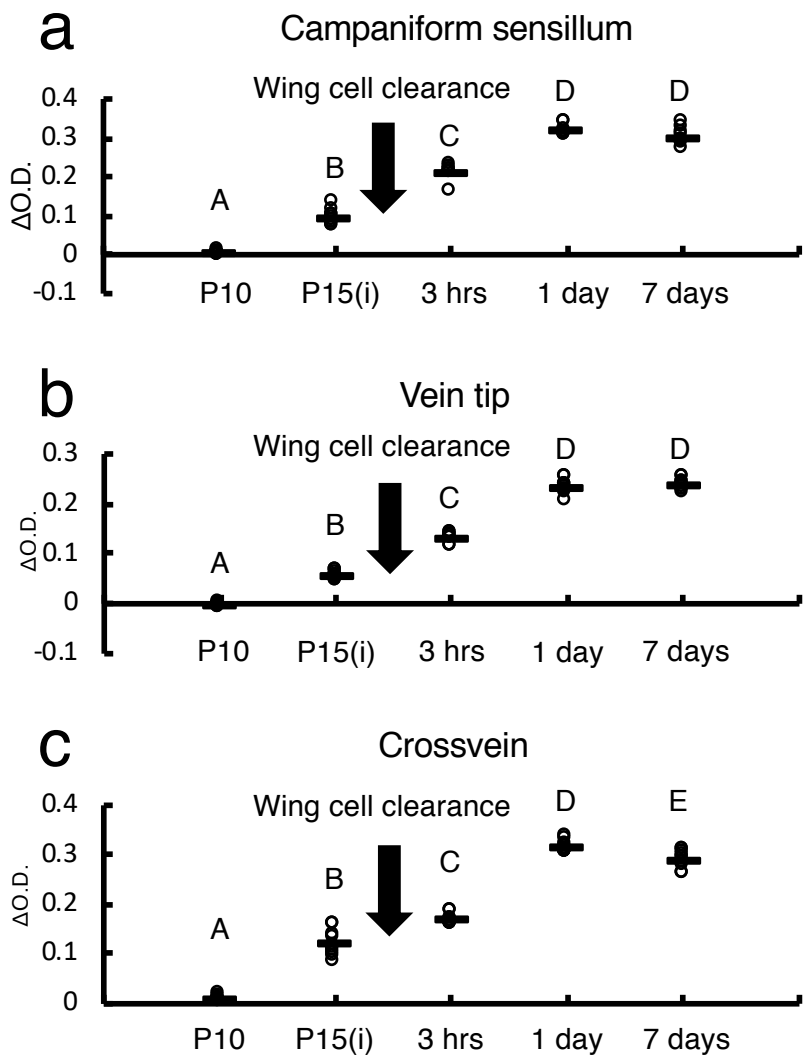


Fig. 1-9
The timing of pigmentation development during pupa and young adult periods. The black arrows indicate the timing of wing cell clearance. Different letters indicate significant differences ($p < 0.05$, Tukey's method). **a** Optical density of pigmentation (ΔOD) around a campaniform sensillum of the third longitudinal vein in pupal stages P10, P15(i), 3 h after eclosion, 1 day after eclosion, and 7 days after eclosion. There were significant differences among stages ($p = 6.80E-36$, one-way ANOVA). **b** ΔOD of wing spots at the tip of the third longitudinal vein. There were significant differences among stages ($p = 3.58E-45$, one-way ANOVA). **c** ΔOD of wing spots around the posterior crossvein. There were significant differences among stages ($p = 2.15E-36$, one-way ANOVA)

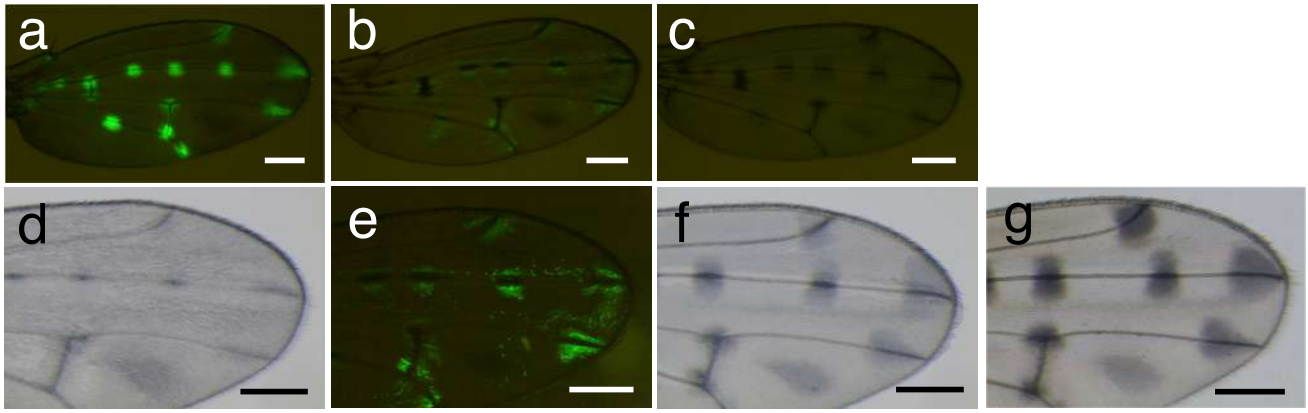


Fig. 1-10
Wing cell clearance observed as cell detachment from the original positions and migration towards the thorax. Flies in **a**, **b**, **c**, and **e** carry *nuclear eGFP* connected with the *yellow* enhancer (*vein spot* CRE). **a** Distribution of EGFP in the wing of an adult fly just after expansion of the wings (green channel). In this stage, the cells with EGFP expression were strictly located in the positions of future spots. **b** A wing of an adult fly 1.5 h after eclosion (green channel). Epithelial cells started to migrate in this stage. **c** A wing of an adult fly 3 h after eclosion (green channel). Almost all the EGFP-labeled cells are retrieved. **d** A wing of an adult fly just after expansion of the wings (bright field). **e** A magnified view of **b**. 1.5 h after eclosion (green channel). **f** A wing of an adult fly 3 h after eclosion (bright field). **g** A wing of an adult fly 1 day after eclosion (bright field). Spots are darker than those at 3 h after eclosion. Scale bars indicate 250 μm.

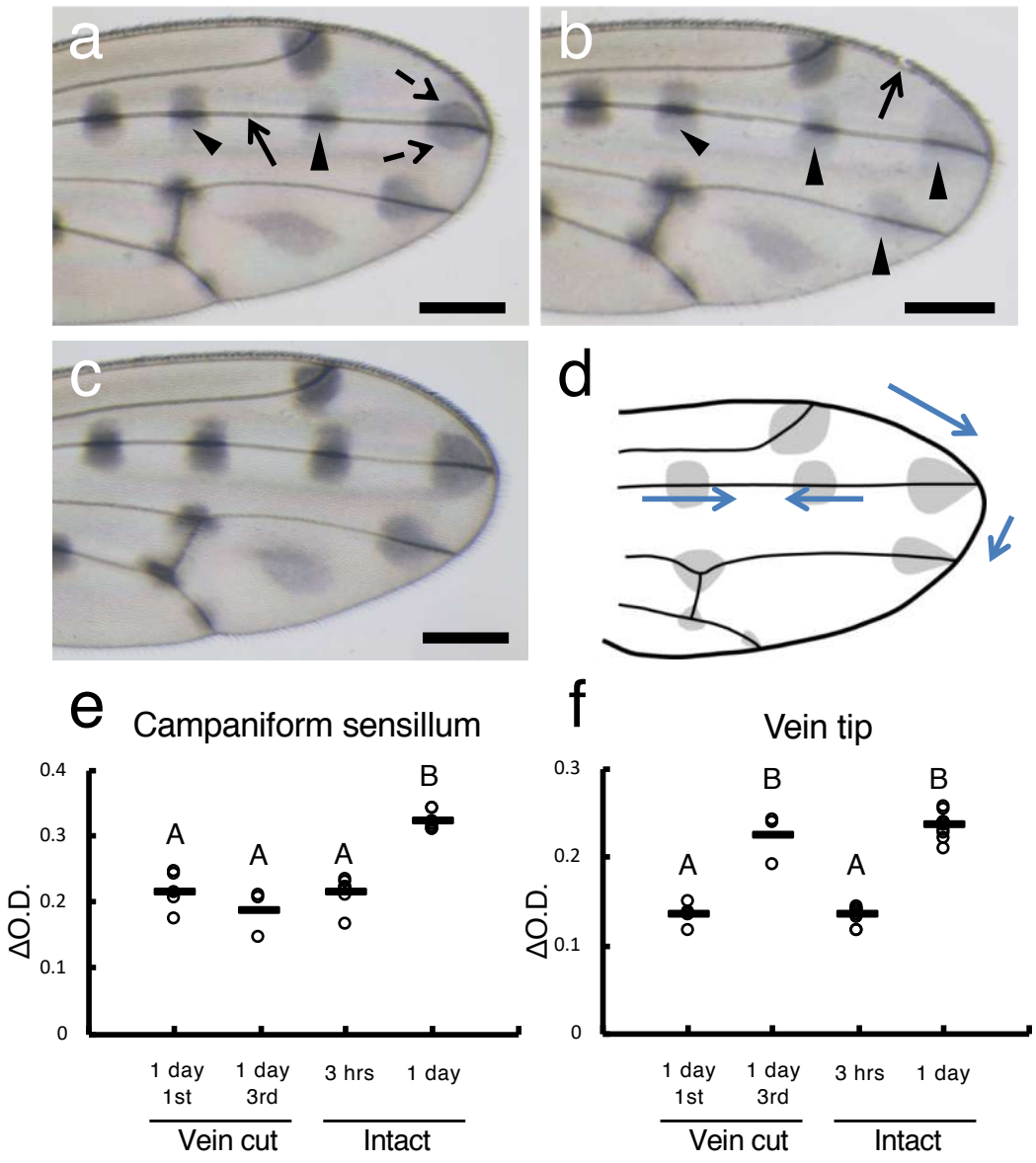


Fig. 1-11
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Fig. 1-11
Vein cutting experiments to show the function of a component transported through veins. **a** The third longitudinal vein was cut with a surgical knife at 3 h after eclosion. The wing was photographed 1 day after eclosion. The solid arrow indicates the site where the vein was cut. Spots around campaniform sensilla on both distal and proximal sides from the cut site were lighter (arrowheads). The spot at the tip of the third longitudinal vein was not lighter, but a defect in the shape was observed (broken arrows). **b** The first longitudinal vein was cut with a surgical knife at 3 h after eclosion. Two spots around campaniform sensilla on the third longitudinal vein and spots at the tip of the third and fourth longitudinal veins were lighter (arrowheads). **c** An intact wing, 1 day after eclosion. **d** The flow of melanin precursors inferred from the results of vein cutting experiments (blue arrows). **e** ΔOD of spots around the campaniform sensillum. There was no significant difference between the vein-cut groups (first and third longitudinal vein) and the intact wings 3 h after eclosion (Tukey's method), indicating the necessity for the first and third longitudinal veins for developing pigmentation around the campaniform sensilla. **f** ΔOD of spots at the tip of the third longitudinal vein. There was no significant difference between the group of the first longitudinal vein-cut and intact wings 3 h after eclosion. There was no significant difference between the group of the third longitudinal vein cut and the intact wings 1 day after eclosion. This result indicates that the first longitudinal vein is necessary to develop full pigmentation in the vein tip, but the third longitudinal vein is not (although it is necessary to make the normal shape of the spot, see **a**). Scale bars indicate 250 μm. Different letters indicate significant differences ($p < 0.05$, Tukey's method).

Table 1-1
The duration of pupal stages of *Drosophila guttifera*. The durations of P14 and P15 are integrated in this table, because precise distinction between P14 and P15(i) is difficult when the puparium is not removed.

| Stage | Duration (hrs) |
|---|---|
| P1-2 | 1.7 |
| P3 | 2.1 |
| P4(i) | 8.8 |
| P4(ii) | 0.3 |
| P5-6 | 5.0 |
| P7 | 31.9 |
| P8 | 9.6 |
| P9 | 10.9 |
| P10 | 11.7 |
| P11 | 4.4 |
| P12(i) | 1.1 |
| P12(ii) | 2.0 |
| P13 | 2.2 |
| P14-15(i) | 28.6 |
| P15(ii) | 1.4 |
| Total | 121.7 |

## Chapter 2

**Transcriptome analysis reveals *wingless* regulates neural development and signaling genes in the region of wing pigmentation of a polka-dotted fruit fly**.

**Abstract**

How evolutionary novelties have arisen is one of the central questions in evolutionary biology. Pre-existing gene regulatory networks or signaling pathways have been shown to be co-opted for building novel traits in several organisms. However, the structure of entire gene regulatory networks and evolutionary events of gene co-option for emergence of a novel trait are poorly understood. In this study, to explore the genetic and molecular bases of the novel wing pigmentation pattern of a polka-dotted fruit fly (*Drosophila guttifera*), I performed transcriptome analyses. As a result, I comprehensively identified the genes associated with the pigmentation pattern. Furthermore, I revealed that 151 of these associated genes were positively or negatively regulated by *wingless*, a master regulator of wing pigmentation. Genes for neural development, Wnt signaling, Dpp signaling, and effectors (such as enzymes) for melanin pigmentation were included among these 151 genes. None of the known regulatory genes that regulate pigmentation pattern formation in other fruit fly species were included. My results suggest that the novel pigmentation pattern of the polka-dotted fruit fly might have emerged through multi-step co-options of multiple gene regulatory networks, signaling pathways, and effector genes, rather than recruitment of one large gene circuit.

The content of this chapter was published in Fukutomi Y, Kondo S, Toyoda A, Shigenobu S, Koshikawa S (2020) *FEBS J.* (doi: 10.1111/febs.15338).

**Introduction**

How do evolutionary novelties emerge? Researchers have tried to unravel the developmental genetic program underlying traits in order to clarify the origins of evolutionary novelty (Shubin et al. 2009). Gene regulatory networks for producing novel traits have been supposed to be composed of a combination of genes forming other traits. One of the most significant current discussions regarding the production of evolutionary novelty is how pre-existing regulatory networks were utilized for this production (Shubin et al. 2009; Wagner and Lynch 2010).

So far, gene regulatory networks or signaling pathways involved in development of novel traits have been scrutinized in several animals. For example, in a horned dung beetle, limb and wing patterning genes are co-opted for horn formation (Moczek and Rose 2009; Hu et al. 2019). In Nymphalid butterflies, components of the

appendage-patterning gene regulatory network, such as *Distal-less*, *wingless*, and *decapentaplegic* signaling, contributed to development of eyespot, another representative novel trait (Nijhout 1980; Carroll et al. 1994; Brakefield et al. 1996; Monteiro et al. 2006; Zhang and Reed 2016; Connahs et al. 2019). In the fruit fly *Drosophila melanogaster*, it was shown experimentally that the gene regulatory network for larval posterior spiracle development was re-used for the posterior lobe, a novel trait observed in male genitalia (Glassford et al. 2015). Many studies have shown or suggested which gene regulatory networks or signaling pathways are necessary for, or involved in, development of novel traits. However, the structure of entire gene regulatory networks and evolutionary events of gene co-option for emergence of a novel trait are poorly understood.

Fruit fly species have been used to study regulatory evolution of pigmentation pattern, and provided many examples of mechanisms underlying phenotypic evolution (Williams et al. 2008; Arnoult et al. 2013; Rebeiz and Williams 2017; Koshikawa 2020). In a polka-dotted fruit fly (*Drosophila guttifera*), which has a novel polka-dotted pigmentation pattern on the wings, a melanin synthesis gene, *yellow*, was expressed in the polka-dotted pattern (Gompel et al. 2005; Werner et al. 2010; Koshikawa et al. 2017). A Wnt signaling gene, *wingless*, was expressed in the centers of pigmentation areas, and positively regulated the expression of *yellow* through an enhancer (Fig. 2-1a, c, e) (Gompel et al. 2005; Werner et al. 2010). Ectopic expression of *wingless* induced ectopic wing pigmentation (Fig. 2-1b, d, f) (Werner et al. 2010). The unique expression pattern of *wingless* seemed to be caused by evolutionary gain of novel enhancer activities (Koshikawa et al. 2015; Koshikawa 2015). In *Drosophila melanogaster*, however, there is no pigmentation around crossveins where *wingless* is expressed. If we assume the ancestral species had *wingless* expression and no pigmentation as in *D. melanogaster*, gain of novel expression pattern of *wingless* alone is not sufficient, for emergence of pigmentation pattern (Werner et al. 2010). Also, expression of the melanin synthesis gene *yellow* is not sufficient to induce pigmentation in the *Drosophila melanogaster* wing (Gompel et al. 2005; Riedel et al. 2011), indicating that expression changes of multiple genes were required for the evolution of pigmentation. Therefore, comprehensive exploration of genes downstream of *wingless*, which include both regulatory and effector genes, is necessary for understanding the emergence of the novel wing pigmentation pattern.

In this study, I comprehensively identified the genes that are expressed in

pigmentation areas and are also regulated by *wingless*, by two successive transcriptome analyses by Quartz-Seq, a highly sensitive method of RNA sequencing. In the first transcriptome analysis, I compared gene expression patterns between pigmentation areas and an unpigmented area and searched for differentially expressed genes (DEGs). In the second transcriptome analysis, I tested whether those DEGs were regulated by *wingless*, the master control gene for wing pigmentation.

## Materials and methods

### Flies

*Drosophila guttifera* is a North American species that belongs to (or is closely related to) the *quinaria* group of subgenus *Drosophila* (Izumitani et al. 2016; Chialvo et al. 2019). The inbred line (A5) was made by ten successive sibling crosses of a wildtype (stock no. 15130-1971.10) obtained from the *Drosophila* Species Stock Center at the University of California, San Diego. Two lines (transgenic lines No. 1 and No.2) of *D. guttifera* were used for transcriptome analyses. Transgenic line No. 1 was established by five successive backcrosses (introgression) of a transgenic line that carries *nuclear eGFP* connected with a *yellow* enhancer (*vein spot* CRE-*nuclear eGFP*, gut 1c+R GFP #12) (Werner et al. 2010) with the A5 inbred line. Transgenic line No. 2 was established by two successive backcrosses of a UAS-*wg* line (Werner et al. 2010) with transgenic line No. 1. These backcrosses aimed to unify the genetic backgrounds to improve the mapping efficiency in the transcriptome analyses. Flies were reared with standard cornmeal/sugar/yeast/agar food at 25 °C (Fukutomi et al. 2018).

### Genome sequencing and gene prediction

For gene prediction, I used sequenced genome of *D. guttifera*. Genome sequencing and genome assembly were conducted by Dr. Toyoda and Dr. Kondo. The information about the constructed libraries (number of reads, total number of base pairs, average insert size) is summarized in Table 2-1. Characteristics of the scaffolds are summarized in Table 2-2.

Gene prediction was conducted with Augustus (Stanke et al. 2004) on the scaffolds of the *D. guttifera* inbred line (A5). The option used in the analysis with Augustus was “--species=fly”.

**Collecting samples for transcriptome analysis**

Pupae at stage P12 (i) or P12 (ii) were used for two successive transcriptome analyses. These stages are just after the stage when *yellow* expression and pigmentation process have started (Fukutomi et al. 2017). The transcriptomes were compared by performing the combination of utilization of fluorescence-marked tissue, repetitive microsurgical samplings, and a sensitive RNA sequencing technology. For the first experiment, individuals from the transgenic line No. 1 which carries *eGFP* connected with an enhancer of *yellow* were used. An EGFP-positive area around a campaniform sensillum on 3rd longitudinal vein (Area 1, Fig. 2-2a), an EGFP-positive area at the tip of 3rd longitudinal vein (Area 2, Fig. 2-2a), and an EGFP-negative area on 3rd longitudinal vein in wings of flies (Area 3, Fig. 2-2b) were separated with a surgical knife under a stereo microscope SZX-16 (Olympus). By using those areas on the same wing vein, I aimed to exclude gene expression changes in regions between wing veins. The width of these tissues was about 50 μm. For the second experiment, an EGFP-positive area and an EGFP-negative area with a width of about 75 μm at the same place in individuals from transgenic lines No. 1 and No. 2 were dissected (Fig. 2-2c, d). From dissected tissues, RNA was collected with an RNeasy Micro Kit (Qiagen) and stored at -80°C. For RNA extraction, 20 dissected tissues were used for one replicate. Five biological replicates for each area were prepared (total: 20 x 5 = 100 tissues). The quality of extracted RNA was examined with an Agilent 2100 bioanalyzer (Agilent Technology).

**RNA sequencing**

The library for RNA sequencing was constructed according to the protocol of Quartz-Seq, a highly sensitive method of RNA sequencing (Sasagawa et al. 2013). This protocol includes two PCR steps. Twenty-one cycles were performed for the first PCR, and eight cycles were performed for the second PCR. RNA sequencing was performed with NextSeq 550 (Illumina).

**Transcriptome analysis and enrichment analysis**

The sequenced transcriptome was mapped to the genome of *D. guttifera* with HISAT2 (Kim et al. 2015). Transcriptome assembly was conducted with StringTie (Pertea et al. 2015). Differentially expressed genes were identified with edgeR (Robinson et al. 2010). An FDR (false discovery rate) of 0.05 was chosen as the threshold to identify DEGs.

DEGs were blasted against the protein database of *D. melanogaster*, obtained from Ensembl (Camacho et al. 2009; Howe et al. 2019). BLAST analysis was performed with Blastx using an E-value < 1e-3. The top hit outcome for each gene was taken as the result of gene annotation. Based on the obtained gene annotation, enrichment analysis was conducted with DAVID (Huang et al. 2007). Genes that could not be annotated was reanalyzed with Blast2GO (database: nr, E-value < 1e-3) (Conesa et al. 2005).

## Results

### Genes expressed in the polka-dotted pattern

I compared gene expression patterns between pigmentation areas and an unpigmented area, and searched differentially expressed genes (DEGs). These areas can be distinguished by GFP label using a transgenic line which carries *eGFP* connected with an enhancer of *yellow* (Fig. 2-1e, f) (Werner et al. 2010). I identified genes upregulated or downregulated commonly in Area 1 (pigmentation area around a campaniform sensillum, Fig. 2-2a) and Area 2 (vein tip, Fig. 2-2a), compared with Area 3 (unpigmented, Fig. 2-2b). Comparison of the gene expression between Area 1 and Area 3 showed that 2333 genes were differentially expressed. Among them, 1390 genes were upregulated (Fig. 2-3a) and 943 genes were downregulated (Fig. 2-3b) in Area 1 in comparison to Area 3. 2582 genes were differentially expressed between Area 2 and Area 3. Among them, 1593 genes were upregulated (Fig. 2-3a) and 989 genes were downregulated (Fig. 2-3b) in Area 2 in comparison to Area 3. Integrating these data, the number of common DEGs was 1035. Among them, 615 genes were upregulated both in Area 1 and Area 2 (Fig. 2-3a), while 420 genes were downregulated in Area 1 and Area 2 (Fig. 2-3b). Consistent with previously reported findings, *wingless* and *yellow* were expressed in the pigmentation areas (Werner et al. 2010; Koshikawa et al. 2015), indicating the high sensitivity and accuracy of the present method of analysis. *wingless* and *yellow* were included in the 615 commonly upregulated DEGs (Fig. 2-4, Table 2-3, Table 2-4, (the complete table is available at https://figshare.com/articles/Genes_differentially_expressed_in_the_pigmentation_areas_of_Drosophila_guttifera_/11888898)).

### Pigmentation pattern-associated genes regulated by *wingless*

Because ectopic expression of *wingless* is known to induce pigmentation, genes sufficient for pigmentation formation in wings must be included in the gene network

downstream of *wingless*. To identify the genes that are under the control of *wingless*, I identified genes upregulated or downregulated when *wingless* was ectopically expressed. Among the 615 common upregulated (Area 1 and 2) DEGs, 78 genes were upregulated by ectopic expression of *wingless* (Fig. 2-3a). In 420 common downregulated (Area 1 and 2) DEGs, *wingless* downregulated 73 genes (Fig. 2-3b). In total, 151 genes associated with the pigmentation pattern were regulated by *wingless* gene. These 151 genes were blasted against the protein database of *Drosophila melanogaster* and 131 genes were annotated. For these 131 genes, enrichment analysis with DAVID resulted 14 functional annotation clusters, and 6 of which were significant (Table 2-5). In the most significant cluster, Gene Ontology (GO) terms "Glycoprotein", "Plasma membrane", "Disulfide bond", "Signal peptide" and "Receptor" were included (Table 2-5). GO terms such as "cuticle pigmentation" and "melanin biosynthetic process" were included in the 3rd significant cluster. 20 genes that could not be annotated were reanalyzed with Blast2GO. Four genes were annotated and remaining 16 genes did not match to any gene in the database.

Among pigmentation pattern associated genes regulated by *wingless*, six genes can be categorized as melanin synthesis-related genes (Walter et al. 1996; Wittkopp and Beldade 2009; Ferguson et al. 2011). Among them, *yellow*, *laccase2*, and *tan* were upregulated in the pigmentation areas (Fig. 2-4a, b, Table 2-3, Table 2-4) and also upregulated by *wingless* (Fig. 2-5a, Table 2-6). *yellow-e*, *yellow-h*, and *silver* (*svr*) were downregulated both in the pigmentation areas (Fig. 2-4a, b, c, d, Table 2-3, Table 2-4) and in the area where *wingless* was ectopically expressed (Fig. 2-5a, b, Table 2-6).

Regulatory genes, such as transcription factors and genes involved in signaling pathways are important to understand the regulatory network controlling the pigmentation pattern. Eight transcription factors were associated with pigmentation and regulated by *wingless*. *Zinc-finger protein interacting with CP190* (*ZIPIC*), *zinc finger protein 28-like*, and Enhancer of split complex genes such as *E(spl)m3-HLH*, *E(spl)m5-HLH*, *E(spl)m7-HLH*, and *lethal (3) malignant brain tumor* (*l(3)mbt*) were upregulated in the pigmentation areas (Fig. 2-4c, d, Table 2-3, Table 2-4) and by *wingless* (Fig. 2-5b, Table 2-6). *Mothers against dpp* (*Mad*), and *Mediator complex subunit 18* (*MED18*) were downregulated in the pigmentation areas and by *wingless*. Two signal ligands, *Delta* (*Dl*) and *Wnt oncogene analog 4* (*DWnt4*, ortholog of human *WNT9A/B,* (Janssen et al. 2010)) were upregulated in the pigmentation areas and by *wingless* (Fig. 2-4c, d, Fig. 2-5b, Table

2-3, Table 2-4, Table 2-6). As receptors of ligands, *saxophone* (*sax*) was upregulated and *frizzled 2* (*fz2*) was downregulated in the pigmentation areas and by *wingless* (Fig. 2-4c, d, Fig. 2-5b, Table 2-3, Table 2-4, Table 2-6). *wingless* itself was not detected in DEG analysis with ectopic *wingless* expression, which is reasonable in my experimental design. My transgenic line ectopically drove the *wingless* gene originated from *D. melanogaster* (Werner et al. 2010) and its transcripts were not mapped on *D. guttifera* genome in the analysis.

**Discussion**

**A large number of genes were specifically regulated in the area of pigmentation formation**

Transcriptome analyses revealed that a large number of genes were specifically regulated in the area of pigmentation formation: 78 genes were upregulated commonly in the pigmentation area and by *wingless*, and 73 genes were downregulated commonly in the pigmentation area and by *wingless*. In the butterfly *Bicyclus anynana*, 132 genes were upregulated and 54 genes were downregulated in relation to eyespot formation (Özsu and Monteiro 2017). In comparison with these numbers of genes in butterflies, the number of genes identified by my results seem reasonable. However, *Drosophila* pigmentation has been thought to be a simple trait, and researchers have tried to explain the evolution of pigmentation by changes of expression of a small number of genes (Gompel et al. 2005; Jeong et al. 2006; Jeong et al. 2008; Rebeiz et al. 2009). In the abdominal tergite of *Drosophila melanogaster,* the combination of *ebony* mutation and ectopic expression of the *yellow* gene can induce ectopic pigmentation (Wittkopp et al. 2002). In *D. melanogaster* wings, however, the same combination resulted in scarcely any ectopic pigmentation (Gompel et al. 2005; Riedel et al. 2011). Those findings are consistent with those of the present study, in which I found many genes that were specifically regulated in the areas of pigmentation formation. This also suggests that experimental reproduction of the gain of pigmentation patterns through overexpression of genes is not trivial.

Although ectopic expression of *wingless* can induce pigmentation, the intensity of induced pigmentation is weaker than that of the natural spotted pigmentation. 537 genes were upregulated and 347 genes were downregulated commonly in Area 1 and Area 2 but not regulated by *wingless* (Fig. 2-3). Thus, they were not essential to make pigmentation, but might have supplemental roles, or they might be unrelated to

pigmentation but have a structural role unique to the pigmented area.

**Known gene regulatory networks of *Drosophila* pigmentation were not responsible for *D. guttifera* wing pigmentation**

Regulation of pigmentation has been studied in multiple *Drosophila* models. The best-studied case was abdominal pigmentation in *D. melanogaster*. Male-specific pigmentation was controlled positively by *Abd-B* and negatively by *bab* genes, and pigmentation common to the two sexes was positively controlled by *omb* (Kopp et al. 2000). In *D. biarmipes* wings, the pigmentation is controlled positively by *Dll*, and negatively by *en* (Arnoult et al. 2013; Gompel et al. 2005). Neither of these genes was included in DEGs in my analysis. Thus, regulatory mechanisms of pigmentation in *D. guttifera* are not a simple co-option of the known gene regulatory network of pigmentation. Then, what kinds of genes are responsible for the emergence of the novel pigmentation pattern in *D. guttifera*?

***wingless* regulated neural development genes and other signaling genes specifically in the pigmentation areas**

I identified 12 regulatory genes (transcription factor genes and signaling factor genes) that were regulated by *wingless* in the pigmentation areas. Among them, four (*Dl*, *E(spl)m3-HLH, E(spl)m5-HLH* and *E(spl)m7-HLH*) genes belonged to neurogenesis genes of GO terms (Fig. 2-6). Because Area 2 (vein tip) did not include any tissue of neural origin, these genes were considered to be expressed in epidermal cells, which is the only cell type present in Area 2. In wing discs of *D. melanogaster*, *Dl* and *E(spl)* complex genes are involved in neurogenesis mediated by Notch signaling (de Celis et al. 1996). During this process, the expression of *wingless* is necessary and sufficient for the expression of *Dl* (Micchelli et al. 1997).

Decapentaplegic (Dpp) and Hedgehog (Hh) signaling can work cooperatively or antagonistically with Wingless signaling in various aspects of *Drosophila* development (Theisen et al. 1996; Basler and Struhl 1994). In the present study, genes involved in Dpp signaling, *saxophone* (*sax*) and *Mothers against dpp (Mad)*, were regulated by *wingless* in the pigmentation areas. *hh* and *patched* of the Hh signaling pathway were up-regulated in the area of pigmentation, but not regulated by *wingless* (Table 2-7).

Wnt signaling genes *fz2* and *DWnt4* were regulated in the pigmentation areas

and by *wingless* (Fig. 2-6). Downregulation of *fz2* in the pigmentation areas of *D. guttifera* might contribute to achieving the proper gradient of Wingless protein, as it is known to do in *D. melanogaster* wing discs (Cadigan et al. 1998). *DWnt4* was expressed in the area surrounding the *wingless* expression region in wings of *D. guttifera* (Koshikawa et al. 2015), suggesting that it might play a role in pigmentation pattern formation.

For the formation of the wing pigmentation pattern of *D. guttifera*, the necessity for a transcription factor gene or signaling factor gene other than *wingless* was suggested in a previous study. When putative binding sites of T-cell factor (Tcf) family proteins (Gustavson et al. 2004) in the *yellow* enhancer were artificially mutated, EGFP expression driven by the mutated enhancer was not altered, indicating the canonical Wnt signaling does not directly give input to the enhancer, and another factor does (Werner et al. 2010). It is possible that the gene which regulates the expression of *yellow* downstream of *wingless* is included in the genes identified in this study. I also found multiple putative binding sites of Tcf family proteins around most of the genes in Table 2-6, both in *D. guttifera* and in *D. melanogaster* by using GenePalette (Smith et al. 2017), suggesting potential connections between *wingless* and these genes, although putative binding sites do not guarantee actual binding or function.

**Known functions of pigmentation genes regulated by *wingless***

In *D. melanogaster*, *yellow*, *tan*, and *laccase2* are known to be effector genes for pigmentation and have been proven to promote melanin pigmentation in *D. melanogaster* (Riedel et al. 2011; Wittkopp et al. 2002; True et al. 2005). Association of these genes with melanin pigmentation patterns was also reported in other insects (Wittkopp and Beldade 2009; Futahashi et al. 2010; Zhang et al. 2017). Therefore it was not surprising to detect these three genes in the present study. The *svr* gene, which encodes a carboxypeptidase and is involved in metabolism of N-acetyl dopamine (NADA) in *D. melanogaster* (Walter et al. 1996; Wright 1987), was downregulated in the area of pigmentation of *D. guttifera*. Expression of Yellow family protein genes such as *yellow-e* and *yellow-h* was also downregulated in the pigmentation areas on the wings of *D. guttifera*, but the molecular functions of these genes have not been identified in any insect. In wings of the butterflies (*Vanessa caudui* and *Heliconius* spp.), another Yellow family

protein gene, *yellow-d*, was upregulated at red pigmentation areas compared with black pigmentation areas (Zhang et al. 2017; Hines et al. 2012). My results together with these previously reported studies suggest that these Yellow protein family genes might play a role in inhibiting melanin pigmentation.

**Evolutionary scenario of pigmentation pattern evolution**

The genes co-opted for the pigmentation formation are highly likely to have been included in the 151 genes identified in this study. This raises the question: What kind of genetic change enabled the evolution of pigmentation patterns? There is an ongoing discussion about whether a large gene circuit is recruited or many genes are individually recruited to form a circuit during the evolution of a novel trait (True and Carroll 2002; Carroll et al. 2013). Examples of co-option of pre-existing circuits consisting of multiple regulatory genes are known from animals and plants (Glassford et al. 2015; Özsu and Monteiro 2017; Keys et al. 1999; Gao and Davidson 2008; Lemons et al. 2010; Nakayama et al. 2012). In the present case, we can ask whether the large gene network for pigmentation regulated by a master control gene, *wingless*, was co-opted all at the same time, or whether individual genes were co-opted one by one to form the current network. To my knowledge, there is no functional evidence of a pigmentation pattern mainly regulated by *wingless* in other *Drosophila* species. Therefore, a "large gene circuit model" does not seem reasonable to explain emergence of the novel pigmentation pattern in *Drosophila guttifera*.

Taking my findings altogether, I suggest that the novel pigmentation pattern of *D. guttifera* was not caused by co-options of a large gene circuit of regulatory genes and effector genes. To test whether the pigmentation pattern has evolved by multi-step co-option, I will have to compare multiple species with and without pigmentation, as well as to test individual gene functions in *D. guttifera*. These investigations will further my understanding of the evolution of pigmentation pattern formation in *D. guttifera*, as an example of the emergence of evolutionary novelties.

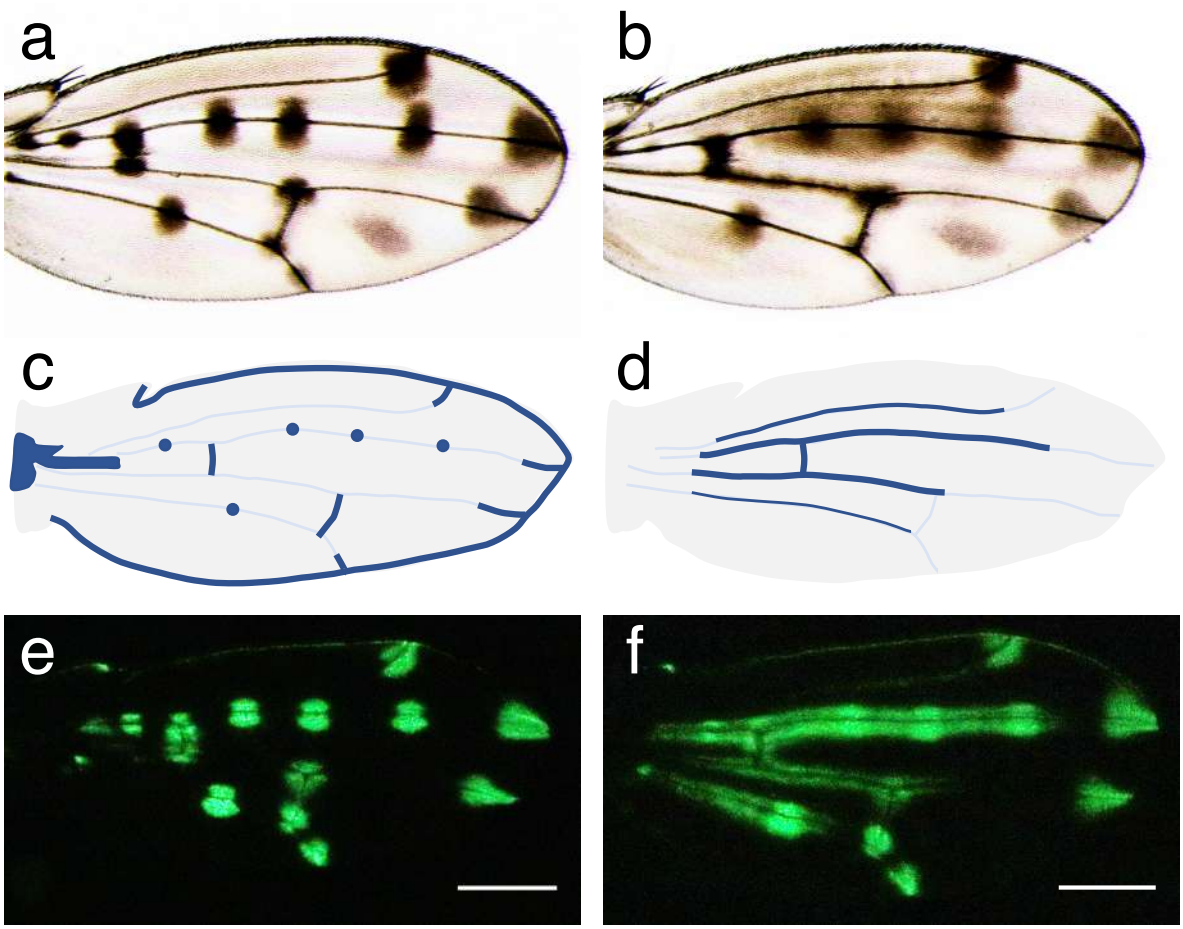


Fig. 2-1
Wings of *Drosophila guttifera* and the expression pattern of *wingless*. **a**: A wing of *Drosophila guttifera*. This picture shows wild type pigmentation pattern. **b**: The wing pigmentation pattern of an individual in which *wingless* is ectopically expressed. **c**: The expression pattern of *wingless* (blue) in a wing of a wild type individual. **d**: The position where *wingless* is ectopically expressed (blue) in the fly shown in b (Modified from Werner et al. 2010). **e**: The expression pattern of EGFP driven by an enhancer of *yellow*. In this individual, the expression pattern of *wingless* is the same as wild type. **f**: The expression pattern of EGFP driven by an enhancer of *yellow* in an individual with ectopic *wingless* expression shown in d. Individuals shown in e and f were from transgenic lines No. 1 and No. 2 for each (see Materials and methods). For experiments, 100 individuals from No. 1 and No. 2 for each were used. The expression pattern of EGFP of 100 individuals from No. 1 and No. 2 were the same as the patterns shown in e and f for each. The scale bar indicates 250 μm.

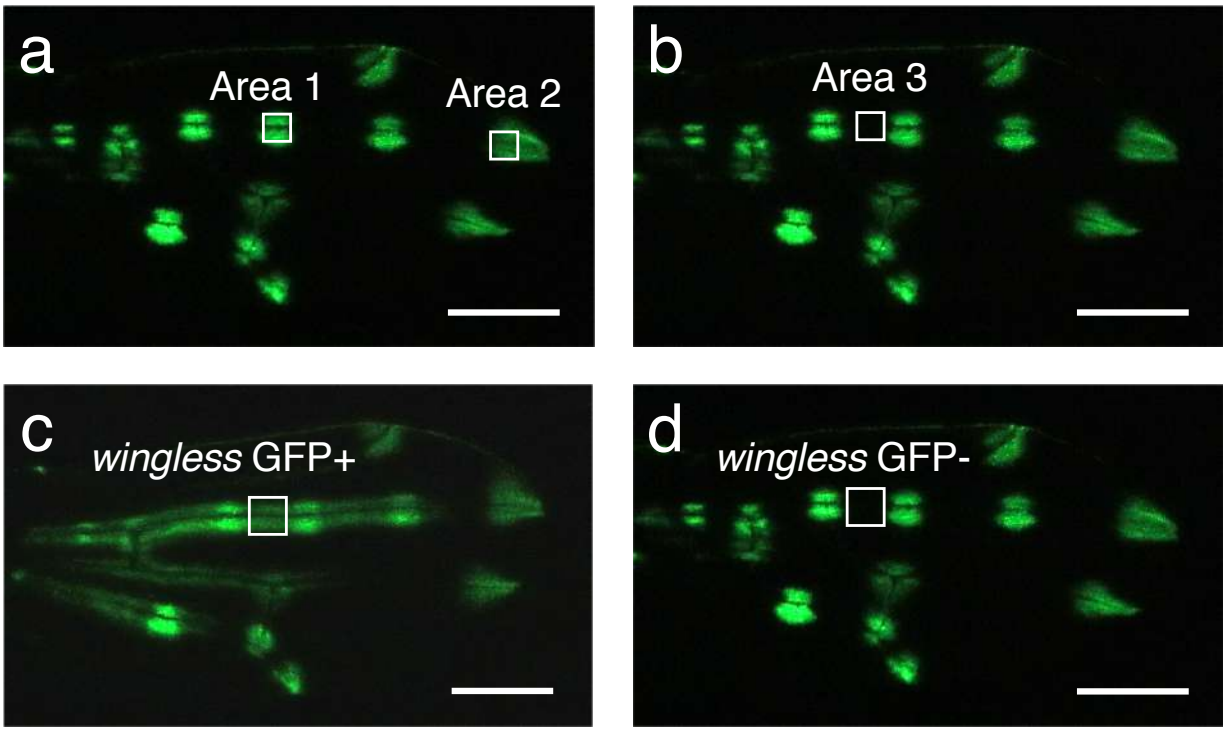


Fig. 2-2
Pupal wing tissues used for transcriptome analyses. Dissected areas are indicated with white boxes. **a**: Area 1 (an EGFP-positive area around a campaniform sensillum on the 3rd longitudinal vein) and Area 2 (an EGFP-positive area at the tip of the 3rd longitudinal vein, which does not include bristles or other tissue of neural origin). **b**: Area 3 (an EGFP-negative area on the 3rd longitudinal vein). **c**: An EGFP-positive area where *wingless* is ectopically expressed. **d**: An EGFP-negative area where *wingless* is not ectopically expressed. For each area, 20 tissues were dissected for one replicate and 5 biological replicates were used for experiments. Scale bars indicate 250 μm.

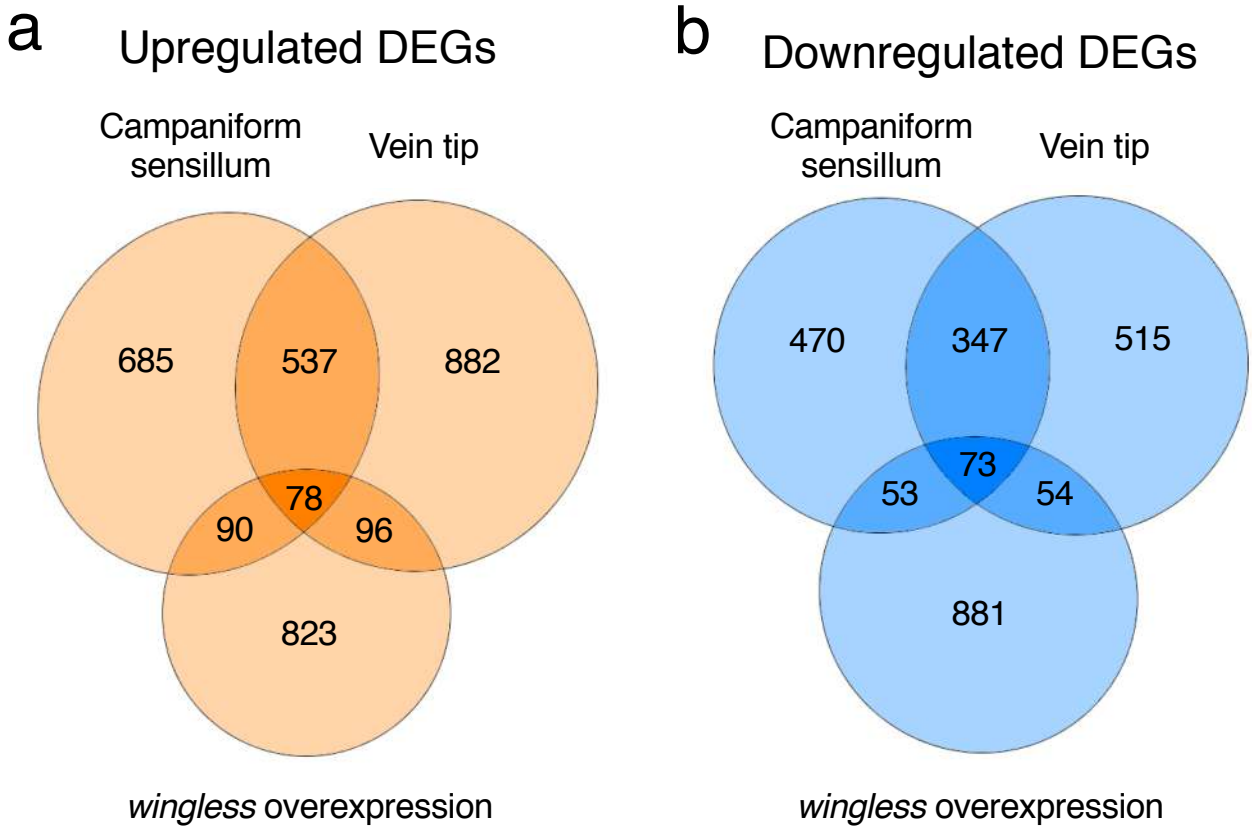


Fig. 2-3
The number of differentially expressed genes (DEGs) detected in transcriptome analyses. **a**: The number of DEGs upregulated at pigmentation areas and an area where *wingless* is ectopically expressed. **b**: The number of DEGs downregulated at the pigmentation areas and an area where *wingless* is ectopically expressed. The circle labeled with "Campaniform sensillum" indicates the result from the comparison of transcriptome between Area 1 (pigmentation area around campaniform sensillum) and Area 3 (unpigmented). The circle labeled with "Vein tip" indicates the result from the comparison of transcriptome between Area 2 (a pigmentation area at the tip of 3rd longitudinal vein) and Area 3. The circle labeled with "*wingless* overexpression" indicates the number of genes differentially expressed where *wingless* is ectopically expressed.

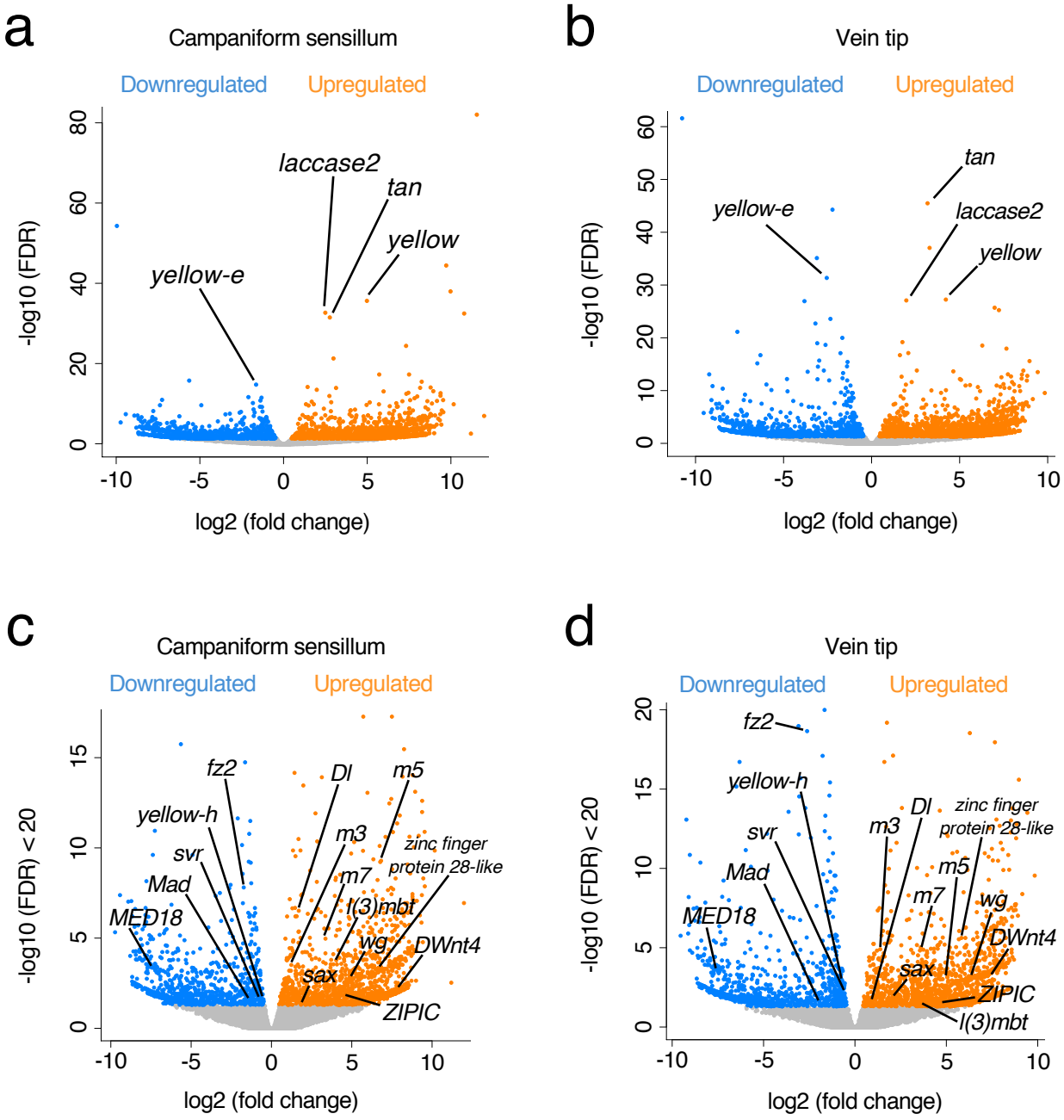


Fig. 2-4
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Fig. 2-4
Volcano plots of the results from the first transcriptome analysis. The horizontal axis indicates fold changes and the vertical axis indicates significance calculated as FDR with edgeR. Orange points indicate upregulated DEGs and blue points indicate downregulated DEGs. **a**: This plot shows genes differentially expressed in Area 1 (a pigmentation area around a campaniform sensillum) compared with gene expression of Area 3 (unpigmented). **b**: This plot shows genes differentially expressed in Area 2 (a pigmentation area at the tip of 3rd longitudinal vein) compared with gene expression of Area 3. **c**: Genes with -log 10 (FDR) < 20 are extracted from a. **d**: Genes with -log 10 (FDR) < 20 are extracted from b. In c and d, *m3*, *m5*, and *m7* indicate *E(spl)m3-HLH*, *E(spl)m5-HLH*, and *E(spl)m7-HLH* respectively.

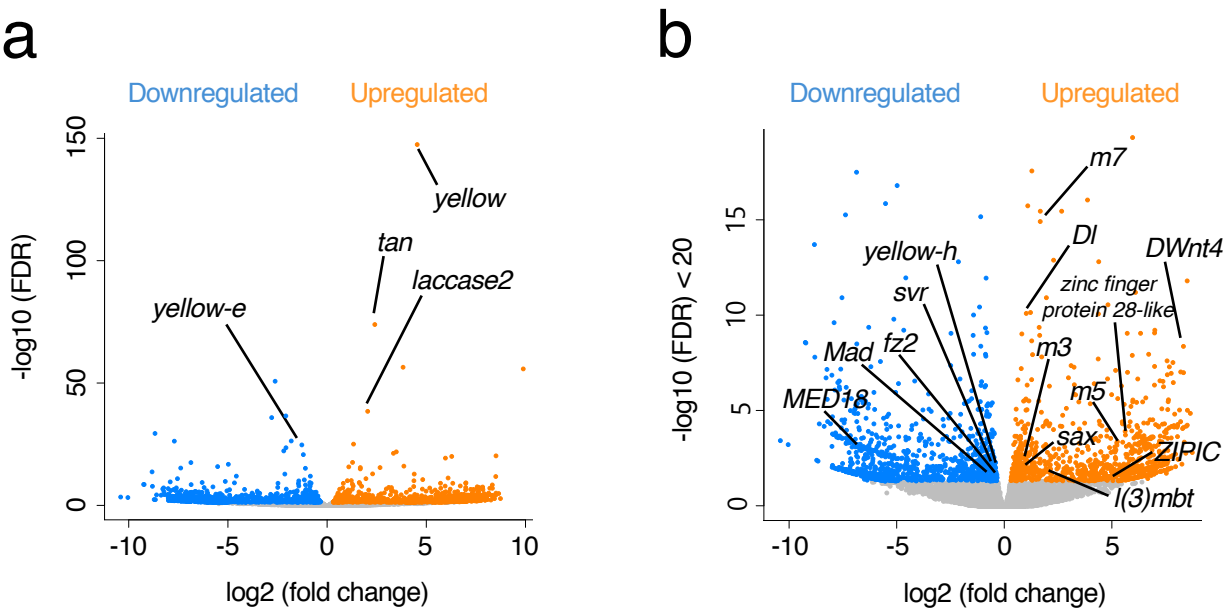


Fig. 2-5
Volcano plots of the results from the second transcriptome analysis. The horizontal axis indicates fold changes and the vertical axis indicates significance calculated as FDR with edgeR. Orange points indicate DEGs upregulated and blue points indicate downregulated DEGs. **a**: This plot shows genes differentially expressed where *wingless* is ectopically expressed. **b**: Genes with -log 10 (FDR) < 20 are extracted from **a**.

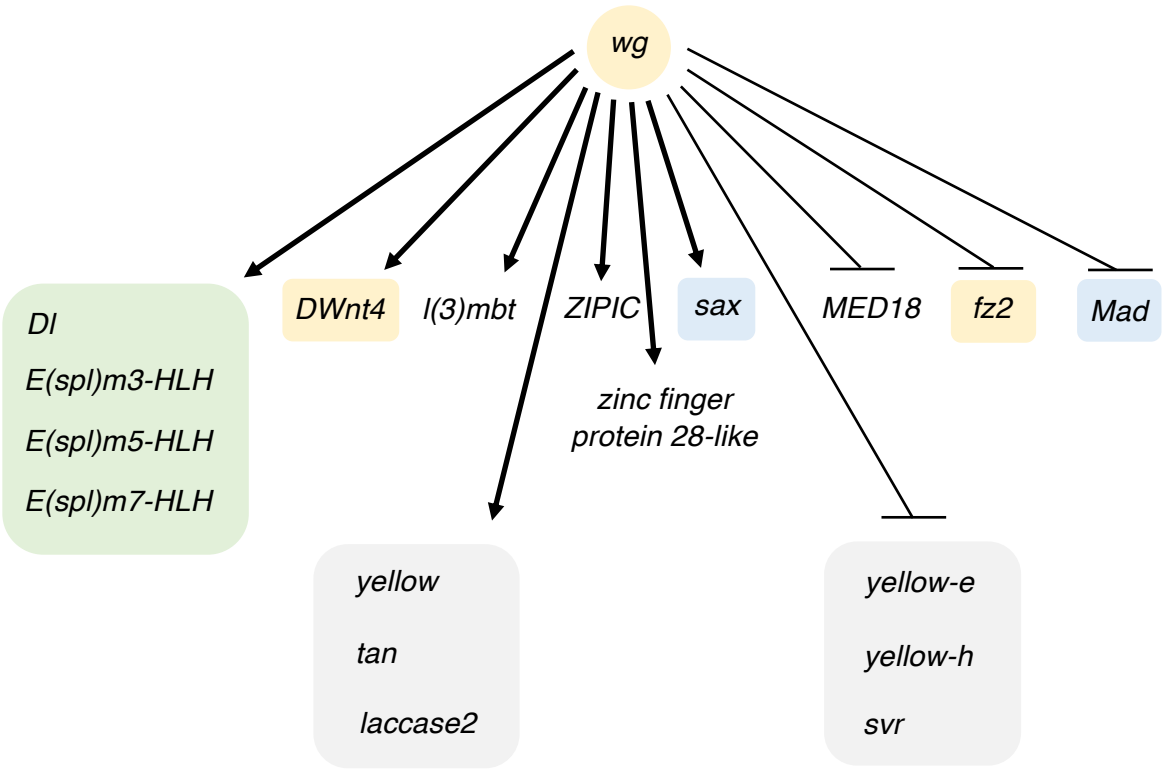


Fig. 2-6
The putative gene regulatory network for formation of the novel wing pigmentation pattern. Green box: Notch signaling gene, grey boxes: melanin synthesis genes, yellow boxes: Wnt signaling genes, and blue boxes: Dpp signaling genes.

Table 2-1
The data of the libraries of *D. guttifera* constructed for genome sequencing. PE400, MP3kbp, and MP5kbp respectively indicate the paired-end library, the mate-pair library (3kb), and the mate-pair library (5kb).

| Library | Number of reads | Total number of base pairs (bp) | Average insert size (bp) |
|---|---|---|---|
| PE400 | 151,389,704 | 22,708,455,600 | 450 |
| MP3kbp | 41,484,414 | 6,222,662,100 | 3,169 |
| MP5kbp | 30,474,280 | 4,571,142,000 | 5,227 |

Table 2-2
Characteristics of the *D. guttifera* genome sequence obtained by *de novo* assembly.

| Characteristics | Measures |
|---|---|
| Total number of base pairs (bp) | 168,421,893 |
| Number of scaffolds | 767 |
| Scaffold N50 (bp) | 1,784,351 |
| Contig N50 (bp) | 182,614 |

Table 2-3
Genes differentially expressed in a pigmentation area around a campaniform sensillum. FC, CPM, and FDR indicate fold change, counts per million, and false discovery rate, respectively.

| | logFC | logCPM | FDR |
|---|---|---|---|
| *y* | 4.97989524 | 13.1819431 | 2.38E-36 |
| *t* | 2.76380354 | 12.0058893 | 3.29E-32 |
| *laccase2* | 2.49456691 | 11.8424645 | 2.15E-33 |
| *yellow-e* | -1.6407385 | 10.3188765 | 1.81E-15 |
| *yelloe-h* | -0.5731037 | 10.5879713 | 0.02343591 |
| *svr* | -0.7948767 | 9.41379963 | 0.00438384 |
| *ZIPIC* | 4.53189996 | 0.48568358 | 0.0208437 |
| *zinc finger protein 28-like* | 6.75852343 | 1.85690936 | 0.0005392 |
| *E(spl)m3-HLH* | 1.23731928 | 8.47601255 | 0.0003062 |
| *E(spl)m5-HLH* | 6.77199195 | 3.84590398 | 6.05E-10 |
| *E(spl)m7-HLH* | 3.36253299 | 4.92651899 | 0.0000107 |
| *Mad* | -1.5333172 | 5.94437046 | 0.02335105 |
| *Dl* | 1.70066765 | 8.69275453 | 3.05E-07 |
| *DWnt4* | 7.88039218 | 2.78881957 | 0.00610713 |
| *sax* | 1.84041069 | 5.74292161 | 0.04887547 |
| *fz2* | -1.7268879 | 8.84471432 | 1.56E-08 |
| *wg* | 4.90156885 | 0.66703408 | 0.00181758 |
| *MED18* | -7.5365154 | 2.49510051 | 0.00031397 |
| *l(3)mbt* | 4.03476224 | 4.54864458 | 0.00016924 |

Table 2-4
Genes differentially expressed in a pigmentation area at the tip of a vein. FC, CPM, and FDR indicate fold change, counts per million, and false discovery rate, respectively.

| | logFC | logCPM | FDR |
|---|---|---|---|
| *y* | 4.21635634 | 12.5960958 | 5.72E-28 |
| *t* | 3.17677306 | 12.5066497 | 3.28E-46 |
| *laccase2* | 1.9708477 | 11.544914 | 8.32E-28 |
| *yellow-e* | -2.5486142 | 10.2771931 | 4.54E-32 |
| *yelloe-h* | -0.6835507 | 10.671958 | 0.00134155 |
| *svr* | -0.6447186 | 9.60117515 | 0.00423173 |
| *ZIPIC* | 4.73395417 | 0.69571295 | 0.0256575 |
| *zinc finger protein 28-like* | 5.82784953 | 1.30102744 | 0.00000242 |
| *E(spl)m3-HLH* | 1.3827603 | 8.72212278 | 0.0000125 |
| *E(spl)m5-HLH* | 5.06729935 | 2.44510302 | 0.00069965 |
| *E(spl)m7-HLH* | 3.60181175 | 5.29016258 | 0.00000958 |
| *Mad* | -2.0456884 | 5.94769958 | 0.01764714 |
| *Dl* | 0.92496862 | 8.28076591 | 0.01422465 |
| *DWnt4* | 7.3910185 | 2.48248069 | 0.00010193 |
| *sax* | 2.05209481 | 6.05221034 | 0.01234576 |
| *fz2* | -2.6177129 | 8.81769459 | 2.23E-19 |
| *wg* | 6.33393063 | 1.65109029 | 0.00062794 |
| *MED18* | -7.5507612 | 2.61383665 | 0.00022139 |
| *l(3)mbt* | 3.71774754 | 4.41678381 | 0.03790313 |

Table 2-5
The results from enrichment analysis with DAVID, showing clusters with enrichment score > 1.3 and Gene ontology terms with *p* value < 0.05 in each cluster (Count indicates the number of genes for each Gene ontology term).

| **Cluster 1 enrichment score: 2.42** | **Count** | ***p* value** |
|---|---|---|
| Glycosylation site: N-linked | 16 | 1.9E-5 |
| Glycoprotein | 16 | 4.9E-5 |
| Plasma membrane | 18 | 8.0E-4 |
| Cell membrane | 11 | 2.9E-3 |
| Topological domain: Extracellular | 10 | 6.1E-3 |
| Disulfide bond | 14 | 7.6E-3 |
| Topological domain: Cytoplasmic | 11 | 9.7E-3 |
| Transmembrane region | 13 | 1.7E-2 |
| Signal peptide | 10 | 4.2E-2 |
| Receptor | 9 | 4.6E-2 |
| **Cluster 2 enrichment score: 2.38** | **Count** | ***p* value** |
| Extracellular matrix | 8 | 3.4E-4 |
| Chitin-based cuticle development | 7 | 2.6E-3 |
| Structural constituent of cuticle | 6 | 3.9E-3 |
| Structural constituent of chitin-based larval cuticle | 5 | 1.7E-2 |
| Insect cuticle protein | 5 | 2.1E-2 |
| **Cluster 3 enrichment score: 2.18** | **Count** | ***p* value** |
| Cuticle pigmentation | 4 | 6.6E-4 |
| Melanin biosynthetic process | 3 | 7.4E-3 |
| Major royal jelly | 3 | 7.8E-3 |
| **Cluster 4 enrichment score: 1.39** | **Count** | ***p* value** |
| Membrane | 42 | 1.2E-2 |
| **Cluster 5 enrichment score: 1.34** | **Count** | ***p* value** |
| ANK repeat | 4 | 3.6E-2 |
| Ankyrin repeat | 4 | 4.7E-2 |
| **Cluster 6 enrichment score: 1.33** | **Count** | ***p* value** |
| Carbohydrate metabolic process | 5 | 1.4E-2 |

Table 2-6
Genes differentially expressed in an area where *wingless* is ectopically expressed. FC, CPM, and FDR indicate fold change, counts per million, and false discovery rate, respectively.

| | logFC | logCPM | FDR |
|---|---|---|---|
| *y* | 4.54116336 | 12.8494004 | 3.6E-148 |
| *t* | 2.40367879 | 12.0035577 | 1.25E-74 |
| *laccase2* | 2.04478128 | 11.4946868 | 3.67E-39 |
| *yellow-e* | -1.2740677 | 11.392683 | 1.96E-25 |
| *yelloe-h* | -0.4516609 | 9.7843299 | 0.00750851 |
| *svr* | -0.6215228 | 9.01017126 | 0.00382779 |
| *ZIPIC* | 5.0147075 | -0.3005216 | 0.0284088 |
| *zinc finger protein 28-like* | 5.61711109 | 2.5793453 | 0.00012374 |
| *E(spl)m3-HLH* | 0.53145394 | 8.24183597 | 0.00380777 |
| *E(spl)m5-HLH* | 5.25665975 | 1.58014564 | 0.00043059 |
| *E(spl)m7-HLH* | 1.67127368 | 5.65074921 | 1.23E-15 |
| *Mad* | -0.8277961 | 4.89916451 | 0.01393637 |
| *Dl* | 1.02257261 | 7.82859549 | 8.12E-11 |
| *DWnt4* | 8.32686641 | 2.16749637 | 4.35E-09 |
| *sax* | 0.91143441 | 5.58205955 | 0.00742031 |
| *fz2* | -0.4058316 | 8.27179547 | 0.02026252 |
| *wg* | 0 | -1.2863604 | 1 |
| *MED18* | -6.8364613 | 0.89990996 | 0.00079404 |
| *l(3)mbt* | 1.93217694 | 4.83757955 | 0.01514216 |

Table 2-7
Data of expression of *hedgehog* (*hh*), *patched* (*ptc*), and *smoothened* (*smo*) in a pigmentation area around a campaniform sensillum, at the tip of a vein, and in an area where *wingless* is ectopically expressed.

| | | logFC | logCPM | FDR |
|---|---|---|---|---|
| | Campaniform sensillum | 4.85624359 | 0.63602448 | 0.04417015 |
| *hh* | Vein tip | 7.03066917 | 2.18407496 | 0.01422465 |
| | wingless overexpression | -4.2417977 | -0.6349855 | 0.0705383 |
| | Campaniform sensillum | 6.41499537 | 3.16486805 | 0.0012656 |
| *ptc* | Vein tip | 5.79689401 | 2.74718533 | 0.02202927 |
| | wingless overexpression | -0.6229317 | 3.57406688 | 1 |
| | Campaniform sensillum | -1.4152405 | 5.26637498 | 0.51827634 |
| smo | Vein tip | -6.8976128 | 4.95229132 | 1.01E-08 |
| | wingless overexpression | 0.52985214 | 4.07845493 | 0.95456002 |

## Chapter 3

## Analysis of necessity of Wingless diffusion for determining the prepattern of wing pigmentation in *D. guttifera*.

**Abstract**

In color pattern formation, diffusion of morphogens has been assumed to determine the prepattern. As a diffusible factor that controls color pattern formation, *wingless*, one of morphogens was identified from the study of wing pigmentation pattern in *Drosophila guttifera* (Werner et al. 2010). However, the necessity of Wingless diffusion (detachment from the membrane of source cells) in determining the patterns of tissues became questionable by experiments on transgenic fruit flies (*Drosophila melanogaster*) whose wild type *wingless* gene was replaced with *NRT-Wg* (membrane-tethered Wingless) gene (Alexandre et al. 2014). In this study, I analyzed the necessity of Wingless diffusion in determining the prepattern of wing pigmentation of *D. guttifera*. In *D. melanogaster*, a part of wing pigmentation pattern of *D. guttifera* can be reconstructed as expression of EGFP driven by a *yellow* enhancer of *D. guttifera* (Werner et al. 2010). I tested whether the EGFP expressing area would be affected by tethering Wingless to the cell membrane in *D. melanogaster*. Based on the obtained result, I deduced that Wingless diffusion is necessary for determining the prepattern of wing pigmentation of *D. guttifera*.

**Introduction**

When mechanisms of color pattern formation are elucidated, we can understand how various animal color patterns have been evolved. In the cases of color patterns of zebras, leopards, and butterflies, it was assumed that morphogens (molecules which determines the pattern of animal body plans in ontogeny; Wolpert 1969) determined the pattern. To explain color pattern formation on mammal furs, the reaction-diffusion model was applied assuming a diffusing activator and an inhibitor which diffuses in a longer range (Turing 1952; Murray 1981; Kondo and Shirota 2009). In eyespots on butterfly wings, it was assumed that a morphogen diffused from a focus (the area where will be the center part of an eyespot in future) and the pattern of an eyespot was determined by the gradient of the morphogen (Nijhout 1980). Involvement of diffusible factors is also assumed in the process of determining the position of a focus (Connahs et al. 2019). There is a case such as the color pattern on zebrafish in which diffusion of morphogen is not thought to be involved (Inaba et al. 2012; Eom et al. 2015; Eom and Parichy 2017), but, in *Drosophila guttifera*, Wingless was identified as a morphogen that induced color pattern formation (Werner et al. 2010). It has been thought that, in *Drosophila melanogaster*, Wingless protein is secreted to the extracellular region and determines the

segment polarity of embryos and the proximal-distal pattern of wings by diffusion (Zecca et al. 1996; Neumann and Cohen 1997; Gilbert 2014). Therefore, in *D. guttifera*, the prepattern of wing pigmentation (wing pigmentation areas in future) was thought to be determined by diffusion of Wingless. Werner et al. (2010) suggested that Wingless protein diffused from campaniform sensilla (Lees 1942) and the area of cells received Wingless signaling became the prepattern of wing pigmentation. *yellow* gene was expressed in cells received Wingless signaling and *yellow* expressing areas in the pupal period could be regarded as wing pigmentation areas in future (Werner et al. 2010). The prepatterns of color patterns in other insects such as twin spots on the dorsal side of silkworms and eyespots on butterfly wings were also assumed to be determined by diffusion of Wingless (Yamaguchi et al. 2013; Özsu et al. 2017).

However, diffusion of Wingless might be unnecessary for determination of patterns in development. NRT-Wg (membrane-tethered Wingless) was artificially made by connecting Neurotactin (a type II membrane protein; Hortsch et al. 1990) with Wingless protein. It was shown that target genes downstream of *wingless* were expressed in only cells adjacent to NRT-Wg expressing cells in *D. melanogaster* (Alexandre et al. 2014). Because flies (*D. melanogaster*) from a transgenic line in which wild type *wingless* gene was knocked out and *NRT-Wg* gene was expressed showed the normal pattern of the body and wings, it was thought that Wingless diffusion might be unnecessary for determining patterns of tissues (Alexandre et al. 2014).

Then, is Wingless diffusion necessary for determining the prepattern of wing pigmentation in *D. guttifera*? In this study, I addressed this question by utilizing *D. melanogaster*. When *eGFP* connected with a *yellow* enhancer is introduced to *D. melanogaster*, a part of wing pigmentation pattern in *D. guttifera* is reconstructed as the expression pattern of EGFP in *D. melanogaster* (Werner et al. 2010). The expression of EGFP is observed around *wingless* expressing position, but whether *wingless* is necessary for the expression of EGFP is unclear. Therefore, in this study, I tested the necessity of *wingless* gene and Wingless diffusion for determination of EGFP expressing areas. For the experiment, I made a transgenic line which carried *eGFP* connected with a *yellow* enhancer and whose wild type *wingless* gene was replaced with *NRT-Wg* gene. Based on the result obtained from the experiment, I evaluated the necessity of Wingless diffusion for determination of *yellow* expressing areas, the prepattern of wing pigmentation in *D. guttifera*.

## Materials and Methods

### Files

I established a line for the experiment in this study from three following transgenic lines.

1. Stock no. 36336 (genotype; *w**; *CyO*, P{2x*Tb*$^{1}$-RFP}*CyO*/*T*(2;3)*ap*$^{Xa}$). This line was provided from Stock center of Indiana University Bloomington. One copy of Chromosome 2 is a balancer chromosome which has *CyO* (*Curly of Oster*) and *Tb* (*Tubby*) (dominant visible markers).
2. A transgenic line provided from Dr. Jean Paul Vincent in Francis Crick Institute. Exon 1 of *wingless* gene is replaced with *NRT-Wg* gene in one copy of Chromosome 2. Another copy is a balancer chromosome which has *CyO* (Alexandre et al. 2014).
3. m256a provided from Dr. Sean B. Carroll. *eGFP* connected with a *yellow* enhancer of *D. guttifera* is inserted in X Chromosome (Werner et al. 2010).

In this study, I compared the EGFP expressing area of flies from m256a and that of flies which have *eGFP* connected with a *yellow* enhancer of *D. guttifera* in X Chromosome and *NRT-Wg* gene homozygously in Chromosome 2. The latter individuals were obtained after more than four crosses.

Cross 1

Males from m256a and virgin females from stock no. 36336 were used in this cross. I selected $F_1$ females which had the expression of EGFP on wings in the pupal period. These $F_1$ females had *eGFP* in one copy of X Chromosome and *Tb* in Chromosome 2.

Cross 2

Virgin females collected in Cross 1 were crossed with males from a line provided from Dr. Vincent. I selected $F_1$ virgin flies (males and females) which had the expression of EGFP in the pupal period and had *Tubby* phenotype of pupae (this means that pupae had *Tb*). Collected $F_1$ flies had *eGFP* in one copy of X Chromosome, *Tb* in one copy of Chromosome 2, and *NRT-Wg* in another copy of Chromosome 2.

Cross 3

Virgin flies collected in Cross 2 were used in Cross 3. I selected $F_1$ virgin flies (males and females) which had the expression of EGFP in the pupal period.

Cross 4

Virgin flies collected in Cross 3 were used in Cross 4. I selected $F_1$ virgin flies (males and females) which had the expression of EGFP in the pupal period. By repeating this cross and selection, I established a transgenic line in which most of individuals have *eGFP* homozygouly (or hemizygously in males) in X Chromosome, *NRT-Wg* gene in one copy of Chromosome 2, and *Tb* or *NRT-Wg* in another copy of Chromosome 2. Flies used in the observation of an EGFP expressing area was obtained by selecting pupae which did not have *Tubby* phenotype.

**Observation of an EGFP expressing area**

To compare EGFP expressing areas, I used pupae from the line established after Cross 4 as the experimental group and pupae from m256a as the control group. Pupae were used when they were P14 (a late pupal stage). Wings were dissected in PBS (phosphate buffered saline, pH 7.4) after removing puparia. Dissected wings were unfolded in distilled water and mounted with 10μl of PBS. For taking photos, SZX16 stereomicroscope (Olympus, Tokyo, Japan) and a DSE-330-A digital camera system (Olympus) were used.

**Results**

The EGFP expressing areas around a posterior crossvein of flies from the experimental group disappeared or became narrower than the EGFP expressing areas of flies from the control group (Fig. 3-1 a, b). This result showed that the EGFP expressing area around a posterior crossvein became narrow when Wingless protein was tethered to the cell membrane. At the same time, it was shown that Wingless signaling is necessary for the expression of EGFP around a posterior crossvein.

**Discussion**

In this study, it was shown that the EGFP expressing area (driven by a *yellow* enhancer of *D. guttifera*) on wings of *D. melanogaster* disappeared or became narrow when Wingless protein was tethered to the cell membrane. It was also shown that

Wingless signaling was necessary for the expression of EGFP. Based on this result, we can deduce that the *yellow* expressing area will become narrow in *D. guttifera* when wild type *wingless* gene is knocked out and *NRT-Wg* gene is expressed. We can also estimate that wing pigmentation areas will become small when Wingless is tethered to the cell membrane because *yellow* expressing areas in the pupal period are regarded as wing pigmentation areas in future (Werner et al. 2010).

The result in this study showed that diffusion of Wingless protein is necessary for determining the EGFP expressing area on wings of *D. melanogaster* driven by a *yellow* enhancer of *D. guttifera*. However, this result did not necessarily show that the EGFP expressing area is determined by free extracellular diffusion of Wingless protein. There is a possibility that some of Wingless protein is transported by "active diffusion" (Akiyama and Gibson 2015). For example, some of Wingless protein might be transported by cytonemes (protrusion of the cell membrane used for transport signal transduction molecules such as Wingless; Ramirez-Weber and Kornberg 1999; Huang and Kornberg 2015). As another example of "active diffusion", some of Wingless protein might be transported by transcytosis (intercellular transport of molecules by repeat of endocytosis and exocytosis). It is suggested that transcytosis of Wingless is necessary for determining the segment polarity of embryos in *D. melanogaster* (Bejsovec and Wieschaus 1994; Moline et al. 1999). Furthermore, a factor other than diffusion of Wingless might be involved in determination of the EGFP expressing areas on wings. There is a possibility that the area might increase by division of cells received Wingless signaling. This mechanism was proposed in the study of DPP signaling transduction in wing discs of *D. melanogaster* (Lecuit et al. 1996), but was denied in the study of Wingless signaling transduction in wing discs (Zecca et al. 1996). This mechanism might occur in determining the EGFP expressing area driven by a *yellow* enhancer of *D. guttifera*.

In future, to make transgenic *D. guttifera* whose *wingless* gene is knocked out and replaced with *NRT-Wg* gene is necessary for understanding how the prepattern of wing pigmentation in *D. guttifera* is determined. To use transgenic *D. melanogaster* in which the expression of EGFP on wings is driven by a *yellow* enhancer of *D. guttifera* is also necessary. It is able to conduct thermoregulated and/or tissue-specific gene knock-out experiments in *D. melanogaster*, but those experiments are not available in *D. guttifera*. Combination of experiments on *D. guttifera* and *D. melanogaster* will facilitate

testing involvement of cytonemes, transcytosis, and cell division.

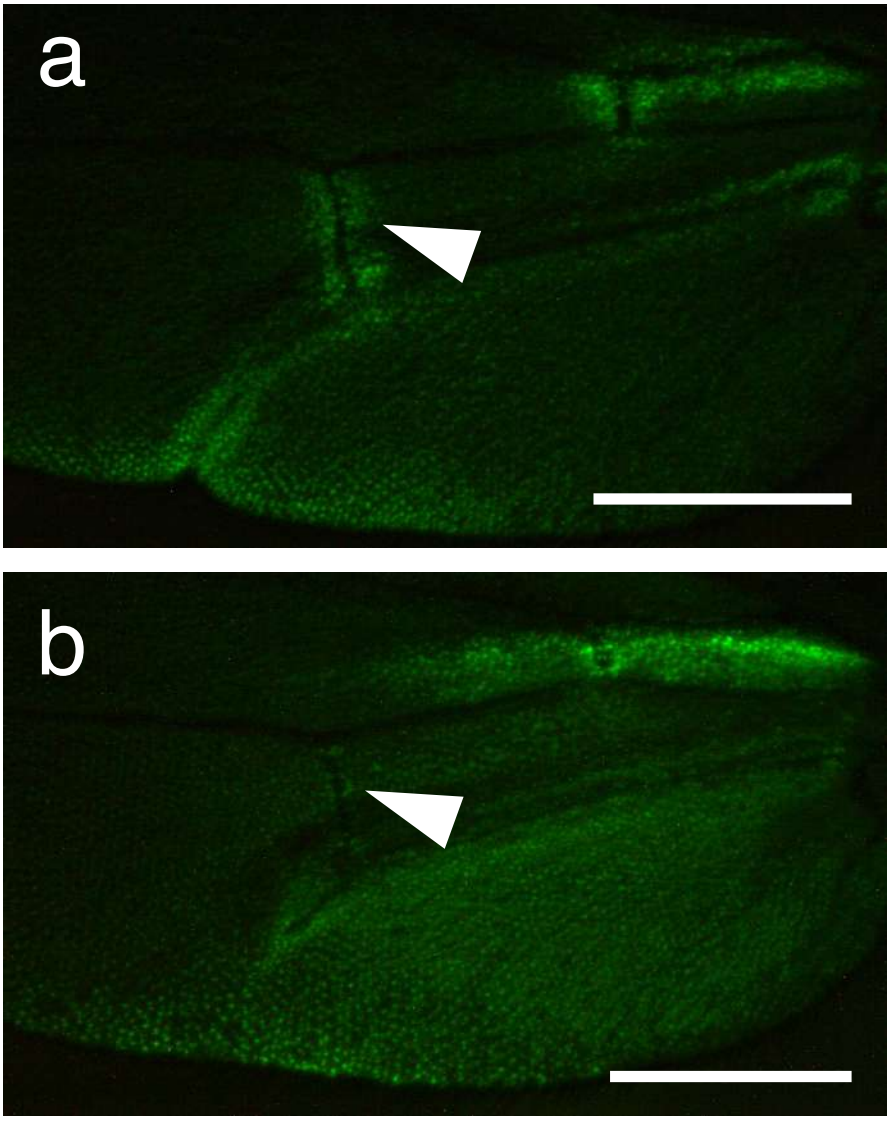


Fig. 3-1
The EGFP expression area around a posterior crossvein in *D. melanogaster*. The expression of EGFP was driven by a *yellow* enhancer of *D. guttifera*. **a** The EGFP expression area of a pupa (stage P14) from m256a which have wild type *wingless* gene (white arrowhead). **b** The EGFP expression area of a pupa (stage P14) whose wild type *wingless* gene was knocked out and replaced with *NRT-Wg* gene (white arrowhead). The area disappeared or became narrower than that of m256a. Scale bars indicate 250 μm.

## General Discussion

In this research, I revealed the process of formation of wing pigmentation in *D. guttifera* (Chapter 1) and comprehensively analyzed genes regulated downstream of *wingless* (Chapter 2). I estimated that diffusion of Wingless might be necessary for determining the prepattern of wing pigmentation of *D. guttifera* (Chapter 3). In Chapter 1, it was suggested that Yellow protein and other extracellular factors contributes to continuing formation of wing pigmentation after Yellow producing cells disappeared. This is consistent with a result mentioned in Chapter 2. Enrichment analysis for GO terms of genes differentially expressed in pigmentation areas downstream of *wingless* showed that GO terms such as "Topological domain: Extracellular" and "Extracellular matrix" were enriched (Table 2-5). The result of enrichment analysis also suggested that extracellular factors contributes to formation of wing pigmentation. Based on the results in Chapter 1 to 3, the mechanism of wing pigmentation pattern formation in *D. guttifera* is estimated as follows: First, *wingless* gene is expressed on wings and Wingless diffusion determines the prepattern. Second, the expression of genes for neural development, genes involved in Wnt and Dpp signaling, and genes for melanin synthesis are regulated by *wingless* and wing pigmentation starts in the later pupal stage (P12 (i)). Third, after eclosion, epithelial cells in pigmentation areas is retrieved from wings and wing pigmentation completes with Yellow protein, other extracellular factors, and substances transported through wing veins.

Then, how does the study of wing pigmentation pattern of *D. guttifera* contribute to understanding developmental mechanisms underlying evolution of color patterns? As mentioned in Chapter 2, *wingless* gene is expressed at crossveins on wings of *D. guttifera* (with wing pigmentation) and *D. melanogaster* (without wing pigmentation). For emergence of wing pigmentation, it might be necessary that genes for neural development, genes involved in Wnt and Dpp signaling, and genes for melanin synthesis become downstream of *wingless* (Chapter 2) (Fukutomi et al. 2020). Another *Drosophila* species, *Drosophila deflecta*, also has wing pigmentation around crossveins and the expression of *wingless* was also observed at crossveins of the species (Werner et al. 2010). In *D. deflecta*, same genes as the case of *D. guttifera* might be regulated downstream of *wingless* in pigmentation areas. Furthermore, the shape of pigmentation areas around a posterior crossvein are different between *D. guttifera* and *D. deflecta*. This difference might be able to be explained by difference of Wingless diffusing distance, but

whether *wingless* controls formation of wing pigmentation in *D. deflecta* has to be tested at first. Other factors might be responsible for the difference of wing pigmentation patterns. As it was suggested that transportation of substances through wing veins could affect intensity and area of pigmentation (Chapter 1) (True et al. 1999; Fukutomi et al. 2017), the difference of wing pigmentation patterns might be explained by this factor. Not only in *Drosophila* species, studying wing pigmentation pattern of *D. guttifera* might contribute to understanding mechanisms of color pattern formation in other insects. Wingless diffusion was assumed to determine the prepattern of twin spots on the dorsum of a silkworm (*Bombyx mori*) and eyespots on wings of a butterfly (*Bicyclus anynana*), but the necessity of Wingless diffusion in prepattern determination has not been tested. Wingless diffusion might be necessary for prepattern determination in these insects.

As a future perspective, to elucidate how morphogen is transported and what concentration of morphogen is necessary to induce expression of downstream genes will be the key point to reveal developmental mechanisms of color pattern formation. As the necessity of Wingless diffusion and the downstream genes of *wingless* was analyzed in the wing pigmentation pattern of *D. guttifera*, it can be an appropriate model to solve these questions. If these two questions are studied in color patterns of many species of insects, that will provide novel insights into the understanding of color pattern evolution.

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