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FUNCTIONAL GENETIC APPROACHES TO PROVIDE EVIDENCE FOR THE ROLE OF TOOLKIT GENES IN THE EVOLUTION OF COMPLEX COLOR PATTERNS IN DROSOPHILA GUTTIFERA

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Author Contribution Statement

The material presented in Chapter 1 of this dissertation was previously published in the journal “Methods and Protocols”, as a collaborative work with Dr. Thomas Werner, Dr. Shigeyuki Koshikawa, and Tessa Steenwinkel. All data were collected at Michigan Technological University in Dr. Thomas Werner’s laboratory. My contributions to this publication included data curation, investigation, methodology development, visualization of results, and protocol validation. The initial draft of the article was written by me and Dr. Werner and revised by me and the rest of the authors (Dr. Thomas Werner, Dr. Shigeyuki Koshikawa, and Tessa Steenwinkel).

The material presented in Chapter 2 of this dissertation was previously published in the journal “Methods and Protocols”, as a collaborative work with Dr. Thomas Werner, Dr. Komal Raja, Tessa Steenwinkel, William Dion, and Nathan Ostlund. All data were collected at Michigan Technological University in Dr. Thomas Werner’s laboratory. My contributions to this article included data curation, investigation, methodology development, visualization of results, and protocol validation. The initial draft of the article was written by me and Dr. Werner with contributions by Will Dion and Tessa Steenwinkel. The article was revised by me and the rest of the authors (Dr. Thomas Werner, Dr. Komal Raja, Tessa Steenwinkel, William Dion, and Nathan Ostlund).

The material presented in Chapter 3 of this dissertation is under review in the journal "Nature Communications", as a collaborative work with Dr. Thomas Werner, Dr. Komal Raja, Peter Nouhan, Tessa Steenwinkel, Evan Bachman, Prajakta Kokate, Alexander McQueeney, Elizabeth Mundell, Alexandri Armentrout, and Amber Nugent. All data were collected at Michigan Technological University in Dr. Thomas Werner's laboratory. My contributions to this article included investigation, data curation and analysis, article editing, and revision. The initial draft of the article was written by Dr. Komal Raja and Dr. Thomas Werner.

The material presented in Chapter 4 of this dissertation was previously published in the journal "Gene Expression Pattern", as a collaborative work with Dr. Thomas Werner, William Dion, Tessa Steenwinkel, Komal Raja, and Prajakta Kokate. All data were collected at Michigan Technological University in Dr. Thomas Werner's laboratory. My contributions to this publication included project supervision, investigation, writing, reviewing, and editing.

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Abstract

Toolkit genes are set of genes that orchestrate the development of basic body plan of animals, and they are highly conserved in all animals. The co-option of the toolkit genes into the pigmentation pathway has led to the evolution of novel species. This study focuses on understanding how the complex color patterns in animals develop by using the *Drosophila* species in the *quinaria* group as models. We developed an mRNA *in situ* hybridization (ISH) protocol, which allowed us to study gene expression patterns in the abdomen of developing pupae of non-model *Drosophila* species (Chapter 2). Through ISH, we found that the pigmentation gene *y* foreshadowed the adult *D. guttifera* abdominal pattern. Following the discovery of the *wingless* gene's (a toolkit gene) expression on the wings of *D. guttifera* by my advisor, Dr. Thomas Werner, we used ISH to screen 110 putative toolkit genes in the abdomen of *D. guttifera* to identify putative upstream activators of the pigmentation gene *y*. We identified five toolkit genes, *wingless* (*wg*), *hedgehog* (*hh*), *abdominal-A* (*abd-A*), *decapentaplegic* (*dpp*), and *zenknullt* (*zen*) that may collectively orchestrate the patterning in the abdomen of *D. guttifera*. Using the transgenic technique for modifying non-model *Drosophila* species (Chapter 1), Dr. Raja Komal (a former Ph.D. student in our lab) deployed the reporter assay technique to investigate the *cis*-regulatory elements (CREs) that control *y* gene expression. Dr. Raja showed that only one CRE (*gut y* spot CRE) controls all six rows of spots, and that this CRE contains a stripe-inducing CRE at its core. In an attempt to provide direct genetic evidence for the roles of toolkit genes in complex pattern formation, we ectopically expressed the cDNAs of the toolkit genes, using the *gut y* stripe CRE as a driver for gene

overexpression. Our intention was to manipulate the adult color pattern on the *D. guttifera* abdomen to change it into a stripe pattern. Unfortunately, the *gut y* stripe CRE could not drive the toolkit genes' expression at a time when it matters for the induction of pigmentation. Unfortunately, no earlier acting abdominal CRE is available for this species to ectopically express our toolkit genes. Furthermore, we performed RNAi knockdown experiments of the toolkit genes, which resulted in gross developmental abnormalities in the fruit flies, causing them to die before the pigmentation patterns could be observed. We suggest that this outcome may be due to the vital roles that these developmental genes play at every stage of *Drosophila* development.

To understand how novel animal color patterns evolved, it is essential to query how the formation of color patterns varies among closely related species. Therefore, we compared the expression patterns of terminal pigmentation genes (*Dopa decarboxylase* (*Ddc*), *tan* (*t*), and *yellow* (*y*)) in three fruit fly species in the *quinaria* species group – *D. guttifera*, *D. palustris*, and *D. subpalustris*. Our results show that the genes *y*, *t*, and *Ddc* are co-expressed in modular and identical patterns in the pupal abdomens in each species, which correlate with the adult abdominal pigmentation pattern.

1 Introduction

Evolution is the heritable changes that occur in organisms over successive generations due to random mutation, genetic drift, and natural, sexual, or artificial selection. This process has given rise to biodiversity at every level of the organismal hierarchy. Evolution of development (evo-devo) is a field of science that studies how developmental processes evolve over evolutionary time to give rise to the amazing diversity of life. One of the significant components of this science is developmental genetics, the tool to study how genes control animals' morphology, behavior, and growth throughout their life cycle. All animals share a highly conserved set of developmental toolkit genes that coordinate the building of animal body plans [1-4].

Pigmentation is one of the easily distinguishable phenotypic features of insects. Melanins are the most widely used pigments in both vertebrates and invertebrates, and they come in different shades and colors. Pigment patterns play numerous important ecological roles, e.g., in adaptation (camouflage and mimicry), reproduction (mating), UV protection, and thermoregulation [5, 6]. Although the biochemical pathway that leads to melanin formation is well understood, there is insufficient knowledge about how complex melanin and other color patterns are generated in animals.

Changes in developmental gene expression are essential to phenotypic evolution; however, the genetic mechanisms responsible for these changes are not yet fully understood. Several studies have shown that spatial alterations in toolkit genes' expression patterns can dramatically result in morphological changes in insects [7-9]. Toolkit genes are crucial developmental genes, which are required for building the basic body plans of animals. The

involvement of toolkit genes in melanin pigmentation of insects was reported in *Drosophila melanogaster* and butterflies. In *D. melanogaster,* the toolkit genes - *abdominal-B* (*Abd-B*), *doublesex* (*dsx*), and *bric-a-brac 1* (*bab 1*) - regulate the black pigmentation pattern in the posterior abdominal segments in males and the dorsal midline shade in females, therefore regulating sexual dimorphism [8, 10]. The evolution of eyespot patterns on butterfly wings are controlled by toolkit genes such as *Notch*, *distal-less*, *spalt*, and *engrailed* [11, 12]. Besides their role in development, toolkit genes have been reported to cause cancer and tumor growth in humans when they become misregulated [13].

My Ph.D. advisor Dr. Thomas Werner was the first to demonstrate that changes in the expression of toolkit genes play a significant role in pigment pattern formation on the wings of *Drosophila guttifera* (*D. guttifera*). He showed that the complex, black-spotted wing pattern of *D. guttifera* is induced by the Wingless (Wg) morphogen through the activation of the *y* gene [14]. In *D. guttifera*, *wg* has evolved novel expression domains in the form of spots across the developing wings, and the *y* gene has evolved a CRE that responds to the Wg morphogen signal. This study corroborated the findings in other *Drosophila* species and butterflies that novel expression patterns of toolkit genes play an important role in the evolution of pigment patterns [8, 10, 11, 12, 15]. Most importantly, the discovery of the involvement of the *wg* gene in the formation of the spot pattern on the wings of *D. guttifera* shows that the development of complex animal color patterns can have a simple underlying logic. The *wg* gene that encodes the Wg morphogen is a famous toolkit gene. The orthologous gene in humans, *Wnt-1*, is a proto-oncogene that causes tumor growth when upregulated [13, 16, 17].

Recent studies in our laboratory have shown that *wg* and additional toolkit genes may activate the *y* gene in the abdomen of *D. guttifera*. In our article under review (Chapter 3 of this thesis), we have shown that the toolkit genes *wingless* (*wg*), *abdominal-A* (*abdA*), *decapentaplegic* (*dpp*), *hedgehog* (*hh*), and *zenknullt* (*zen*) are expressed in a modular fashion in the abdomen of *D. guttifera* [18]. The *wg, dpp*, and *hh* genes are known proto-oncogenes in humans, i.e., they can cause cancer if they are misregulated [13, 16, 17].

The pigmentation biosynthesis pathway – How animals develop their color patterns

In fruit flies, melanin synthesis depends on the enzyme Dopa Decarboxylase (Ddc) and Pale. Tyrosine is converted to dopa through the action of Pale, and Ddc catalyzes the conversion of dopa into dopamine. The fate of dopamine is decided by several other proteins. Dopamine can be converted into black or brown melanin by the action of the Yellow protein and phenol oxidases, respectively. Dopamine can also form *N*-β-alanyl dopamine (NBAD) through the activity of Ebony, which forms a yellow-tan pigment by phenol oxidases. The protein Tan converts NBAD into dopamine, thus acting opposite of Ebony. Several studies have revealed the roles of the genes *y*, *t*, *Ddc,* and *ebony* (*e*) in pigment formation in *Drosophila* species. While *y*, *t*, and *ebony* (*e*) encode proteins that synthesize black, brown, and yellow pigments in *Drosophila* species, respectively, the pigments are incorporated into the developing cuticle during late pupal and early adult stages of fruit flies [19-23]. The expression of these pigment-forming genes is controlled by the *cis*-regulatory elements (CRE) of these genes, which dictate the spatio-temporal expression patterns as well as the distribution of the pigments on the adult cuticle [8, 21, 22]. The expression of the *y*, *t*, and *e* genes has been shown to be regulated by several

upstream activator genes (transcription factors). Modifications to the activator genes may affect the pigmentation patterns in fruit flies, thereby leading to the diversification of pigments in animals [7, 9, 14].

The role of *cis*-regulatory elements *and trans*-factors in gene expression

The regulation of gene expression in eukaryotes is controlled by several mechanisms, which include the structural properties of the DNA, the interactions of proteins called transcription factors, and the presence/absence of the transcription factors' binding sites [24]. In the eukaryotes, basal transcription factors and RNA-polymerase II bind to the promoter of a protein-coding gene, which initiate the transcription of a gene. However, the binding of the transcription factors to the promoter are not enough for transcription to take place. Therefore, additional factors, which may include the toolkit proteins, must bind to non-coding regions (enhancers/repressors) on the DNA, also known as the *cis*-regulatory elements (CRE) [1, 2, 4]. CREs are regions of non-coding DNA that contain a multitude of transcription factor binding sites that interact with the promoter to determine **when, where, and how much** a gene is transcribed. The CREs can be located anywhere on the same chromosome but are usually found within a few kb upstream or downstream of the promoter [25]. Most eukaryotic genes contain multiple CREs, allowing modular gene expression in time and space throughout the development of an animal.

The *trans*-acting regulatory proteins known as transcription factors bind to the *cis*-regulatory DNA sequences to activate and sustain transcription. The absence of a transcription factor from an enhancer or a repressor region will leave its binding site

unpopulated, which results in the down-regulation or up-regulation of the target gene, respectively [25].

Germline mutations cause heritable changes in organisms, which may lead to phenotypic diversity within and between species [26]. Evolutionary changes may be caused by mutations in the non-coding DNA sequences, such as in CREs. Changes in the CREs are driven by random mutation, which either lead to the loss, modification or gain of transcription factor binding sites [7, 14, 27]. Mutations in *cis*-regulatory elements can lead to novel gene expression patterns, provided the transcription factor that binds to the novel binding site is present in a given cell. However, in the absence of transcription factors, changes in the CRE will not produce a novel expression pattern; instead, they can remain cryptic until transcription factors gain new expression domains, which would be a change in *trans* [1, 2, 4, 27, 28].

Evolution in *cis*-regulatory elements and *trans*-factors play a major role in the diversification of fruit fly pigmentation patterns

Most species of *Drosophila* possess a combination of light and dark colors that are spatially distributed across the wings and abdomens. Commonly, the colors are either black or dark on a tan or yellow background. The dark pigmentation is usually shaped in the form of stripes, spots or body shades, depending on the spatial regulation of gene expression during development [22]. Variations in the expression of any individual gene may occur as a result of changes to its *cis*-regulatory sequences or the availability or deployment of the transcription factors that act upon these elements, or the combination of both [29].

Therefore, any genetic changes that affect the CREs and transcription factors of pigmentation genes may result in the diversification of species.

Studies have shown that the pigment pattern on the abdomen of an adult *D. melanogaster* is controlled by the coordinated actions of the pigmentation genes *y*, *t*, and *e* [30]. The expression patterns of the downstream pigmentation genes *y*, *t*, and *e* are controlled by their CREs and several upstream activators (transcription factors and toolkit genes). The diversification of the black color patterns on the wings and abdomens of *Drosophila* species may be due to mutations that affect CREs of the *y* gene or changes in the deployment of *trans*-factors that control them, or both [7, 30, 31, 32].

There are generally two mechanisms at work that can lead to the evolution of novel traits: evolution in *cis* and evolution in *trans*. Common to both is that a specific gene, for example a "paintbrush gene" that creates black pigment, changes its expression pattern, thereby changing the distribution of black pigment in the animal. But how is this change in the expression pattern of the "paintbrush gene" accomplished: 1) It could be due to evolution in *cis* to the "paintbrush gene", which means that CREs of that gene have mutated, thus leading either to gains or losses of transcription factor binding sites. These changes would directly affect the spatio-temporal expression patterns of the "paintbrush gene", thereby leading to color pattern changes. 2) Sometimes, however, expression changes of the "paintbrush gene" can happen even if its CREs remain unchanged. Such a scenario is called evolution in *trans*. Hereby, the real change is the novel deployment of *trans*-factors in cells, in which the unaltered CREs are "waiting" to become bound by that *trans*-factor, so that the CREs of the "paintbrush gene" now can bind to these factors and alter the gene

expression of the “paintbrush gene”. It is interesting to note that although the last example is a true scenario of evolution in *trans* from the viewpoint of the “paintbrush gene”, it is also an example for evolution in *cis* (or *trans* again) from the viewpoint of the gene that encodes the *trans*-factor protein, simply because the *trans*-factor also has to change its expression pattern in order to experience its novel deployment.

No matter if *cis* or *trans* changes have led to expression changes of the “paintbrush gene” in our example, when a new connection is established in a cell that links a new developmental pathway to a target gene, we say that the target gene has co-opted a new pathway, i.e., it got under the control of a new pathway that now determines the expression pattern of the “paint brush gene” in novel ways, leading to, in this example, new color patterns. A typical example for evolution in *cis* is the differences in abdominal pigmentation of two closely related species, *D. melanogaster* and *D. subobscura*. *D. subobscura* has darker pigmentation throughout each abdominal tergite, as compared to the abdominal tergites of *D. melanogaster*. The CRE sequences that drive the expression of the *y* gene in *D. melanogaster* and *D. subobscura* are found in a similar region, located upstream of the transcription start site of *y*. However, mutations that bring about changes in the *y* CREs between *D. melanogaster* and *D. subobscura* have been implicated in the spatial distribution and intensity of the abdominal pigmentation pattern [30]. Another study that compared the abdominal pigmentation between *D. yakuba* and *D. santomea* reported that the reduction in male abdominal pigmentation in *D. santomea* is correlated with the loss of *t* gene expression, caused by the mutational inactivation of a *t* CRE [29].

Evolutionary changes in *trans* have also been shown to contribute to the evolution of pigmentation patterns in *Drosophila* species. The *trans*-factors can be morphogens (acting as extracellular ligands), receptors, intracellular signaling proteins, and transcription factors that usually regulate hundreds or thousands of downstream target genes. A good example is the study conducted by my advisor Dr. Werner. He has shown that the toolkit gene *wingless* (*wg*) is sufficient and necessary in the generation of the 16-spotted pattern in *D. guttifera*. This study showed that the complex wing spot pattern evolved by changes in the deployment of the Wingless morphogen at the sites where the black spots on the adult wing [14].

The drosophilids of the *quinaria* species group as models for investigating the evolution of complex color patterns in animals

In order to understand how complex color patterns evolve in animals, I chose the drosophilids in the *quinaria* species group – such as *D. guttifera*, *Drosophila deflecta* (*D. deflecta*), *Drosophila palustris* (*D. palustris*), and *D. subpalustris* (*D. subpalustris*) - because these species show modularity and complexity of coloration on their abdomens. Although *D. melanogaster* has been widely studied and genetic tools are available to easily study this species, it only displays a simple abdominal pigmentation pattern, which makes it unsuitable to study complex pattern evolution. *D. guttifera*, however, is endowed with beautiful black melanin spots on its wings, six rows of longitudinal black spots on the abdomen, and dark stripes on the thorax. The six abdominal spot rows are divided into three rows on each side of the abdomen, separated by a dorsal midline shade. Each row of spots is designated as dorsal (closest to the dorsal midline), median, and lateral (closest to

the ventral part of the abdomen) [33, 34]. *D. guttifera* and *D. deflecta* are two closely related species. Unlike *D. guttifera*, *D. deflecta* lacks the dorsal midline shade and the vague intersegmental (tergite) dark stripes. In *D. palustris*, the dorsal row of spots and the dorsal midline shade are absent, whereas *D. subpalustris* lacks the dorsal midline shade as well as the dorsal and median rows of spots (Figure 1). Among the *Drosophila* species in the *quinaria* group, *D. guttifera* has emerged as a model to study complex pattern development [14, 18, 35, 36, 37, 38]. The genome of *D. guttifera* has been sequenced (unpublished), and we have developed the tool to genetically modify this organism, which has provided us the opportunity to study the mechanisms that underlie the development of complex color patterns in this species [38].

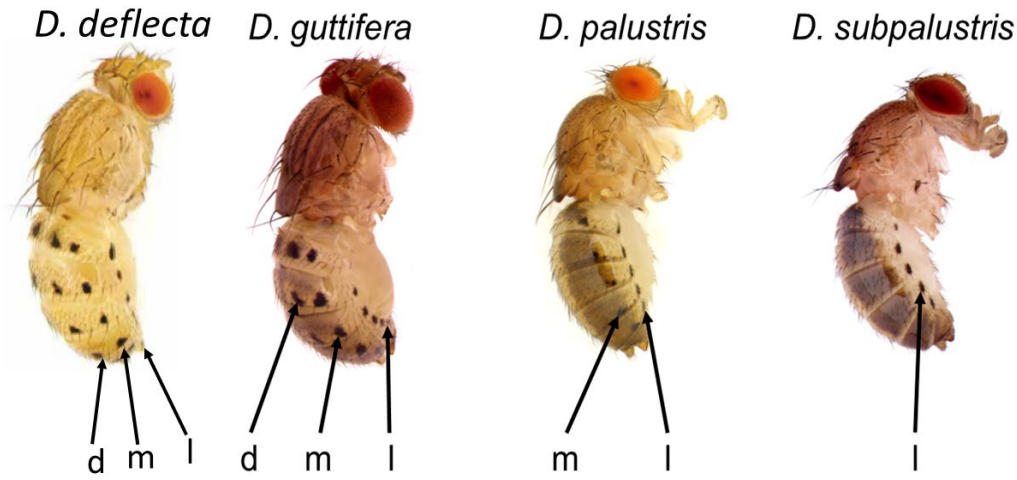

Figure 1.0. Spot pattern complexity in the *quinaria* species group. Four members of the *quinaria* group are shown from a lateral view. The dorsal (d), median (m), and lateral (l) rows of spots are labeled. Images are from Werner *et al.* [33].

This project, funded by the National Institute of Health, focuses on studying how complex color patterns evolve in fruit flies to better understand how bigger animals, such as leopards, zebras, cheetahs, etc. develop their color patterns. Understanding the mechanism underlying the activities of developmental genes during pattern development will further our knowledge of how complex patterns in animals evolve. The evolution of complex abdominal spot patterns, as observed among *Drosophila* species in the *quinaria* group, seems to occur through the gain or loss of spot rows, the gain or loss of the dorsal midline shade, and/or as a result of stripe repression. We also observed that the diversity in abdominal color patterns among *Drosophila* species in the *quinaria* group may have evolved by deviating from the *D. guttifera* ground plan (Figure 3 in Chapter 3). Therefore, we hypothesize that variations in these patterns are due to differences in the deployment of certain developmental genes that are involved in pigmentation. Through *in situ* hybridization experiments, we examined the mRNA expression patterns of pigmentation genes and candidate developmental genes during the pupal stage of *D. guttifera*, *D. deflecta*, *D. palustris*, and *D. subpalustris*.

We investigated the expression patterns of three downstream pigmentation genes, *y*, *t*, and *Ddc*, on the abdomens of *D. guttifera*, *D. palustris*, and *D. subpalustris*. Our data show that *y*, *t*, and *Ddc* are co-expressed in identical patterns in pupal abdomens. These gene expression patterns perfectly correlate with the adult abdominal pigmentation patterns in all three species. Also, our study shows that the abdominal color pattern diversity among the *quinaria* group members *D. guttifera*, *D. palustris*, and *D. subpalustris* is modular. In order to understand the molecular pathways that control the activation of the downstream

pigmentation genes, we performed *in situ* hybridization for 110 toolkit genes. We found five toolkit genes (*dpp, wg, hh, zen* and *abd-A*) that foreshadow the *D. guttifera* abdominal spot pattern in distinct subsets. We reported that the expression of *wingless* (*wg*) precisely foreshadowed the six rows of black spots, *dpp* expression foreshadowed the dorsal and median pairs of spot rows, and that *abd-A* expression correlated with the lateral pair of spot rows and the dorsal midline shade, while *hh* and *zen* were also expressed along the dorsal midline of the abdomen. In *D. palustris, wg* expression was also found to prefigure the lateral and median spot rows, as well as the lateral spot row in *D. subpalustris*, prefiguring all spot rows that are present in each of the species, which, importantly, differ among species. These findings suggest that the *wg* gene may be the activator of the patterns in *D. palustris* and *D. subpalustris* because its expression is showing on the rows where future adult spots will appear. It also suggests that the abdominal patterns have evolved due to changes in *trans*, these species likely have the *y* spot CRE but lack the *trans* landscape for making additional spot rows.

Next, we hypothesized that the developmental candidate genes (*wg*, *abd-A*, *dpp*, *hh*, and *zen*) may activate the *y* gene to orchestrate the biosynthesis of the black spots on the abdomen of *D. guttifera*. In order to test our hypothesis, we ectopically expressed these developmental genes by using the *gut y* stripe CRE that was earlier reported in our lab, to drive the expression of the cDNAs of *wg*, *abd-A*, *dpp*, and *hh*. Our aim was to change the adult color pattern on the *D. guttifera* abdomen into a stripe pattern. Also, we knocked down the mRNAs of the above-mentioned developmental genes by RNA interference technique, through a ubiquitous heat shock driver, with the aim to selectively delete

modular components of the color pattern in adult flies. Our goal was to provide genetic evidence for the role of developmental genes in assembling the complex abdominal color pattern of *D. guttifera*.

Finally, this study leveraged on the diversity in the abdominal color patterns of *D. guttifera*, *D. deflecta, D. palustris*, and *D. subpalustris* to provide evidence for the involvement developmental genes in color pattern development. The use of these species to understand how complex color patterns evolve in nature is promising. Although we can successfully establish transgenic lines of *D. guttifera* and *D. deflecta*, the development of genetic tools to manipulate *D. palustris* and *D. subpalustris* will still need to be developed to advance our understanding of the mechanisms underlying complex color patterns formation in animals.

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1 Chapter 1

THE MAKING OF TRANSGENIC *DROSOPHILA GUTTIFERA*

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1.1 Abstract

The complex color patterns on the wings and body of *Drosophila guttifera* (*D. guttifera*) are emerging as model systems for studying evolutionary and developmental processes. Studies regarding these processes depend on overexpression and downregulation of developmental genes, which ultimately rely upon an effective transgenic system. Methods describing transgenesis in *Drosophila melanogaster* (*D. melanogaster*) have been reported in several studies, but they cannot be applied to *D. guttifera* due to the low egg production rate and the delicacy of the eggs. In this protocol, we describe extensively a comprehensive method used for generating transgenic *D. guttifera*. Using the protocol described here, we are able to establish transgenic lines, identifiable by the expression of enhanced green fluorescent protein (EGFP) in the eye disks of *D. guttifera* larvae. The entire procedure, from injection to screening for transgenic larvae, can be completed in approximately 30 days and should be relatively easy to adapt to other non-model *Drosophila* species, for which no white-eyed mutants exist.

1.2 Introduction

Our ability to genetically manipulate any organism of choice has become a powerful tool for studying gene function. The introduction of a foreign DNA into the genome of an organism to produce germline transformations is known as transgenesis. Until recently, genetic manipulation in *Drosophila* has been largely achieved by transposase-mediated transgenesis [1,2]. In this technique, transposases catalyze the integration of a transposon into the genome of an organism in an unpredictable manner [3]. However, in recent years, significant improvements in fly genetic techniques have been reported, particularly the integration of transgenes into the genome in a site-specific manner through the use of different integrases and recombinases [4–7]. These techniques have been used to manipulate a variety of species [7–9], but arguably, most of these studies have been conducted in *D. melanogaster*. Many non-model *Drosophila* species offer a variety of life history and morphological traits that are absent in *D. melanogaster* [10]. Thus, our transgenic protocol will enable researchers to study new phenomena beyond the commonly used model organism.

Drosophila guttifera is a rare mycophagous species in the quinaria species group, native to the Midwest and Southeast of the USA [10]. *D. guttifera* has recently become a useful model for pigmentation studies [11]. Most studies carried out on this species are focused on unraveling the functional mechanism for the formation of polka-dotted patterns on the wings of this species. For example, Werner et al. (2010) reported that the wing spots are induced by the Wingless morphogen, and the unique polka-dotted expression pattern of *wingless* is due to the evolution in *cis*-regulatory elements of the *wingless* gene [12–14].

Furthermore, Fukutomi et al. (2017) conducted a study measuring the timing of melanin deposition in wings, using a transgenic *D. guttifera* line [15]. Ongoing and future studies in the field of color pattern development depend on the overexpression and downregulation of genes, which ultimately rely on an effective and efficient transgenesis system.

Previously, a review highlighting strategies for making transgenic *D. melanogaster* was published [16]. However, this protocol cannot be directly applied to *D. guttifera* transgenesis for the following reasons: (1) The egg production rate of *D. guttifera* is one to two orders of magnitude lower than that of *D. melanogaster*, and thus, a large fly population is needed; (2) *D. guttifera* flies die quickly from the ethanol fumes produced by the yeast required for egg-laying, requiring proper ventilation during the egg collection process; (3) *D. guttifera* females stick their eggs into the substrate, making them virtually impossible to collect from an agar surface; (4) *D. guttifera*'s eggs are covered by a thin proteinaceous chorion, which causes rapid embryonic desiccation in this species; (5) there is no white-eyed mutant available for *D. guttifera*, which is why larvae have to be screened for fluorescent markers; and (6) besides the *piggyBac* transposon, most other tested transposons do not result in transgenic *D. guttifera* [12,14,15,17]. For these reasons, critical steps must be deployed to successfully transform *D. guttifera*.

Here, we provide a detailed approach for creating transgenic *D. guttifera*. This protocol is accompanied by a video (Video S1) and troubleshooting steps, which makes it easy for researchers in the field to follow. To the best of our knowledge, this protocol is the first to describe how to make transgenic flies in a non-model *Drosophila* species. It is our

expectation that this protocol can be adopted by researchers who might be interested in experiments involving transgenesis in other non-model insect species.

1.3 Experimental design

1.3.1 Required materials and equipment

1.3.1.1 Materials

- Plexiglas egg-laying cage measuring 300 mm × 200 mm × 200 mm
- Lamp
- Instant yeast (Instaferm)
- Sponges
- Medium-sized Petri dishes (100 mm × 15 mm)
- 1-L plastic beakers for wet chambers
- 2-L plastic jar
- 5-L beaker
- Egg collection filter with Corning Netwell insert (fine-filter, New York, NY, USA)
- Egg collection filter with Corning Netwell insert (coarse-filter, New York, NY, USA)
- Squeeze bottle
- Micro cover glass (18 mm × 18 mm)
- Microslide (25 mm × 75 mm, 1 mm thick)
- Small brush (to move egg masses)
- Very fine brush (to line up individual eggs)

- Halocarbon oil 700 (Sigma-Aldrich, cat. no. H8898, Albany, NY, USA)
- Halocarbon oil 27 (Sigma-Aldrich, cat. no. H8773, Albany, NY, USA)
- 10-mL syringe
- 50-mL Falcon tube
- FHC capillary tube (Borosil 1.0 mm × 0.75 mm ID/Fiber with Omega dot fiber)
- Nitrogen gas
- CO_2 gas
- Fly food vials
- Cornmeal-sucrose-yeast medium (fly food) [10]
- Aluminum foil
- Two pairs of forceps
- Spatula
- Cotton plugs
- Moist chamber
- Anti-fungus paper (see section 6.1)

1.3.1.2 Equipment

- Flaming/Brown micropipette puller Model P-97 (Sutter Instruments, Novato, CS, USA)
- Microinjector (Narishige IM 300, Amityville, NY, USA)
- Needle-holder (Narishige, Amityville, NY, USA)
- Micromanipulator (Narishige, Amityville, NY, USA)

- Inverted microscope (Olympus CKX31, Center Valley, PA, USA)
- Dissecting microscope
- Water bath (Thermo Scientific, HAAK S3, Waltham, MA, USA)
- Mercury burner (Olympus U-RFL-T, Center Valley, PA, USA)
- Imaging microscope (Olympus SZX16, Center Valley, PA, USA) with fluorescence filters for GFP and DsRed

1.4 Methods

1.4.1 The Egg-laying cage

The egg-laying cage is made of Plexiglas that allows good aeration through the nylon entrance at the front and the plastic mesh at the back of the cage. Aeration is necessary to prevent the accumulation of toxic ethanol fumes produced by the yeast. The cage measures 300 mm × 200 mm × 200 mm and holds approximately 10,000 flies from 20 bottles. Before a clean cage can be populated, six small (35 mm × 10 mm) plastic Falcon Petri dish bottom halves are taped upside-down (using double-sided tape) onto the bottom of the cage. They will serve as “tables” for the bigger cornmeal-sucrose-yeast medium plates and must be spaced out evenly. Medium-sized empty dishes can be used to see if the spacing is good. Without these “tables”, the cornmeal-sucrose-yeast medium plates would crush lots of flies on the ground with every food exchange (Figure 1A, B).

Feed the flies in the cage every 2–3 days with six cornmeal-sucrose-yeast medium plates at a time, which are medium-sized (100 mm × 15 mm) Petri dish bottom halves, filled to the top with cornmeal-sucrose-yeast medium. Right before feeding the flies, wipe off any

excess water from the plates with a paper towel and sprinkle with some dry baker's yeast. When replacing old food plates with new plates, follow the three rules below to minimize the killing of flies:

(1) Knock the plate gently against the round Petri dish "table" to remove any flies before taking out a plate. Use the thumb and middle finger to hold the dish, while holding the index finger in place to prevent the food from falling out.

    (a) If the gap between the food and the Petri dish is large due to excessive drying of the food, then bump (knock the flies) towards the large gap. The food will not move much downwards, and no fly will be trapped.

    (b) If the gap is narrow, bump towards the side where the food still sticks to the plastic. This prevents most of the flies in the gap from being squeezed to death.

(2) One out, one in! Always take only one plate out at a time and replace immediately with a fresh plate. If there are flies on the food "table" in the cage, they can be gently pushed away with the front edge of the fresh food plate when putting it down. Then repeat this step with the remaining plates.

(3) Freeze and discard the used food as the flies developing from it will be of poor quality and a source of fungus infestations.

An egg-laying cage can be in operation for up to 10 weeks before the flies must be transferred to a new cage. If fungus grows (pay attention to places where the food comes

in contact with the wall), change the cage earlier. Perform the following steps to move the flies to a new cage:

(1) Take out all cornmeal-sucrose-yeast medium plates, close them, and continue working at a fly bench with CO_2 gas.
(2) Cover an area with unfolded paper towels to your right and put a 5-L beaker on it.
(3) Have a new cage with a fresh entrance ring plus nylon ready.
(4) Put the populated cage to the left of the new cage (not on the towels), the entrance facing upwards to prevent CO_2 from leaking out of the cage through the back wall.
(5) Open the CO_2 gas, lead it through the nylon to anesthetize the flies, and then close the CO_2 gas supply.
(6) Remove the ring with the nylon of the old cage and pour the flies into the 5-L beaker.
(7) Flies that fall on the towels can now be thrown into the beaker as well.
(8) Gas the old cage again to blow loose the remaining living flies and shake the cage over the beaker.
(9) Pour the flies from the beaker into the new cage.
(10) Close the freshly filled cage, bring it to its designed place in the lab and supply the flies with food.
(11) Clean the old cage and the nylon with tap water. Do not use soap or bleach. Rinse with distilled water.

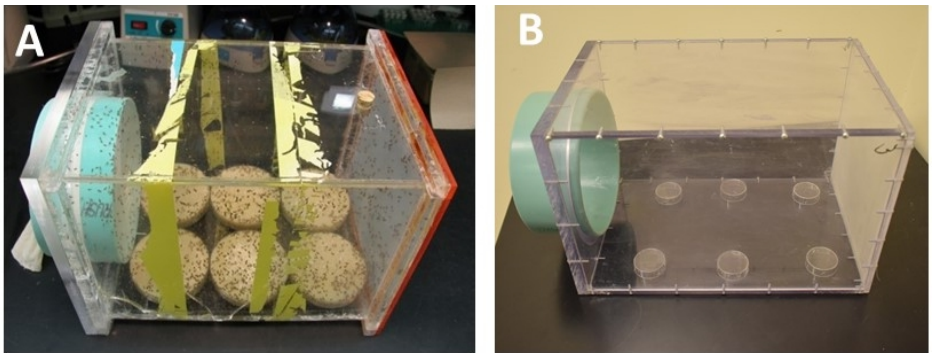

Figure 1.1. The egg-laying cage measuring 300 mm × 200 mm × 200 mm. (A) An egg-laying cage holding ~10,000 flies, which contains six plates of cornmeal-sucrose-yeast medium. (**B**) An empty egg-laying cage showing the six small (35 mm x 10 mm) plastic Petri dish bottom halves taped upside-down onto the bottom of the cage using double-sided tape.

1.4.2 Egg collection and preparation for microinjections

D. guttifera females lay comparably few eggs within a given period. For this reason, the cage setup described above is used to obtain at least 200–300 freshly fertilized eggs per hour from the population of ~10,000 mixed-sex flies. Keep the cage on the lab bench at room temperature and normal lab humidity with a 40-W lamp placed ~30 cm above it, which is timed to turn on at 6 a.m. and off at 6 p.m. In the cage, females need to be reared on fresh food with dry yeast at all times in order for their ovaries to develop and to achieve a continuous egg production. The age of the females varies, as fresh flies are provided from the bottle cultures every week. If the food is in poor condition, females will retain their fertilized eggs inside of their bodies and lay them when the eggs are too

old for microinjections. Supply the cage with fresh cornmeal-sucrose-yeast medium 3–4 times a week and especially one day before injections are carried out.

Eggs that are laid in the morning are often already cellularized and tend to have a thicker chorion, which is disadvantageous for injections. The egg quality increases later during the day. The best time to start the egg-laying is 11 a.m. Harvest the eggs in 1-hour cycles, starting at noon. It is advisable that one-person lines up two slides (~200 eggs) during a 1-hour cycle, while another person injects them. All wash steps of the eggs are done with distilled water.

⚠ **CRITICAL STEPS:** Never wash *D. guttifera* eggs with ethanol, because it seems to harden the chorion and prevents the injection needles from penetrating it, and never leave the cage
without food.

Make a starter yeast paste from instant baker's yeast grains one day before using it, and on the morning of injection, mix it again with additional dry yeast and water. Keep the yeast paste at room temperature during the injections; it should smell a bit like fermented fruit. At 11 a.m., replace all cornmeal-sucrose-yeast medium plates in the cage with four moist sponges, which are to be placed into medium-sized (100 mm × 15 mm) Petri dish bottom halves and covered with a thin layer of fresh, active yeast paste (Figure 2). A second set of four sponges will be needed for the next round.

The best way to prepare the sponges is to first make them too wet and then to carefully squeeze off the excess water with two thumbs pressing and moving from the top to the

bottom throughout the surface of the sponges, until no drops are running out easily. The sponges should be dry enough that no water runs out when flies are bumped off from them during removal from the cage but wet enough to supply the flies with enough liquid. Sponges that are too dry seem to result in thicker coronas of the eggs (the thickening near the entry site of the injection needle), while sponges that are too wet cause the accumulation of liquid in the cage. The yeast paste is then supplied evenly over the sponge surface. Cover the sponges with lids before placing them into the cage to prevent stray flies from laying eggs on them.

Allow the females to lay eggs on the 4 sponges for one hour. The flies prefer to sit on the sponge part that is closest to the cage wall. Sponges that are placed too far away from the wall are often ignored by the flies.

Figure 1.2. The yeast paste is evenly applied with a flat spatula over the sponge surface.

To collect the eggs, remove the sponges one by one from the cage and immediately replace them with sponges covered with fresh yeast paste. Gently squeeze out the eggs-

containing sponges one by one in a 2-L jar filled with 1 L of distilled water (repeatedly bend the sponges from the edge outward to avoid crushing the eggs, and the eggs will drop out of the pores), then allow the eggs to sink to the bottom of the 2-L jar for 2 min. While waiting, wash the sponges and Petri dishes with distilled water. The sponges should be squeezed hard while washing to kill any possible remaining eggs. After the four used sponges are washed, put fresh yeast paste on them and store them in a drawer (away from contaminating flies) until they are used again. At the end of the injection day, wash the sponges thoroughly and place them on a few paper towels on the bench for drying.

Assemble—from top to bottom—a funnel, a coarse-filter basket, and a fine-filter basket (Figure 3A). Next, pour the upper half of the egg collection water into the sink (the eggs are at the bottom of the jar). Then swirl and pour the remaining water with the eggs into the funnel/filter system. The coarse-filter traps sponge fibers and flies, while the fine filter collects the eggs (Figure 3B). Rinse the 2-L jar once with 100–200 mL of distilled water, then pour it through the filter system to collect the remaining eggs. With a squeeze bottle of distilled water, wash the eggs off the upper filter and allow them to slip through the coarse mesh into the fine filter below. After obtaining a sufficient number of eggs in the lower filter, remove the baskets from the funnel and wash the eggs thoroughly with distilled water from a squeeze bottle. Then, put the basket with the washed eggs into a bottom half of a small Petri dish, which is halfway filled with distilled water. Line up the eggs under a dissection scope.

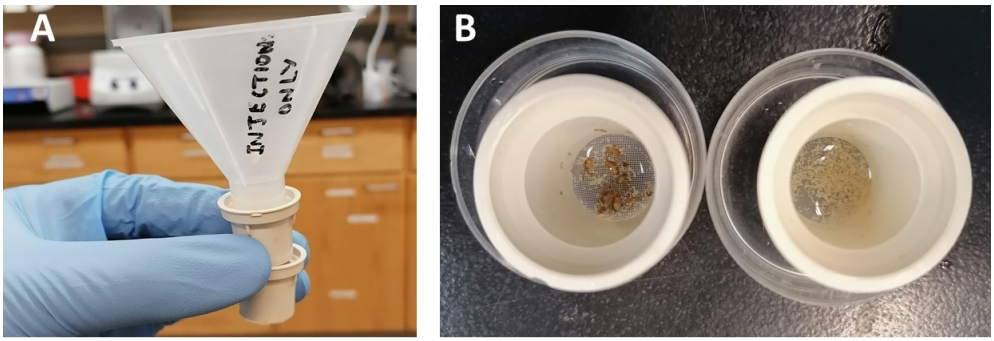

Figure 1.3. Egg collection with a funnel and two filters. (**A**) The funnel is placed on top of the coarse filter, and the fine filter is held underneath to collect the eggs. (**B**) The coarse filter (left) mainly traps the flies and sponge fibers, while the fine filter (right) retains the eggs.

1.4.3 Lining up the eggs for microinjections

Place a micro cover glass (18 mm × 18 mm) onto a microslide (25 mm × 75 mm, 1 mm thick) with a small droplet of water so that it sticks. Dry off the edges with a piece of tissue. The washed eggs are now transferred from the basket onto the cover glass as a clump, using a small brush. It is essential that the eggs are kept wet at all times. During lining up, always pick only the white and structureless-appearing eggs. It is advisable to put a clump of more than 100 eggs into the upper middle of the cover glass. Then, use a very fine brown-haired brush, make it wet, briefly dry it on a tissue paper, pull the first egg into position, dry the brush, pull the next egg to extend the line, and so on.

Align the first 10 eggs with a relatively dry (very fine) brush, or otherwise, the surface tension will make it impossible to position them correctly. Then, gently move the clump of ~100 eggs down while extending the line of selected eggs downwards, otherwise you

lose sight of your egg clump, and the eggs may dry out and die by accident. In the end, the row consists of about 100 eggs, which shall have their posterior end towards the right edge of the cover glass. The row of eggs should be positioned about 5 mm away from that edge. Keep the eggs moist at all times by supplying water with the brush to the clump of unaligned eggs, as well as the top, middle, and end of the row of aligned eggs. When the row is completed, remove all unused eggs and let the row dry, while carefully monitoring the eggs under the microscope. The row can now be rearranged slightly U-shaped to make them fit better to the cornmeal-sucrose-yeast medium surface later. When the egg line appears completely dry, wait about 3–5 more seconds (depending on the humidity of the room) and then quickly cover them with the Halocarbon oil mixture (7 parts of type 700 + 1 part of type 27), supplied by a syringe (Figure 4). The Halocarbon oil stops further desiccation but allows breathing. The halocarbon oil mix must be prepared at least a day before you can use it, so plan ahead.

⚠ **CRITICAL STEPS:** If the eggs become too desiccated (oil added too late), they die and become deformed when the needle pokes against them. If, however, the oil is put on too early, the eggs will not stick to the glass and move away when the injection needle is poked against them. Only a few seconds lay between both extremes. Do not try to inject a slide with swimming or dead eggs. Instead, save your needles for a better slide.

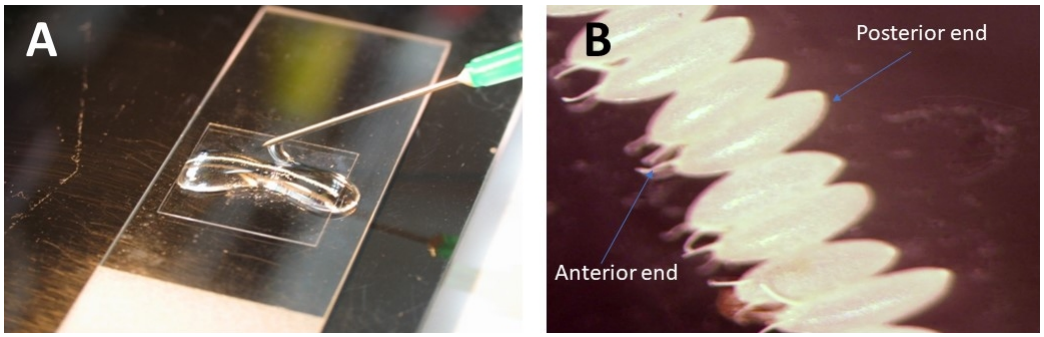

Figure 1.4. *D. guttifera* embryos lining-up. (**A**) Adding the halocarbon oil mixture to a line of eggs. (**B**) The anterior ends of the eggs contain the filaments (left), while the posterior ends point to the right.

⚠ **CRITICAL STEPS:** Note the following important timelines: egg-laying in the cage = 1 h, egg collection and lining up = 20 min, injecting one slide containing about 100 eggs = 15 min. It is important to ensure that the eggs are in the syncytial blastoderm stage during injection, which lasts for about 2 h or less at room temperature.

1.4.4 Preparation of transgene DNA

(1) Follow the manufacturer's instructions on the isolation of the plasmid DNA containing the transgene of interest from an overnight *Escherichia coli* culture. In this described experiment, we use the Invitrogen HiPure Plasmid Midi-Prep Kit by Thermo Fisher Scientific (Waltham, MA, USA).

(2) Add 3.5 mL of isopropanol to the eluted DNA in a Corex glass vial.

(3) Close the Corex vial with Parafilm, invert 10 times, take away the Parafilm, and wipe off any drops with Kimwipes.

(4) Centrifuge at 11,000 rpm for 30 min at 4 °C.

(5) Mark the area of the DNA pellet with a marker, decant, and invert the vial on a paper towel.

(6) Add 350 µL of sterile MQ water and dissolve the DNA pellet by vortexing.

(7) Collect the plasmid DNA in a 1.5-mL Eppendorf tube.

(8) Add 35 µL of 3 M of sodium acetate (pH 5.5).

(9) Add 875 µL of 200-proof ethanol and invert 10 times.

(10) Spin the DNA at 14,000 rpm for 20 min at 4 °C on a bench-top centrifuge.

(11) Remove supernatant and wash with 300 µL of 70% ethanol by inverting the tube 10 times.

(12) Centrifuge again at 14,000 rpm for 10 min at room temperature.

(13) Remove supernatant and centrifuge for 1 min.

(14) Remove all ethanol and dry the pellet for 5 min.

(15) Dissolve the DNA in 50–100 µL of elution buffer, measure the DNA concentration, and add more elution buffer if necessary, to a final concentration of 1 µg/µL.

1.4.5 Preparation of the injection cocktail

(1) On ice, add 20 µL of transgene-containing *piggyBac* construct (1 µg/µL) to a 1.5-mL test tube, 5 µL of the *piggyBac* helper plasmid (phspBac ([1 µg/µL)), and 15 µL of sterile distilled water. Note: We do not add food color, because it is not necessary for the visualization of the injected material.

(2) Centrifuge the DNA mixture for 20 min at maximum speed, and transfer the upper 39 µL of it to a fresh tube (do not touch the bottom of the tube with the pipette tip to prevent transferring undissolved material that would clog the needle). Spin the DNA

again for 20 min and transfer 38 µL of the injection mix into another fresh tube. This DNA is now clear of debris and can be used to load the needles. The *piggyBac* system pBac (backbone)/phspBac (*piggyBac* helper) results in 1 transformation event every 50 *D. guttifera* eggs with an empty vector and 1 every 500 eggs with an 8-kb insert [18].

1.4.6 Needle preparation and microinjections

Use a Flaming/Brown micropipette puller Model P-97 and FHC capillary tubing (Borosil 1.0 × 0.75 mm ID/Fiber with Omega dot fiber) to obtain the standard needles. We currently use the following parameters, but these parameters can change based on the filament, machine, and humidity of the room: P = 500, Heat = 496, Pull = 125, Vel = 10, Time = 186 [19]. Do not use gloves when pulling needles, but wash your hands before touching the capillaries! Extreme care should be taken to avoid touching the platinum heating filament of the machine with the glass capillaries. Load the needles at least one hour before using them with approximately 0.5 µL of the DNA mix, and store them in a moist chamber at 4 °C for a few days. Use an automated injection system based on nitrogen pressure, a micromanipulator for holding the needle, and an inverted microscope for viewing.

The very tip of every needle must first gently be broken off before the first injection can happen. To do this, carefully touch the chorion of an egg with the needle tip, while continuously pushing the "BALN" or “INJ” button (Figure S1), until the DNA flows out. (Different models may have different button names; please refer to the user manual.) Ensure that the needle is not badly broken, as this may cause the granular cytoplasmic

content to ooze out of the egg (Figure 5A). Inject the eggs with a very fine needle tip into the posterior-most part (Figure 5B) by hitting either the “INJ” button or the foot pedal (it is a matter of preference).

In order to prevent the needle from getting clogged, the “BALN” or “INJ” button or the foot pedal should be hit frequently, while the needle tip is outside of the eggs. If the needle is clogged, carefully rub the tip of the needle against an egg while holding the BALN button pressed. Additionally, moving the eggs away from the needle at high speed can help make the DNA flow again.

⚠ **CRITICAL STEP:** The needles must be changed when the eggs start leaking out a lot of granular cytoplasm, whereas small clear droplets are acceptable and normal.

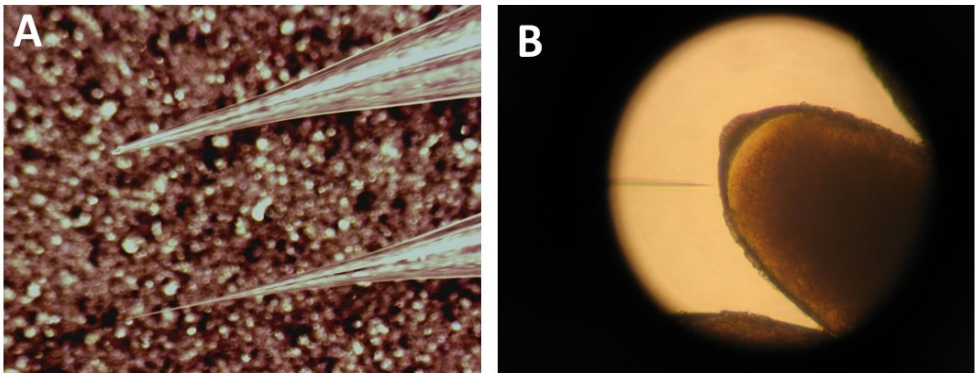

Figure 1.5. Needle qualities and correct injection site. (**A**) The top needle is a badly broken needle, while the second needle is the standard needle. Note that the best needle is the one that has a beveled tip with a small opening. (**B**) The transgene can be injected through the posterior part of the egg.

1.4.7 Post-injection treatment and heat shock of the eggs

After injecting all eggs on a slide, remove the slide from the microscope stage, remove the cover glass from the slide, and briefly let the oil drop off. Quickly wipe off some excess oil against the microscope slide. Stick the cover glass twice into a fresh cornmeal-sucrose-yeast medium vial, thereby carving out a small trench of food. Then place the posterior ends of the eggs with the cover slip into the trench without pushing the eggs too hard against the food. Close the vial with a cotton ball and squeeze enough distilled water into the cotton ball to provide immediate moisture (do not make them dripping-wet but sufficiently moist). Place the vial vertically into a wet chamber (a beaker with 5-mm-high water on the ground and 2 wet paper towels stuck against the inner wall). Hold up to 8 vials with injected eggs together with a rubber band (Figure 6). Avoid water flowing into the vials by keeping the cotton balls low in the vials, so that they do not touch the wet paper on the side of the wet chamber. Close the chamber with aluminum foil, label the lid, and store it at room temperature.

On the next morning (16–18 h after injection), heat shock the eggs at 40 °C for 90 min in the water bath (use a rubber band to hold the vials together, and submerge the vials as deep as needed to get the food level under water; do not let any water get into the vials!). After the heat shock, put the vials back into the chamber (now remove the wet paper towels that lined the wall). Place the foil lid back on, and store the chamber (with some water at the bottom) at room temperature for roughly a week. Check daily for problems (mold or flooded vials) and the appearance of the first pupae.

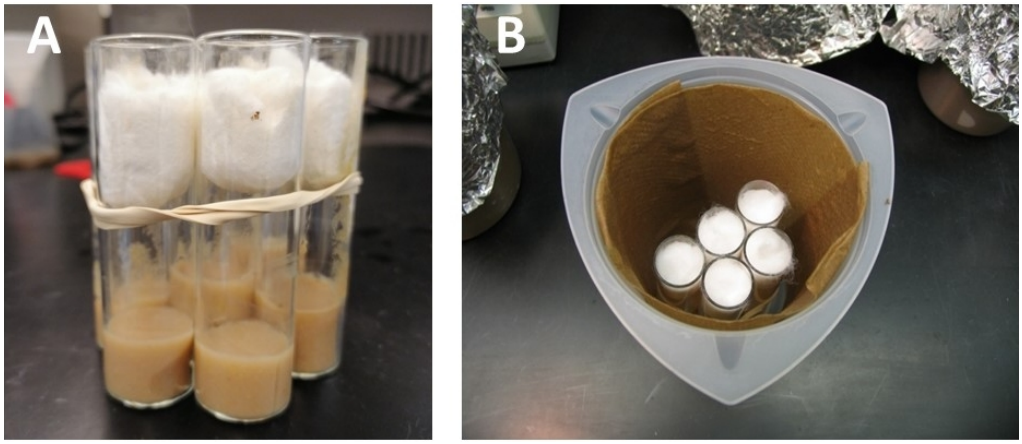

Figure 1.6. Keeping injected embryos moistened. (**A**) Vials are bundled together with a rubber band. (**B**) A wet chamber for the vials containing injected eggs.

1.4.8 After-care and fly crosses

Look at the moist chamber every day to ensure it remains moistened. When most pupae have formed and no more wandering larvae are seen, collect the pupae from the cotton plugs with a brush and two pairs of forceps (Figure 7A). Making the cotton balls very wet helps to easily detach the pupae. Do not leave much cotton left on the pupae; however, a few fibers can remain attached. Collect the pupae temporarily on a wet tissue paper. Take a clean and empty glass vial ("hatching vial"), place a long piece of anti-fungus paper along one side of the wall, and moisten the paper with a squirt bottle (remove excess water with a paper towel afterwards so that no free water is in the vial, or else the pupae will drown). Transfer the collected pupae with a moist brush from the wet tissue paper onto the anti-fungus paper in the vial. Then, collect the remaining pupae of the same construct as described above, and combine them with the others in the same hatching

vial. Close the hatching vial with a fresh cotton ball (push the ball deep enough down to prevent it from picking up water), and store it angled upwards (on a small plastic lid from a pipette box) in a moist chamber (Figure 7B).

⚠ **CRITICAL STEP:** Moisten the anti-fungus paper inside the glass vials whenever needed, and never let the anti-fungal paper dry out. Check the chamber twice daily.

Start collecting wild-type virgin flies of both sexes for backcrosses as soon as you see the first pupae forming from the injection experiment vials. The collected virgins are stored in cornmeal-sucrose-yeast medium vials with a few grains of dry baker's yeast and must be bumped to fresh vials every 3–4 days (or they will get stuck in slimy food). They will be sexually mature when your first injection survivors hatch. The hatched flies from the injections (the injection survivors) are collected once a day in the late afternoon on a CO_2 pad and immediately crossed with wild-type virgins of the opposite sex. Use cornmeal-sucrose-yeast medium vials with a few grains of baker's yeast, and combine 3 males or 10 females of the injection survivors with 15 wild-type virgins of the opposite sex in one vial. The lumping of injection survivors into the same cross is done to produce enough larvae per vial (to keep microorganisms in the culture down). Bump the crosses to fresh food every 3–4 days. Look at your crosses and their offspring every day. Screen for transgenic larvae 3 days after the parents have been removed from the vials.

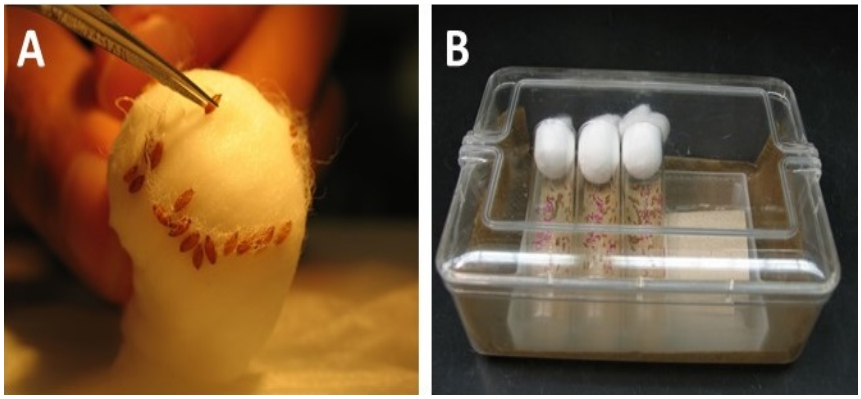

Figure 1.7. Collection of pupae and after-care. (**A**) Pupae are collected from the cotton plugs with fine forceps. (**B**) Pupae collected on moist anti-fungus paper in a hatching vial and stored angled upwards in a moist chamber.

1.4.9 Screening for transgenic larvae

The larval enhanced green fluorescent protein (EGFP) screen is carried out in the fly room with the main lights switched off as follows:

(1) Switch on the UV lamp 10 minutes prior to screening.

(2) Microscope settings: light filter = “GFP”, bottom filter = “Oblique”, objective = 1× shutter open, camera tract closed, and all visible microscope lights off (check that the bottom light is off as well!).

(3) Repeat the following steps until all vials have been screened:

(a) Remove the larvae from a food vial by squirting distilled water (squeeze bottle) and stirring with a rough brush; then, pour the larva/food soup into a medium-sized Petri dish

bottom half.

(b) Collect positive larvae (Figure 8) with a pair of forceps into 2-mL tubes filled with 1-mL cornmeal-sucrose-yeast medium (briefly angle the food by centrifugation, allowing the larvae to be removed more easily from the collection forceps). Wash and screen each vial 3 times for transgenic larvae.

(c) Poke two holes into each lid (not too big that the larvae can escape) after putting larvae into the 2-mL food tubes (only add up to 10 larvae per vial). Label each vial with the construct and line information.

(d) Discard screening soup with negative larvae and food debris into an empty 5-L beaker. Rinse the Petri dish with distilled water after screening each vial.

1.5 Expected results

The perfect timepoint for screening for transgenic larvae is 3 days after the parents have been removed from the vials, as all larvae would have hatched, but the pupae would not have formed yet. EGFP can only be detected in larvae but not in eggs, pupae, or the adult flies. Look for EGFP in the larval eye disks, Bolwig organs, brain, and/or in the anal plates (Figure 8) [20]. EGFP expression is usually strong and clearly visible in all larval instars. Finding multiple positive larvae per vial is not uncommon. Note that many transgenic lines are obtained in later screening sessions, so it is worth the time and patience bumping the crosses for 2 or 3 weeks before declaring them negative.

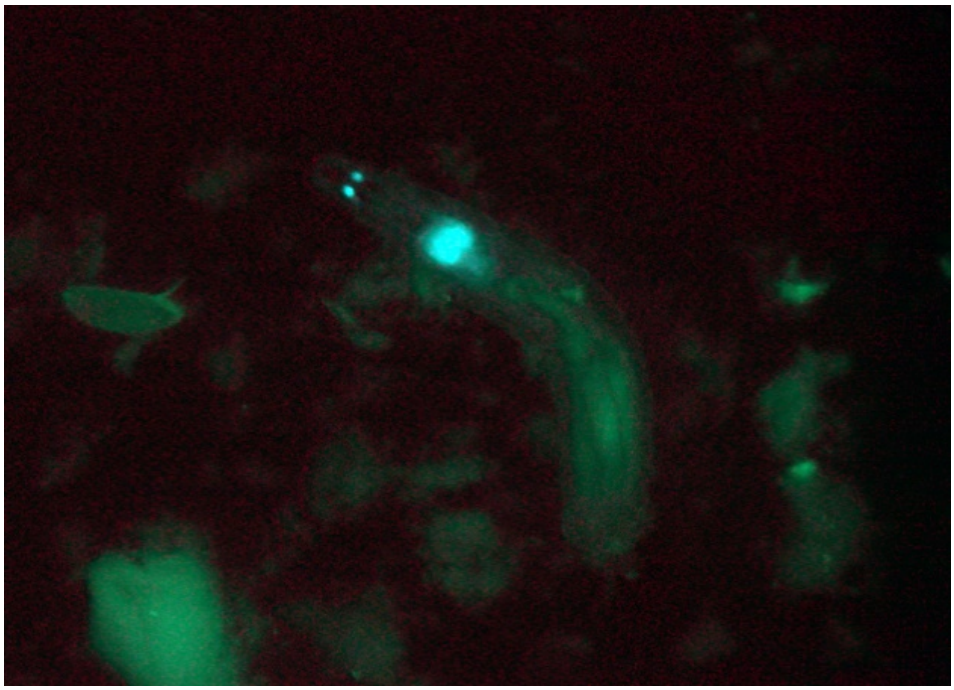

Figure 1.8. A transgenic *D. guttifera* larva showing enhanced green fluorescent protein (EGFP) expression in the Bolwig organs (two small glowing dots on the left) and the eye disks/brain (larger blob to the lower right).

1.6 Troubleshooting

Table 1.1: Troubleshooting guide (summarizes a variety of problems that we have encountered in the past. The table explains the reasons underlying the problems and offers appropriate actions to work around each problem).

Problem	Reason	Solution
Few larvae hatch.	Bad injection needles.	Make sure you use standard needles. Strictly follow the instructions under Section 3.6.
	Over-desiccation of eggs during lining up.	Add injection oil within 3–5 s after eggs appear completely dry under the microscope.
	Toxic DNA.	Use highly pure DNA (no mini-preps). Additional isopropanol precipitation after the first extraction of DNA is necessary.
	Too-high heat shock temperature.	The heat shock temperature for D. guttifera transformation is 40 °C; however, if this temperature is causing strong reduction in the survival rate, reduce the temperature by 1–2 degrees but never go below 37 °C.
	If the Halocarbon oil mixture is suffocating and killing the eggs. This does not normally happen, but we have seen this problem in our lab.	Use Halocarbon oil 27 only or GEM extra virgin olive oil. We are using extra virgin olive oil in our lab with high success.
Few surviving adults.	Having only a few injection-surviving larvae causes bacterial growth and/or mold formation in the food.	Ensure a high injection survival rate. At least 10–20 larvae can keep the food mold-free.
	Desiccation of pupae due to dry chamber and/or dry cotton plugs.	Always keep the chamber and the cotton plugs
No transgenic larvae.	Poor DNA quality.	Always clean the DNA by isopropanol precipitation or prepare new DNA samples.
	Insufficient heat shock temperature.	Always ensure the heat shock temperature is not below 37 °C. Check the temperature of your water bath with a calibrated thermometer.

1.7 Reagents

1.7.1 Anti-fungus paper

Dissolve 2 g of sorbic acid and 0.6 g of methyl paraben in 200 mL of 95% ethanol. Roll ~20 paper towels and stick them into a 1-L glass beaker. Slowly pour the anti-fungus solution over the towels to soak them evenly. Unfold the towels and let them dry completely. Anti-fungus paper does not go bad and can be stored for years.

1.7.2 Halocarbon oil mixture

Use a 50-mL Falcon tube, add 35 mL of Halocarbon oil 700 and 5 mL of Halocarbon oil 27. Homogenize the mixture on a nutator overnight.

1.8 Conclusions

The starting point for this protocol was the standard method that was optimized for *D. melanogaster*. We quickly learned that "whatever works for *D. melanogaster* does not work for/kills *D. guttifera*." It took therefore several years of innovations and deviations from the original protocol to make *D. guttifera* survive the procedure from the beginning to the end and to obtain the first transgenic animals. Key innovations are (1) establishing a large cage population (this compensates for the low egg-lay rate), (2) having good cage aeration (this prevents the accumulation of ethanol fumes, which become quickly lethal for ethanol-susceptible species), (3) using sponges as egg-lay surfaces (this allows for easy egg collections, even when females tend to stick their eggs into substrates), (4) no ethanol wash step to visualize the interior of the eggs before injection (this prevents the egg chorion from becoming rock-hard), and (5) washing larvae out of the food for transgenic screening (no need for a white-eyed mutant). We believe that our method, developed for a very delicate species, should be useful to transform most drosophilid species that can be reared in the laboratory on a standard fly-food medium, such as *Drosophila tripunctata*, *Drosophila quinaria*, *Drosophila subquinaria*, and *Drosophila deflecta*. For recommendations on collecting and rearing many non-model species, please refer to our book [10].

1.9 Supplementary Materials

The following are available online at **https://www.mdpi.com/2409-9279/3/2/31/s1**, Figure S1: The Narishige IM 300 Microinjector, Video S1: The Making of Transgenic *Drosophila guttifera*.

Figure S1.1. The Narishige IM 300 Microinjector

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1.11 Author Contributions

All authors have read and agree to the published version of the manuscript.

Conceptualization, Shigeyuki Koshikawa and Thomas Werner; **data curation,** Mujeeb Shittu and Thomas Werner; **formal analysis,** Shigeyuki Koshikawa and Thomas Werner; **funding acquisition,** Shigeyuki Koshikawa and Thomas Werner; **investigation,** Mujeeb Shittu, Tessa Steenwinkel, Shigeyuki Koshikawa and Thomas Werner; **methodology,**

Mujeeb Shittu, Tessa Steenwinkel, Shigeyuki Koshikawa and Thomas Werner; **project administration,** Thomas Werner; **resources,** Shigeyuki Koshikawa and Thomas Werner; **supervision,** Thomas Werner; **validation,** Mujeeb Shittu, Tessa Steenwinkel, and Thomas Werner; **visualization,** Mujeeb Shittu, Tessa Steenwinkel, and Thomas Werner; **writing—original draft,** Mujeeb Shittu and Thomas Werner; and **writing—review and editing,** Mujeeb Shittu, Tessa Steenwinkel, Shigeyuki Koshikawa and Thomas Werner. All authors have read and agreed to the published version of the manuscript.

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2 Chapter 2

RNA *IN SITU* HYBRIDIZATION FOR DETECTING GENE EXPRESSION PATTERNS IN THE ABDOMENS AND WINGS OF *DROSOPHILA* SPECIES

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2.1 Abstract

RNA *in situ* hybridization (ISH) is used to visualize spatio-temporal gene expression patterns with broad applications in biology and biomedicine. Here we provide a protocol for mRNA ISH in developing pupal wings and abdomens for model and non-model *Drosophila* species. We describe best practices in pupal staging, tissue preparation, probe design and synthesis, imaging of gene expression patterns, and image-editing techniques. This protocol has been successfully used to investigate the roles of genes underlying the evolution of novel color patterns in non-model *Drosophila* species.

2.2 Introduction

RNA *in situ* hybridization (ISH) is a method used to detect and localize specific mRNA transcripts in cells, tissues, and whole organisms. Early ISH procedures used radioactively labeled RNA probes that hybridized with denatured DNA in tissues. The RNA-DNA hybrids were then detected by autoradiography[1,2]. A significant technical advancement to this method was the development of non-radioactive labeling systems that facilitated colorimetric visualization, which allowed for gene expression patterns to be observed in intact *Drosophila* embryos[3]. mRNA distribution patterns can now be detected by treating fixed tissues with digoxigenin-labeled RNA probes, to which F_{ab} fragments of anti-digoxigenin attached to alkaline phosphatase are bound. The addition of 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 4-nitroblue tetrazolium chloride (NBT) chromogenic solutions then results in the formation of a purple crystalline deposit, wherever the probe has bound to its target mRNA. The results, indicative of the gene expression patterns of

interest, can be observed under a regular stereo microscope[3,4]. The non-radioactive ISH method is sensitive, easier, and safer.

ISH is a very powerful molecular tool used in research and diagnosis. The non-radioactive RNA ISH technique has been applied to studies in developmental biology, evolutionary biology, and cancer biology[5-8]. It has played a significant role in the detection of mRNA expression in humans, mice, insects, and in viral RNA detection[9-14].

Detailed protocols to perform RNA ISH in *Drosophila* embryos can be found in the published literature[4,15,16]. However, these protocols cannot be adapted for pupal wings and abdomens because additional critical steps are required for processing pupal tissues. Especially during the early pupal stage, RNA ISH can be very difficult to perform due to the fragility of the newly forming adult tissues. Although several studies have used the RNA ISH technique to detect and characterize gene expression patterns on pupal wings and abdomens of various *Drosophila* species[6,7,17,18], no protocol describing this technique in *Drosophila* pupae has been published in any scholarly journal.

Here we provide a protocol suitable for model and non-model *Drosophila* species alike, detailing the steps of probe design and synthesis, pupal tissue dissection, the ISH process, and imaging techniques. We have applied this protocol to investigate the gene-regulatory networks governing the development and evolution of pigmentation patterns in pupal abdomens and wings of a variety of *Drosophila* species, such as *D. melanogaster*, *D. guttifera, D. deflecta, D. palustris,* and *D. subpalustris*[5,6,19]. The users of this protocol

should pay close attention to the pupal tissue processing steps, as the outcome of an RNA ISH critically depends on the tissue quality.

2.2.1 Overview

The major steps involved in ISH for *Drosophila* abdomens and wings are outlined in the flowchart (**Fig. 2.1**). First, species-specific PCR primers are designed to amplify a partial coding region of the gene of interest, using genomic DNA as a template. The PCR product is then cloned into the vector pGEM®-T Easy. A region containing the PCR product with flanking T7/Sp6 sites is PCR-amplified from this vector and used as a template for the synthesis of the DIG-labeled antisense RNA probe by *in vitro* transcription. Abdominal epidermis preparations involve pupal dissection, the removal of unwanted tissues by pipetting, fixation of the epidermal cell layer, and tissue storage. For wing preparations, the order of wing dissection and fixation depends on the pupal stage. In younger pupae, the wings are fixed before they are separated from the body, while in older pupae, the fixation step follows the separation of the wings. The important steps in the ISH procedure provided here are xylene treatment, tissue rehydration, fixation, proteinase K treatment, second fixation, prehybridization, probe addition, anti-DIG-AP F_{ab} treatment, and NBT/BCIP staining.

2.2.2 Application of this protocol

ISH has been integral to the field of evolutionary developmental biology in that it is a valuable technique to study the emergence of novel traits, such as the color patterns of butterflies and fruit flies[17,18,20,21]. This protocol allows for the detection of gene

expression patterns in abdomens and wings of different *Drosophila* species. We have used this procedure in model and non-model *Drosophila* species to study toolkit and terminal pigmentation genes involved in color pattern formation, such as *D. melanogaster*, *D. guttifera, D. deflecta, D. palustris,* and *D. subpalustris*[5,6,19]. No specialized skills are required to use this protocol; we have had undergraduate students generate high-quality results in our lab. We believe that this protocol will facilitate the study of novel gene expression patterns in rare and unstudied fruit flies, which can be collected and brought into the lab using the two new field guides to drosophilid species[22,23].

2.2.3 Advantages and limitations

Our protocol allows for the detection of developmental toolkit genes' involvement in color patterning in *Drosophila* species. The most important advantage of this ISH protocol is that it enables the detection of gene expression patterns in very early pupal abdomens (as soon as the epidermal layer has formed, i.e., from P7 onwards), as well as early wings that cannot be dissected without prior fixation (from stage P5ii onwards). This procedure requires basic laboratory equipment to generate high-quality ISH results[5,19]. However, this method is not without limitations. One of the limitations of the ISH technique is that it is semi-quantitative, and although it can detect and visualize spatial gene expression patterns, it is less accurate in determining quantitative gene expression differences than RNA-seq-based methods. Also, the final staining outcome of an ISH technique depends on the duration of the staining reaction, probe concentration, and fixation conditions, therefore making it sometimes difficult to generate the desired

result in one attempt. Furthermore, the development of background staining is a limiting factor for how long a staining reaction can continue, which causes problems when a gene is only weakly expressed.

2.3 Experimental Design

2.3.1 Probe design and synthesis

The process starts with designing species-specific PCR primers to amplify a partial protein-coding region from a single exon, using the GenePalette software[24] as described in **BOX 1**. Primers are 18-25 bases in length and are designed to yield products of 200-500 bp in size. When comparing expression patterns of a gene among different *Drosophila* species, we used the multiple alignment tool in GenePalette to identify the most highly conserved regions of the open reading frame. For the probe design, we chose a region that did not contain indels among the species; thus, the PCR products for the same gene for different species would have the same length. The PCR products (inserts) were then cloned into the vector pGEM®-T Easy, which contains the Sp6 and T7 promoters required for *in vitro* synthesis of antisense RNA probes. The *E. coli* DH-*5α* competent cells were transformed with the cloned vector in order to generate several clones, after which we performed the colony PCR to isolate the positive colonies. The positive colonies were cultured overnight, and the cloned plasmids were extracted (mini-prep DNA). In order to determine the orientation of the insert in the vector, we carried out insertion direction PCR. Depending on the direction of insertion, we used the Sp6/T7 RNA polymerase to synthesize antisense RNA probes.

2.3.2 Pupal staging

We collected wandering third-instar (L3) larvae in a 10-cm Petri dish with moist tissue paper covering the bottom. After the larvae began to pupate, we followed the progression of the pupal stages under a dissection scope. We adopted the description by Bainbridge and Bownes[25] and Fukutomi *et al.*[26] for *Drosophila* pupal stage determination (**Fig. 2.2**).

2.3.3 Abdominal epidermis preparation

Once the pupae reached the desired stage, they were placed in groups of ten onto a double-sided tape fixed to a microscope slide to cut them and clean the epidermal tissue. We performed two types of cuts: 1) the pupae were placed with their ventral side facing the tape and then cut longitudinally between both eyes (dorsal cut) and 2) one of their lateral sides faced the tape, and the cut ran longitudinally through the pupae, separating the dorsal from the ventral half (lateral cut) (**Fig. S2.1**). Dorsal cuts are best suited to examine lateral patterns of gene expression, while lateral cuts allow for the visualization of the dorsal and ventral regions of the abdomen. The cut pupae were then washed with phosphate-buffered saline (PBS), fixed in paraformaldehyde, and stored in 100 % ethanol at -20 ºC until further processing by ISH.

2.3.4 Pupal wing preparation

Wing preparations are possible from stage P5ii onward. It is important to note that wings from stages P5ii to P8 are too fragile to be dissected without prior fixation. These early pupae were pulled out of their puparia in PBS with a pair of fine forceps, followed by cutting off the head and the tip of the abdomen with a small pair of surgical scissors. The

resulting carcass tubes were cleaned by pipetting PBS through them to remove the inner organs. The carcasses were then fixed overnight at 4 °C, after which the hardened wings were dissected from the pupae with two pairs of fine forceps. Pupae of stage P9 and older have sturdier wings, which allows the fixation process to follow dissection, instead of preceding it. These older pupae were placed with the ventral side down on a glass slide with double-sided tape. The pupae were pulled out of their puparia by the head and submerged in a glass-viewing dish in distilled water. The wings were then carefully extracted from the pupal membrane and allowed to inflate, followed by fixing them with paraformaldehyde at room temperature for 30 min. Regardless of the pupal stage, the extracted wings were washed twice with methanol and twice with 100 % ethanol after the fixation step and then stored in ethanol at -20 ºC until the ISH procedure was performed.

2.3.5 ISH of *Drosophila* abdomens and wings

On the first day of the ISH, the processed tissues (pupal abdomens or wings) were carefully transferred into the wells of a glass-viewing dish, using a cut 1-mL pipette tip. They were then treated with xylenes to remove any fatty tissue, re-hydrated, fixed, washed, treated with proteinase K, washed, post-fixed, and prehybridized. After the prehybridization, DIG-labeled RNA probes were added, and the samples were incubated at 65 °C for 18 h to 3 d. On the second day, any unhybridized probe molecules were washed away to reduce background staining. The washed tissues were incubated in anti-DIG-AP F_{ab} fragments at 4 °C overnight. On the third and final day, the tissues were treated with NBT/BCIP staining solution to detect the dark-purple hybridization pattern. This reaction took place in the dark. The staining progress was observed under a

dissecting scope every 20 min to avoid overstaining. After the staining reached its desired intensity, the staining solution was washed off with staining buffer, then with PBT, and the gene expression patterns were observed under a stereo microscope. Several images of the tissues were taken at slightly different focal planes and Z-stacked with Helicon Focus software. A minimum of three days is required to perform the ISH experiment in *Drosophila* using this protocol.

2.4 Materials

2.4.1 Reagents

- Distilled H_2O
- Taq 2× MeanGreen Master Mix (Syzygy Biotech, www.integratedscientificsolutions.com)
- Agarose (Dot scientific inc., cat. no. AGLE500)
- TAE buffer (see REAGENT SETUP)
- 1 mM dATP (Sigma-Aldrich, https://www.sigmaaldrich.com/)
- 10× PCR buffer (Sigma-Aldrich, cat. no. P2192)
- Taq Polymerase (Thermo Scientific, cat. no. EP0401)
- Gel extraction kit (Thermo Scientific, cat. no. K0692)
- pGEM®-TEasy vector system (Promega, cat. no. A1360, store at -20 °C)
- *DH-5α* competent cells (Thermo Scientific, cat. no. EC0112)
- LB medium (see REAGENT SETUP)
- Culture media preparation (see REAGENT SETUP)

- Ampicillin (Sigma-Aldrich, cat. no. A0166, store at -20 °C)
- IPTG (Fisher scientific, cat. no. BP1755-1, store at -20 °C)
- X-GAL (Sigma-Aldrich, cat. no. 7240-90-6, store at -20 °C)
- Plasmid mini-prep kit (Thermo Scientific, cat. no. K0503)
- M13F and M13R vector primers (Integrated DNA Technologies, www.idtdna.com) (see **Supplementary table S1** for primer sequences)
- DIG RNA labeling kit (SP6/T7) (Roche, cat. no. 11175025910, store at -20 °C)
- DNA gel-loading buffer (6X) (Thermo Fisher Scientific, cat. no. R0611 store at 4 °C)
- Linear acrylamide (AMRESCO, cat. no. K548, store at 4 °C)
- Sodium acetate (Sigma-Aldrich, cat. no. 127-09-3)
- 100 % Ethanol (DECON laboratories Inc, cat. no. 64-17-5)
- Hybridization buffer (see REAGENT SETUP)
- PBS (see REAGENT SETUP)
- Fixation buffer (see REAGENT SETUP)
- Xylenes (Sigma-Aldrich, cat. no. 534056) CAUTION Harmful by inhalation and in contact with skin.
- Methanol (Sigma-Aldrich, cat. no. 38460) CAUTION Methanol is poisonous. It should not be inhaled, swallowed or be allowed to touch the skin.
- PBT (see REAGENT SETUP)
- Proteinase K (Sigma-Aldrich, cat. no. P2308, store at -20 °C)
- Anti-digoxigenin-AP Fab fragment (Roche, cat. no. 11093274910, store at 4 °C)

- Staining buffer (see REAGENT SETUP)
- Staining solution (see REAGENT SETUP)
- NBT (Promega, cat. no. S380C, store at store at -20 °C) CAUTION Toxic.
- BCIP (Promega, cat. no. S381C, store at 4 °C) CAUTION Toxic.
- Trizma Base (Sigma-Aldrich, cat. no. T6066)
- Glacial acetic acid (Sigma-Aldrich, cat. no. 320099)
- EDTA (Sigma-Aldrich, cat. no. E0399)
- 37% HCl (Sigma-Aldrich, cat. no. 320099) CAUTION Toxic when inhaled, causes irritation to the respiratory tract, and causes skin burn.
- Tryptone (Sigma-Aldrich, cat. no. T7293)
- Yeast extract (Sigma-Aldrich, cat. no. Y1625)
- NaCl (Sigma-Aldrich, cat. no. S3014)
- NaOH (Sigma-Aldrich, cat. no. S5881) CAUTION Causes severe skin burns and eye damage.
- Agar (Sigma-Aldrich, cat. no. A6686)
- Formamide (Sigma-Aldrich, cat. no. 221198) CAUTION Suspected of causing cancer.
- Salmon sperm DNA (Invitrogen, cat. no. 15632-011)
- Heparin sodium salt (Sigma-Aldrich, cat. no. H3393)
- Tween 20 (Sigma-Aldrich, cat. no. P1379)
- Triton X-100 (Sigma-Aldrich, cat. no. X100)
- K_2HPO_4 (Sigma-Aldrich, cat. no. P2222)

- KH_2PO_4 (Sigma-Aldrich, cat. no. P5655)
- Sodium citrate (Sigma-Aldrich, cat. no. S1804)
- Deoxycholic acid (Sigma-Aldrich, cat. no. D2510)
- 16 % Paraformaldehyde (Electron microscope sciences, cat. no. 15710)

  CAUTION Causes skin and eye irritation. Suspected of causing genetic defects and may cause cancer.
- $MgCl_2$ (Sigma-Aldrich, cat. no. M8266)
- Gel slick (Lonza, cat. no. 50640)

2.4.2 Equipment and supplies

- Thermocycler (Eppendorf AG, cat. no. 6325)
- Incubator (Thermo Fisher Scientific, cat. no. 6246)
- Centrifuge (Eppendorf AG, cat. no. 5424)
- Refrigerated centrifuge (Eppendorf AG, cat. no. 5404R)
- Water bath (Thermo scientific, cat. no. 1521038, model HAAKE S3)
- Incubator shaker (New Brunswick, model I-SERIES 24)
- Vortex mixer (Labnet, cat. no. S0100, model VX100)
- Electrophoresis power supply (Fisher scientific, cat. no. FB1000)
- Dissecting scope (Olympus, cat. no. SZ51)
- Digital heating block (Apollo instrumentation)
- Stereo Microscope (Olympus, www.olympus-lifescience.com, model SZX16)
- DP72 camera (Olympus, www.olympus-ims.com, model U-TV1X-2)

- UVP Transilluminator (BioDoc-It™ Imaging System, www.uvp.com)
- Digital monochrome printer (Mitsubishi, www.uvp.com, model P95DW)
- Pipettors (Fisherbrand, cat. no. 14-388-100)
- Blunt forceps (Dumont #2) (Inox-Med.Bio, cat. no. 11223-20)
- Fine forceps (Dumont #5) (Inox-Med.Bio, cat. no. 11254-20)
- Surgical scissors (F.S.T, cat. no. 91500-09)
- 1.5-mL Eppendorf tubes (Fisher Brand, cat. no. 05-408-141)
- 2-mL Eppendorf tubes (Fisher Brand, cat. no. 05-408-132)
- Falcon tubes (Fisher scientific, cat. no. 14-959-70C)
- 20-mL Scintillation vial (DWK Life Sciences Wheaton)
- 20-mL glass centrifugation tube (Pyrex, cat. no. 9825)
- Glass-viewing dish (Pyrex spot plates 9 concave depressions 22 mm O.D. x 7 mm deep) (Fisher scientific, cat. no. 13-748B)
- Medium-sized Petri dish (VWR, cat. no. 25384-302)
- Microscope slides 75 x 25x 1 mm (VWR Vista vision, cat. no 16004-368)
- Permanent double-sided tape (Scotch, cat. no 38-8507-5367-3)
- Clean razor blade (VWR, cat. no. 55411-050)
- Medium-sized paintbrush (Liner 44278 Plaid®Xuancheng, China)
- Kleenex paper (Fisher scientific, cat. no. 06-666-11)
- Kimwipes® disposable wipers (Sigma Aldrich, cat. no. Z188964)
- Helicon Focus software (http://www.heliconsoft.com/heliconsoft-products/helicon-focus/)

2.4.3 Reagent setup

- **20× TAE** Add 1600 mL distilled water and a stir bar to a 2-L beaker, add 193.6 g of Trizma Base, 46 mL Glacial Acetic Acid, 1.5 g EDTA. Stir until everything dissolves. Adjust the pH to 8.0 with about 26 mL of 37% HCl. Add distilled water to make 2 L and stir a little more.
- **LB medium** Add 1800 mL of distilled water and a stir bar to a 2-L beaker. Add 20 g of Tryptone, 10 g Yeast extract, and 20 g NaCl. Set pH to 7.5 with about 700 µL 5 M NaOH. Adjust volume with distilled water to 2 L and stir a little more. Fill into 1 L bottles with about 600 mL, then autoclave.
- **500-mL ampicillin agar plates** Add 7.5 g of agar into 500 mL LB medium in a 1-L bottle and autoclave. Let it cool down until you can touch the bottle with your hands. Add 1 mL of ampicillin (at 100 mg/mL) per liter of agar to obtain a final concentration of 200 µg/mL.
- **Hybridization solution** Add 200 mL of formamide and 100 mL 20× SSC. Set pH to 5.5 (check with color strips) and filter-sterilize. Then, add 4 mL of salmon sperm DNA (10 mg/mL), 40 mg heparin, 400 µL Tween 20, and 96 mL H_2O. Mix thoroughly and store at -20 °C.
- **10× PBS** Add 1800 mL of distilled water and a stir bar to a 2-L beaker. Add 21.4 g of K_2HPO_4, 10.3 g KH_2PO_4, and 163.6 g NaCl. Adjust volume to 2 L and stir a little more. Fill into 1-L bottles with about 600 mL, then autoclave. Note: The pH is at around 6.5 now. Prepare 1× PBS by making 1:10 dilution in distilled water. This will cause the pH to go up to 7.0 - 7.2. This is where the pH should be.

- **PBT** Add 100 mL of 10× PBS, 1 mL Triton X-100, and 900 mL distilled H_2O.
- **20× SSC** Add 700 mL of distilled water and a stir bar to a 1-L beaker. Add 175.3 g of NaCl and 88.2 g sodium citrate. Adjust pH to 7.0 with about 2 drops of 37 % HCl. Adjust the volume with distilled water to 1 L and stir. Fill into two 1-L bottles, then autoclave.
- **Staining buffer (50 mL)** Add 1 mL of 5 M NaCl, 2.5 mL 1 M $MgCl_2$, 2.5 mL 2 M Tris pH 9.5, 50 µL Tween 20, and 44 mL distilled H_2O. Make fresh staining buffer each time you perform an *in situ* and mix thoroughly before use.
- **Staining solution (400 µL)** Add 2.8 µL of NBT (50 mg/mL) and 1.4 µL BCIP (50 mg/mL) into 400 µL of freshly prepared staining buffer. Make fresh staining solution when required. Keep in the dark.
- **Fixation buffer for abdominal ISH (40 mL)** To a clean 50-mL Falcon tube, add 4 mL of 10× PBS, 85 µL 5 M NaOH, 20 mL sterile distilled H_2O, and 80 mg deoxycholic acid. Vortex until the milky color (undissolved deoxycholic acid) disappears, then add 10 mL of 16 % paraformaldehyde. Fill up to 40 mL with sterile distilled H_2O. Store at 4 °C for maximum of one-month.
- **Fixation buffer for wings ISH (PBT + 4 % PFA)** To a clean 50-mL Falcon tube, add 4 mL of 10× PBS, 10 mL 16 % paraformaldehyde, 400 µL Triton X-100, and add 25.6 mL sterile distilled H_2O. Store at 4 °C for maximum of one month.

2.5 Procedure

2.5.1 A-tail genomic PCR

Timing **4 h**

1. Prepare a reaction mix according to the table below.

Reagent	**Volume per reaction (µL)**
Taq 2× MeanGreen Master Mix	12.5
Forward primer (10 pmol/µL)	1.25
Reverse primer (10 pmol/µL)	1.25
Genomic DNA	0.25
d H_2O	9.8
Total	25.0

2. Amplify the PCR product according to the appropriate cycling conditions (**Table 2.2**).
3. Run the PCR product through a 1 % (wt/vol) agarose gel in 1× TAE buffer by electrophoresis and visualize it under UV light.
4. If the size of the PCR product matches the expected size, perform a gel extraction.

2.5.2 Gel extraction and purification of PCR products

Timing **30 min**

5. On a table-top UV light, cut out the gel slice containing the DNA fragment, using a clean razor blade and place it into a 2-mL Eppendorf tube.
6. Add 1:1 volume of binding buffer to the gel slice (vol:wt).

7. Incubate at 60 ºC until the gel slice is completely dissolved.
8. Transfer the solubilized gel solution to the purification column. Centrifuge for 1 min and discard the flow-through.
9. Add 700 µL of wash buffer to the column. Centrifuge for 1 min and discard the flow-through.
10. Centrifuge the empty column for 1 min to completely remove the wash buffer.
11. Transfer the column into a 1.5-mL Eppendorf tube. Add 30 µL of elution buffer and incubate at room temperature for 1 min.
12. Centrifuge for 1 min to collect the DNA fragment in the 1.5-mL tube.
13. Store the purified DNA at -20 ºC.

PAUSE POINT Purified PCR products can be stored at -20 ºC for one month.

CRITICAL STEP MeanGreen master mix adds A-tails to the PCR products. In case you use a PCR master mix that does not add A-tails, add the A-tails after performing the gel extraction, according to the table below. Also, note that A-tails may fall off after one month of storage at -20 ºC.

Reagent	**Volume per reaction (µL)**
PCR product	7.0
dATP/dNTP (10mM)	1.0
10× PCR buffer	1.0
Taq Polymerase (5 U/ µL)	1.0
Total	10

14. Incubate in a thermocycler at 72 ºC for 45 min. Store at -20 ºC.

2.5.3 Ligation

Timing **18 h (overnight)**

15. To ligate the A-tailed PCR product with the pGEM®-T Easy vector, use the reaction mix in the table below.

Reagent	**Volume per reaction (µL)**
A-tailed PCR product	3.5
2× ligation buffer	5.0
pGEM-TEasy vector (50 ng)	0.5
T4 DNA ligase	1.0
Total	10

16. Incubate the ligation reaction at 4 ºC overnight.

CRITICAL STEP The pGEM®-T Easy vector features T-overhangs essential for T/A cloning of an A-tailed DNA fragment. Also, this vector contains the SP6/T7 promoters, which will later flank the insert after an additional PCR reaction.

2.5.4 Transformation of DH-5α cells and colony PCR

Timing **18 h (transformation) and 4 h (colony PCR)**

17. Add 2 µL of the raw ligation product to 50 µL of *E. coli* DH-5α competent cells in a 1.5-mL Eppendorf tube.

18. Place the tube on ice for 45 min.

19. Heat shock the mixture at 42 ºC for 1 min in a water bath.
20. Immediately transfer the tube back to the ice and leave it for 5 min.
21. Add 200 µL of LB medium to the cells.
22. Incubate the culture at 37 ºC in an incubator shaker for 1 h at 200 r.p.m.
23. Prepare an ampicillin agar (200 µg/mL) in a medium-sized culture medium plate. The plate should be pre-made at least a day before use and stored at 4 ºC.
24. Mix 100 µL of the IPTG and 50 µL of the X-Gal solutions and spread them evenly on the bacterial agar plate for the blue/white colony selection.
25. Add 50-100 µL of the bacterial culture (step 22) onto the agar plate.
26. Incubate the plate at 37 ºC for 18 h.
27. Pick 12 white colonies with a small pipette tip and independently suspend the bacteria in 10 µL of dH_2O in 1.5-mL Eppendorf tubes. The white colonies should contain inserts, while the blue colonies likely contain empty, self-ligated vector.
28. Immediately perform a colony PCR to confirm the presence of the correct insert in these clones, as tabulated below. Use the cycling conditions described in **Table 2.3**.

Reagent	Volume per reaction (µL)
10× PCR buffer	1.0
dNTP mix (10 mM)	0.5
Internal forward primer (10 pmol/µL)	0.5
Internal reverse primer (10 pmol/µL)	0.5
Taq polymerase (5 U/µL)	0.2
d H_2O	15.3
Bacterial suspension	2.0
Total	20

2.5.5 Culturing the positive colonies

Timing **19 h**

29. Mix 6 µL of ampicillin (2 mg/mL) with 3 mL LB medium in a sterile glass tube.

30. Inoculate the tube with 5 µL of the bacterial colony suspension (Step 27).

31. Mix thoroughly.

32. Incubate at 37 ºC in an incubator shaker for 18 h at 200 r.p.m.

PAUSE POINT After the incubation is completed, the plates can be stored at 4 ºC for up to 7 d.

2.5.6 Plasmid extraction from a positive clone (mini-prep)

Timing **30 min**

33. Pour ~1.8 mL of the cultured cells (containing the cloned plasmid) into a 2-mL Eppendorf tube.

34. Centrifuge at 15,000g for 30 s at room temperature and discard the supernatant.
35. Add 250 µL of the resuspension buffer and vortex to resuspend the cell pellet.
36. Add 250 µL of the lysis buffer and mix by inverting the tube 10 times.
37. Add 350 µL of the neutralization buffer and mix by inverting the tube 10 times.
38. Centrifuge for 5 min at 15,000g at room temperature.
39. Pour the supernatant into the spin column and centrifuge for 1 min at 15,000g at room temperature.
40. Add 500 µL of the wash buffer to the spin column. Centrifuge for 1 min at 15,000g at room temperature and discard the flow-through. Repeat this step.
41. Transfer the spin column into a clean 1.5-mL Eppendorf tube.
42. Add 30 µL of the Elution buffer to elute the plasmid DNA. Incubate for 2 min at room temperature and centrifuge for 2 min at 15,000g at room temperature.
43. Store the mini-prep DNA (containing the cloned-plasmid) at -20 ºC.

PAUSE POINT The DNA can be stored at -20 ºC indefinitely.

2.5.7 Insertion direction PCR

Timing **3 h**

44. Carry out the insertion direction PCR for each mini-prep DNA to determine the orientation of the insert in the pGEM-TEasy vector to choose the correct RNA polymerase that will synthesize an antisense probe.
45. Set up two PCR reactions simultaneously for each DNA clone, using the following primer pairs: (i) the M13F primer plus the gene-specific internal

forward primer; and (ii) the M13F primer plus the gene-specific internal reverse primer.

Reagent	**Volume per reaction (µL)**
10× PCR buffer	2.0
dNTP mix (10 mM)	0.5
M13F (vector primer) (10 pmol/µL)	0.5
Internal gene-specific primer (forward or reverse) (10 pmol/µL)	0.5
Taq polymerase (5 U/µL)	0.2
dH_2O	16.2
Mini-prep DNA (insert in pGEM-TEasy)	0.1
Total	20

46. Perform the PCR reactions according to the cycling conditions described in **Table 2.4**.

47. Perform gel electrophoresis of the PCR products, using a 1 % (wt/vol) agarose gel in 1 × TAE and visualize the bands under UV light.

CRITICAL STEP If the primer pair M13F/internal reverse shows a PCR band, use Sp6 polymerase to make an anti-sense probe. However, if the primer pair M13F/internal forward shows a PCR band, use T7 polymerase to make an anti-sense probe. Only one of the primer pairs should produce a clear PCR band.

2.5.8 RNA probe synthesis

Timing **5 h**

48. PCR-amplify the cloned insert using the mini-prep DNA as a template (Step 43). Use the M13F and M13R primer pair, as tabulated below.

Reagent	**Volume per reaction (µL)**
Taq 2× MeanGreen Master Mix	12.5
M13F (vector forward primer) (10 pmol/µL)	1.25
M13R (vector reverse primer) (10 pmol/µL)	1.25
Mini-prep DNA	0.1
d H_2O	9.8
Total	25.05

49. Amplify according to the cycling conditions described in **Table 2.4**.
50. Run the electrophoresis of the PCR product, using a 1 % (wt/vol) agarose gel in 1× TAE.
51. Extract the DNA band and elute it in 30 µL Elution buffer, as described in Steps 5-13.
52. Measure the DNA concentration.

CRITICAL STEP For a high probe yield, use 0.05 – 0.1 µg/µL of the DNA as a template for the *in vitro*-transcription reaction.

53. Prepare the anti-sense RNA probe reaction mix as tabulated below.

Reagent	Volume per reaction (µL)
Purified PCR product (0.05 – 0.1 µg/µL)	6.5
10× NTP labeling mixture	1.0
10× Transcription buffer	1.0
Protector RNAse inhibitor	0.5
Sp6 RNA polymerase or T7 RNA polymerase	1.0
Total	10

54. Incubate at 37 °C for 2 h.

CRITICAL STEP Remember to use the correct RNA polymerase based on the insertion direction PCR result (Step 44 - 47). (Recall: If the primer pair M13F/internal reverse shows a PCR band, use Sp6 polymerase to make an anti-sense probe. However, if the primer pair M13F/internal forward shows a PCR band, use T7 polymerase to make an anti-sense probe).

2.5.9 RNA probe quality check and preparation

Timing **2 h**

55. Check the quality of the synthesized RNA probe by running 1 µL of the newly synthesized probe (Step 54) alongside 1 µL of the purified PCR (Step 51) on a gel.

56. Add 2 µL of the gel loading buffer into 9 µL of H_20, then add 1 µL of the probe.

57. Vortex, then briefly spin down.

58. Load the 12 µL on a 1 % agarose gel and perform electrophoresis in 1× TAE buffer. An example of a gel image showing a successfully transcribed probe is shown in **Figure 2.3**.

59. Precipitate the remaining 9 µL of the synthesized probe as shown below.

Reagent	**Volume per reaction (µL)**
Probe (from step 51)	9
Linear Acrylamide (5 µg/µL)	1
3 M Sodium acetate pH 5.5	1
200 Proof Ethanol	22.5

60. Incubate at -20 °C for 20 min.

61. Spin at 4 °C for 30 min.

62. Pipette off the supernatant and discard.

63. Air-dry the probe-containing pellet for 5 min.

64. Dissolve the pellet in 50 – 100 µL of the pre-hybridization buffer on ice by carefully pipetting the liquid up and down.

CRITICAL STEP Depending on the probe yield, use 100 µL of pre-hybridization solution to dissolve the pellet when you see a solid probe band on the gel or use 50 µL if the band is rather faint.

PAUSE POINT The RNA probe can be stored for about two years at -20 ºC.

2.5.10 *Drosophila* pupa collection and processing for abdominal ISH

Timing **4 h**

CRITICAL STEP The following instructions are critical for Steps 65 – 132: 1) Look through the dissecting scope during all pipetting steps. 2) Washes are with 1 mL of organic solutions for 5 min, and 10 min for aqueous solutions, unless otherwise indicated. 3) Washes involving hybridization solution are 500 µL. 4) Do not agitate the samples too much during washes; only gently move the liquid in and out of the pipette when removing liquid. 5) After washes, remove waste from the wells of the glass viewing dish in 150 µL increments and with the same 200-µL tip. 6) Successive washes are done under a running clock.

65. Collect wandering third-instar (L3) larvae from the *Drosophila* culture bottle and place them in a Petri dish with moist Kleenex paper on the bottom.
66. Store the Petri dish in a moist chamber to prevent the larvae from drying out.
67. Wait until the pupae are at the desired stage (**Fig. 2.2**).
68. Use a dissecting scope set to 20× magnification to clearly see the key features of the pupal stages.
69. Prepare a glass slide with a piece of double-sided tape on it (dissection platform).
70. Fill the well of a glass-viewing dish with 1 mL of freshly prepared 1× PBS.

71. Use blunt forceps (type #2) to gently remove the pupae from the Petri dish (one at a time) and immediately transfer them onto the dissection platform.

72. Lay the pupae with their ventral side facing the tape and cut longitudinally between both eyes (dorsal-cut) or lay them of their lateral side and cut longitudinally through the pupae, separating the dorsal from the ventral half (lateral-cut) (**Fig. S2.1**). Perform only one type of cut in a session.

73. With a razor blade, immediately dissect each pupa lengthwise (starting with the one first placed on the tape). This is best accomplished with a single rapid cut from the anterior to the posterior end of the pupae.

74. Using a medium-sized paintbrush, transfer a small amount of 1× PBS from the glass-viewing dish to each dissected pupa to dissolve them from the tape.

75. Transfer the pupal halves with the brush into the well of a glass-viewing dish filled with 1× PBS.

CRITICAL STEP Dissect 10 pupae within 2 min and then transfer them into 1× PBS immediately. Label each end of the razor blade and use each end to dissect 50 – 60 pupae, after which the blade is too blunt and should be discarded.

76. With a pair of surgical (sharp-pointed) forceps (type #5), grasp an individual pupa half anteriorly (by the head) and gently wash away the internal organs with 1× PBS.

77. Use a pipettor to gently flush 1× PBS over the internal organs without touching the epithelial layer of the pupa with the pipette tip (**Fig. S2.2**). Prevent the

epidermal tissue from becoming detached from the puparium at this time, as the puparium provides mechanical protection throughout the process.

CRITICAL STEP You must ensure to keep the epithelial cell layer intact throughout the washing steps. Therefore, apply low pressure from the pipettor by setting a 20-µL pipettor to 8.5 µL for pupal stages P7, P8 or to 15 µL for pupal stage P9. For stages P10 and older, use a 200-µL pipettor set to 25 µL. Too much pressure or excessive washing will lead to the loss of epithelial cells.

78. Immediately transfer the washed pupa halves into a well with 1× PBS.
79. Remove the 1× PBS solution from the well.
80. Add 1 mL of fixation buffer to the pupa halves.
81. Incubate at room temperature for 1 h.
82. Rinse the fixed pupae 3 times with 1× PBS.
83. The fixed samples may be used to perform ISH immediately or stored for later use. If storage is the goal, equilibrate the pupa halves through a dilution series of 1× PBS:100 % ethanol (3:1, 1:1, 1:3) for 20 min in each solution at room temperature.
84. Rinse once and wash once in 1 mL 100 % ethanol. With a cut 1-mL tip, transfer the pupae into a 2-mL Eppendorf tube and store in 100 % ethanol at -20 ºC.

PAUSE POINT The processed pupae can be stored at -20 ºC for 1 year.

2.5.11 ISH of *Drosophila* abdomens and wings

Timing **3 d**

85. Take the processed wings (described in **Box 4**) and the pupa halves from the -20 °C freezer.
86. Transfer the pupa halves with a pipette and a cut 1-mL tip into a glass-viewing dish.
87. Transfer the wings with a pipette and a cut 200-µL tip into a glass-viewing dish. Ensure that the glass-viewing dish is gel slick coated **BOX 3**.
88. Wash once with 100 % ethanol.
89. Incubate for 30 min with 1 mL 1:1 xylenes:ethanol (vol/vol) in a fume hood.
90. Rinse once and wash 5 × with 100 % ethanol.
91. Wash 2 × with methanol (wings only).

CAUTION Incubate and discard all xylenes-containing washes in the fume hood. Xylenes can constitute a serious health hazard.

92. Equilibrate the tissues through a dilution series of 1× PBS:100 % ethanol (1:3, 1:1, 3:1) (vol/vol) and incubate at room temperature for 20 min in each solution (skip this step for wing ISH).
93. Rinse once and wash 3 × with PBT.

94. Fix the tissues for 30 min in 1 mL of fixation buffer at room temperature. Note that the fixation buffers for wing and abdomen ISH are different; therefore, see REAGENT SETUP for fixation buffer preparation.

95. Rinse once and wash 5 × with PBT.

96. Replace the last PBT wash with 1 mL of the Proteinase K solution.

    CRITICAL STEP Freshly prepare the Proteinase K solution on ice and use it within the same hour or two. For abdominal ISH, dilute 1 µL of Proteinase K stock [10 mg/mL in PBS] in 99 µL of PBT. Take 4 µL of this dilution and add it to 1 mL of PBT. For wing ISH, mix 0.4 µL of Proteinase K [10 mg/mL in PBS] with 1 mL of PBT.

97. Incubate the tissues at room temperature for 10 min.

    CRITICAL STEP Incubate the wings and abdomens (P11 to P15) in Proteinase K for 20 min to increase the tissue permeability and reduce background staining.

98. Rinse 2 × with PBT.

99. Wash 2 × with PBT.

100. Post-fix the tissues for 30 min in 1 mL of fixation buffer at room temperature.

101. Wash 5 × with PBT.

102. Wash in 1:1 PBT:hybridization solution (vol/vol).

103. Wash 3 × with hybridization solution at room temperature.

104. Pipette the tissues into a 2-mL Eppendorf tube with a cut 1-mL tip.

105. Set the heating block to 80 °C.
106. Prehybridize the tissues in 500 µL of the hybridization solution and incubate at 65 °C for 1 h.
107. Dilute the probe (1:500) by adding 1 µL of the probe to 500 µL of hybridization solution in a 2-mL Eppendorf tube.
108. Incubate the diluted probe for 5 min in the heating block at 80 °C.
109. Immediately put the probe on ice to prevent secondary RNA structure formation.
110. Remove as much of the hybridization solution in (Step 104) without damaging the tissues and replace with the diluted probe on ice.
111. Incubate at 65 °C for >18 h to a maximum of 3 d, and gently swirl every couple of hours (not necessary during the night).

Day 2

112. Pre-heat 3 mL of hybridization solution per sample to 65 °C on a dry-heating block.
113. Transfer the samples back into a clean glass-viewing dish by pipette, using a cut 1-mL tip for abdomens and a cut 200-µL tip for wings.
114. Rinse once with pre-heated hybridization solution.
115. Incubate in pre-heated hybridization solution at 65 °C for 1 h.
116. Wash 3 × at 65 °C for 30 min in pre-heated hybridization solution.
117. Prepare 1.5 mL of 1:1 PBT:hybridization solution (vol/vol).
118. Wash 2 × with 1:1 PBT: hybridization solution (vol/vol) at room temperature.
119. Wash 5 × with PBT.

120. Pipette the tissues with the 5th PBT wash into a 2-mL Eppendorf tube, using a cut 1-mL tip for abdomens or a cut 200-µL tip for wings.
121. Remove most of the PBT (leave about 50 µL PBT in the tube) and place the tissues on ice.
122. On ice in a 2-mL Eppendorf tube, add 0.2 µL of the Roche α-DIG AP Fab fragments to 1,200 µL of PBT to result in a 1:6,000 dilution.
123. Add 300 µL of the 1:6000 diluted Roche α-DIG-AP F_{ab} fragments to each sample and incubate at 4 ºC overnight.

Day 3

124. Wash 5 × with PBT.
125. Wash 3 × with staining buffer (see REAGENT SETUP).
126. Remove the epidermal tissue layer from the puparium (outer tan shell). This step is for abdominal ISH only.
127. Prepare the staining solution: add 2.8 µL of NBT (50 mg/mL) and 1.4 µL of BCIP (50 mg/mL) to 400 µL of staining buffer. Mix and keep in the dark.
128. Replace the last wash with 400 µL of staining solution.
129. Incubate in the dark at room temperature.
130. Check for signal development (purple stain) every 20 min.
131. Stop staining after the expression patterns look good, rinse once, and wash 2 × with staining buffer.
132. Rinse once and wash 2 × with PBT.
133. The tissues are now ready to be imaged. Use the information in **Box 2** to image the tissues.

CRITICAL STEP The tissues are very fragile and brittle at this stage; therefore, minimize physical contact during imaging.

TIMING

Steps 1 – 4, A-tail genomic PCR: 4 h

Steps 5 – 13, Gel extraction and PCR product purification: 30 min

Steps 15 – 16, Ligation: 18 h (overnight)

Steps 17 – 28, Transformation of *DH-5α* cells and colony PCR: 24 h (overnight)

Steps 29 – 32, Culturing the positive colonies: 18 h (overnight)

Steps 33 – 43, Plasmid extraction from a positive clone (mini-prep): 40 min

Steps 44 – 47, Insertion direction PCR: 3 h

Steps 48 – 54, RNA probe synthesis: 5 h

Steps 55 – 64, RNA probe quality check and precipitation: 2 h

Steps 65 – 84, *Drosophila* pupa collection and processing: 4 h

Steps 85 – 133, *Drosophila* abdominal or wing ISH: 3 d

2.6 Troubleshooting guide

Table 2.1. A table summarizing the problems that we have encountered in the past with appropriate actions to solve each problem.

Step	Problem	Possible cause	Solution
3	No PCR product	Poor primer design	Ensure the primers are made from the highly conserved region. Check the primer design
		Low-quality genomic DNA	Check the genomic DNA on agarose gel
		Poor cycling conditions	Adjust the PCR conditions as needed
		Failed ligation	Ensure A-tail are added to the PCR products before ligation with the pGEM-TEasy vector. Note that the A-tails may fall off by keeping the PCR product at -20 ^{o}C for one month
26	No positive colonies	No insert or wrong insert in the pGEM-TEasy vector	Perform a PCR with the internal primers to check that the correct insert is in the vector
		Wrong primer pairs are used	Ensure to use the internal forward versus M13F, and internal reverse versus M13F primer pairs

47	Lack of PCR product after insertion direction PCR	The species-specific internal primers' melting temperature does not match the M13F vector primer	Design species-specific internal primers with melting temperatures between 45 ºC and 50 ºC
58	Agarose gel analysis showing no RNA probe	Poor probe synthesis reaction	Repeat probe synthesis reaction. Avoid RNAse interference
133	Purple staining is visible but weak	Low gene expression or inadequate staining time	Stain the tissue longer (overnight). Ensure the tissues are adequately permeabilized with the Proteinase K solution
	High background staining	Over-staining	Check for signal every 30 min
		Poor tissue treatment	Use the appropriate Proteinase K concentration, and ensure correct duration of treatment
	No *in situ* signal	Gene may not be expressed	Always perform positive and negative controls to rule out bad reagents.
		Sense probe used instead of antisense	Use the appropriate RNA polymerase to synthesize the anti-sense probe

		RNAse might have destroyed the mRNA or the RNA probe	Avoid talking, coughing, and sneezing into the sample. Always use gloves when performing ISH

2.7 Anticipated results

The images of the gene expression patterns on *Drosophila* wings and abdomens generated using this protocol are shown in **Figures 2.4 - 2.8**. This protocol has been used to generate quality ISH images for toolkit genes during early pupal stages on wings and abdomens of non-model *Drosophila* species[6,19] (**Fig. 2.4 and Fig. 2.5**). The pupal dissection steps determine the final orientation of the pupa, whereby improper dissection can lead to the loss of important features, which might affect the image quality and interpretation. Through pupa abdominal ISH, we have shown that the *yellow* gene is expressed in all six rows of spots foreshadowing the adult *D. guttifera* and *D. quinaria* patterns (**Fig. 2.6 and Fig. 2.7**). It may be technically challenging to use one ISH image to show gene expression patterns in all the six rows of spots on the abdomen of some *Drosophila* species in the *quinaria* species group. Therefore, the mastery of the anatomy of the pupa and proficiency in making different types of cuts are very important to ensure that all spot rows are revealed in at least two ISH images. For example, the mid-cut pupa used for abdominal ISH shows the lateral, median, and/or dorsal rows of spots, which prefigure the spots in the lateral region of the adult fly's abdomen (**Fig. 2.6a, 2.8c**), while the lateral cut shows the dorsal and median rows of spots foreshadowing the spots in the dorsal region of the adult fly's abdomen (**Fig. 2.6b, 2.8a**). However, the lateral cut may

occasionally reveal all six rows of spot as shown in **Figure 2.8b**. Interestingly, we have shown this protocol to work in a wide range of rarely studied fruit flies in the *quinaria* species group, such as *D. deflecta, D. guttifera, D. recens, D. quinaria, D. subpalustris*, and *D. palustris.* This protocol contains the necessary information to facilitate the study of novel gene expression patterns in rare and unstudied fruit flies in the future.

BOXES/TABLES/FIGURES

BOX 1 Primer design strategy to amplify specific coding regions of non-model *Drosophila* species

1. Find the gene of interest in the *D. melanogaster* genomic sequence, using GenePalette software[24] (Genome Tools > Entrez Nucleotide Query).
2. Do a local BLAST search, using the coding region of *D. melanogaster* as the query sequence against the *D. guttifera* genome. We perform BLAST search in our lab using "BlastStation-Local software".
3. To the GenePalette *melanogaster* file, add the *D. guttifera* sequence as a sequence comparison (Sequence > Add a Sequence Comparison). Set the comparison word size so that you can see what parts of the *D. guttifera* sequence match with the *D. melanogaster* sequence.
4. Choose an exon that gives the best alignment result to design the primers.
5. Naming the primers depends on the transcription direction, which is indicated by an arrow on the graphical representation of the gene organization in the GenePalette file (**Extended Data Figure 3)**. Therefore, if the arrow is facing the left, the forward primers will go from the right to the left and reverse primers will go from the left to the right. However, if the arrow is facing the right, forward primers will go from the left to the right and reverse primers will go from the right to the left.
6. The gene-specific primers should contain 18 - 25 bases with a Tm of 55 - 60 ºC, GC content of 50 ± 15 %, and give rise to a product between 200 – 500 bp in length.
7. The primers will be used for PCR amplification from genomic DNA, and the products will be cloned into pGEM-TEasy.
8. Design internal primers (internal forward and internal reverse) that do not overlap with the primer sequences already made. They should point inwards and allow the amplification of a shorter "internal PCR product" inside the "external PCR product". The internal forward primers go in the same direction as the outer forward primers, and the internal reverse primers go in the direction of the outer reverse primers. The internal primers will be used to determine the insertion direction in pGEM-TEasy and to confirm that the PCR product is from the correct gene.

   CRITICAL STEP The annealing temperatures for the internal primers should be between 45 ºC and 50 ºC. because the primers will be used with the pGEM-TEasy vector primers M13F and M13R, which also have such low annealing temperatures.

BOX 2 Imaging the wing and abdomen ISH results

1. Place a stained wing or abdomen into 300 μL of PBT in a well.
2. Use the Olympus SZX16 stereoscope with an SDF PLAPO 1 × PF objective lens and a DP72 camera to image the abdomen.
3. Take images of the lateral, dorsal, and ventral views of the abdomen, depending on the location of the expression pattern.
4. Take several images by slowly tuning the fine focus adjustment knob between the photo shots, starting from the top-most piece of tissue that comes into focus and progressing downwards, until the lowest-laying part of the tissue goes out of focus.
5. Z-stack the raw images with Helicon Focus software.
6. Use the "curves" function in Adobe Photoshop to reduce the background noise and maintain the natural colors of the image. Do not bend the "line" to avoid altering the ratios of the original result.

BOX 3 The gel slick coating

1. Use a 200-μL pipette with tip to take up a drop of gel slick
2. Dispense the drop into the first well of a glass-viewing dish.
3. Then dip a clean Kimwipes paper into the drop and rub it into the remaining wells.
4. Use another wipe to smear out any excess gel slick, only leaving a very thin coating in the wells.

BOX 4 *Drosophila* pupa processing for wing ISH

Processing pupae of stages P5ii – P8

1. Collect wandering larvae in a Petri dish with a wet tissue paper on the bottom.
2. Check the time when most puparia have formed and collect P5ii – P8 pupae.
3. Place a 15-mL and a 50-mL Falcon tube with 1× PBS on ice.
4. Ensure the glass-viewing dish is coated with gel slick.
5. In a glass-viewing dish, put a batch of 5 pupae into 1× PBS and take them by the head out of their puparium. The heads may get destroyed, which is fine.
6. Cut off the heads and the tip of the abdomens without squeezing the body (avoid liquid getting pushed into the wings). Then, hold the pupae by the thorax and use a 200-µL pipette to carefully suck and blow out the guts through the open abdomen.
7. Collect the empty carcasses in a 2-mL Eppendorf tube filled with 1.5 mL of fixation buffer on ice.
8. Clean out the glass-viewing dish with distilled water and proceed with the next batch of 5 pupae.
9. Fix overnight at 4°C. On the next morning, place the tube with the fixed carcasses on ice.
10. Pipette about 5 carcasses into a glass-viewing dish and remove the pupal membrane from the wings.
11. Carefully rip off the wings with a small piece of thorax still attached and collect them in a scintillation vial containing about 4 mL of methanol at room temperature.
12. After all wings are dissected, pipette them all back into the clean glass-viewing dish and wait additional 5 min.
13. Wash 2 × with methanol.
14. Wash 2 × with 100 % ethanol.
15. Store the wings in 100 % ethanol at -20 °C.

Processing pupae of stage P9 and older

16. Dissect pupal wings in dH_2O at room temperature in a glass-viewing dish coated with gel slick.
17. Allow the wings to inflate.
18. Place the wings in 1.5 mL of fixation on ice in a 2-mL Eppendorf tube for 30 min.
19. Wash 2 × with methanol.
20. Wash 2 × with 100 % ethanol.
21. Store the wings at -20 °C in 100 % ethanol.

CAUTION Allow methanol waste to evaporate in the hood or pour it down the drain and flush for 1 min. Do not inhale, swallow, or allow methanol to touch your skin.

Table 2.2: Cycling conditions for genomic DNA amplification

Reaction	**Temperature**	**Time**	**Cycle number**
Initial Denaturation	95 ºC	5 min	35 cycles
Denaturation	92 ºC	30 sec	
Annealing	X ºC	30 sec	
Extension	72 ºC	1 min per kb	
Final Extension	72 ºC	5 min	

Table 2.3: Cycling condition for colony PCR

Reaction	**Temperature**	**Time**	**Cycle number**
Initial Denaturation	95 ºC	5 min	35 cycles
Denaturation	92 ºC	30 sec	
Annealing	X ºC	30 sec	
Extension	72 ºC	1 min per kb	
Final Extension	72 ºC	10 min	

Table 2.4: Cycling condition for insertion direction PCR

Reaction	**Temperature**	**Time**	**Cycle number**
Initial Denaturation	95 ^{o}C	5 min	35 cycles
Denaturation	92 ^{o}C	30 sec	
Annealing	45 ^{o}C	30 sec	
Extension	72 ^{o}C	1 min per kb	
Final Extension	72 ^{o}C	5 min	

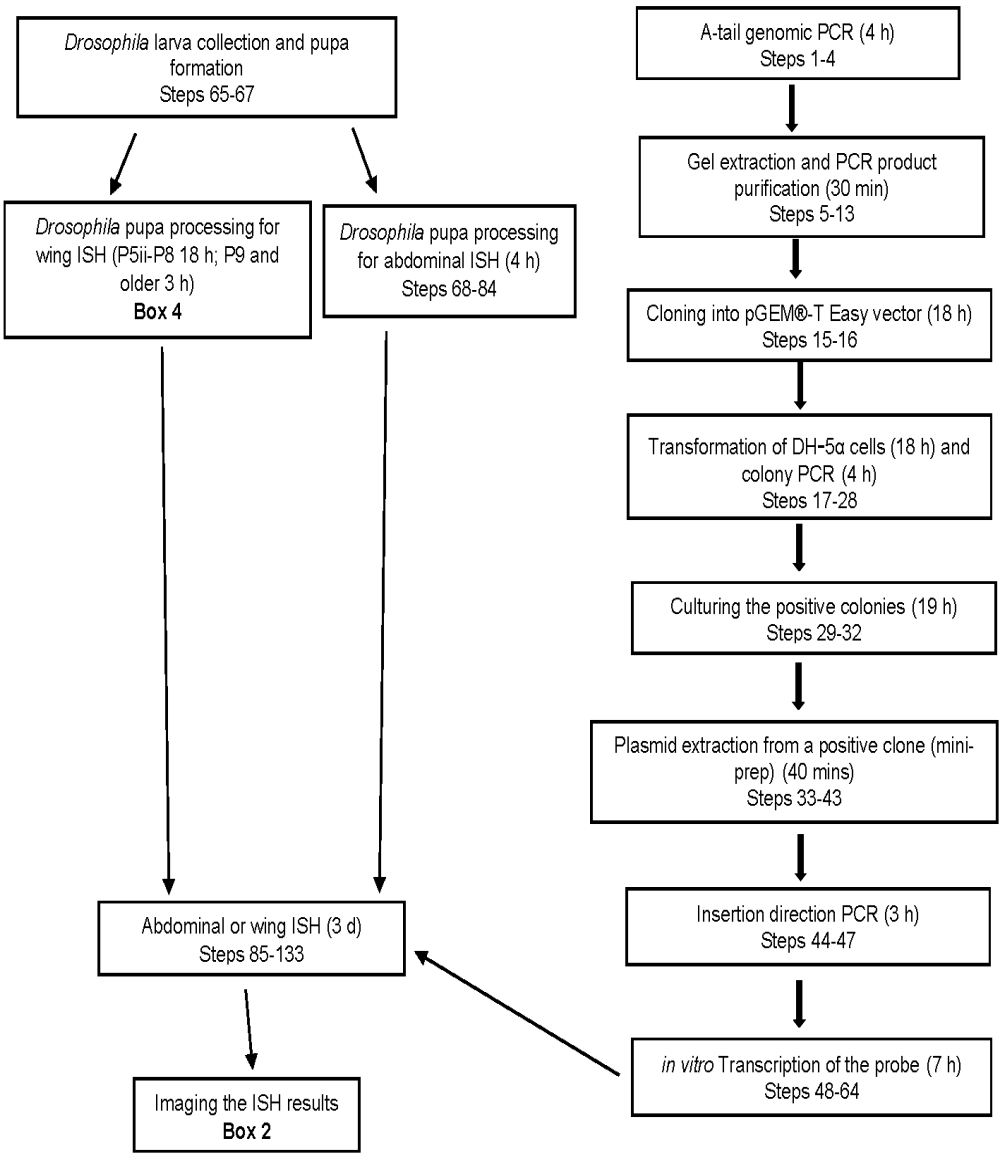

Figure 2.1. Overview of the ISH procedure in the wings and abdomens of *Drosophila* species. The major steps are shown sequentially in the boxes linked by arrows. The duration of each step is indicated in parentheses inside the boxes. Boxes on the right side describe the probe preparation steps, and boxes on the left side illustrate the sample preparation and ISH steps.

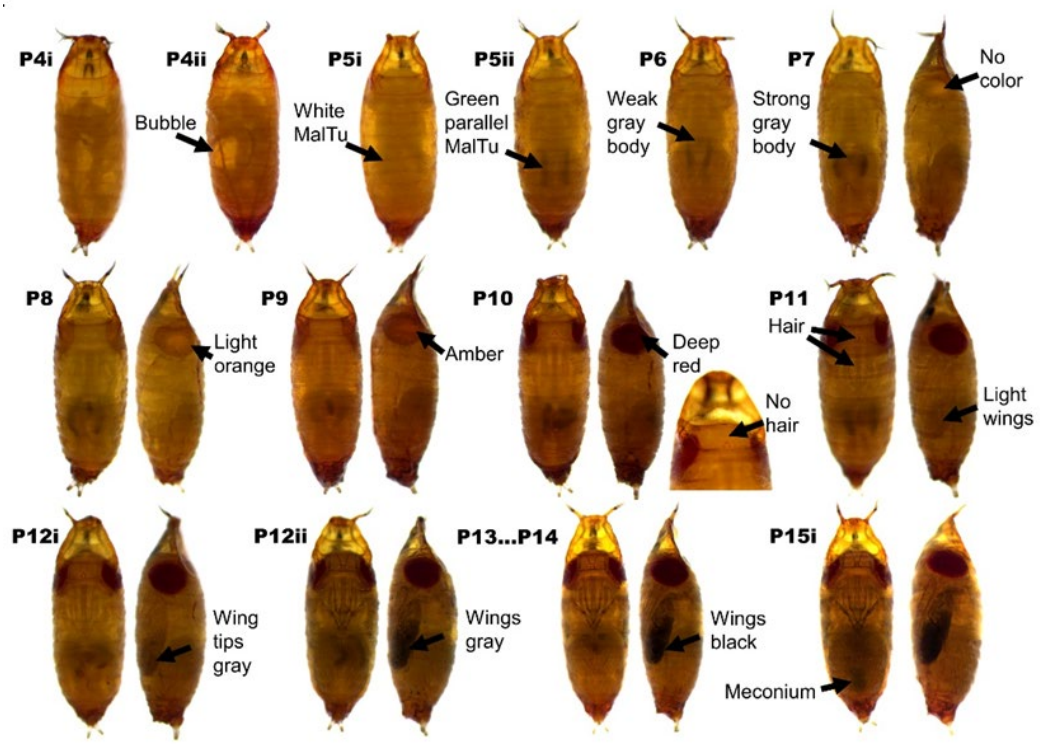

Figure 2.2. *D. guttifera* pupal developmental stages are labeled as described by Bainbridge and Bownes[25] for *D. melanogaster* and adopted for *D. guttifera* by Werner *et al.*[6]. (MaITu stands for Malpighian tubule).

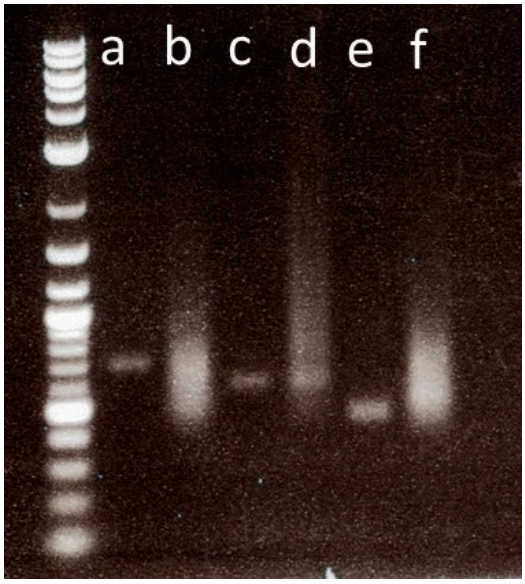

Figure 2.3. Probe quantity determination. An agarose gel image showing the quantity of anti-sense RNA probes. (**a, c, e**) represent PCR templates for the probes' synthesis. (**b, f**) High-quantity probes showing thick probe smears (**d**) Low-quantity probe.

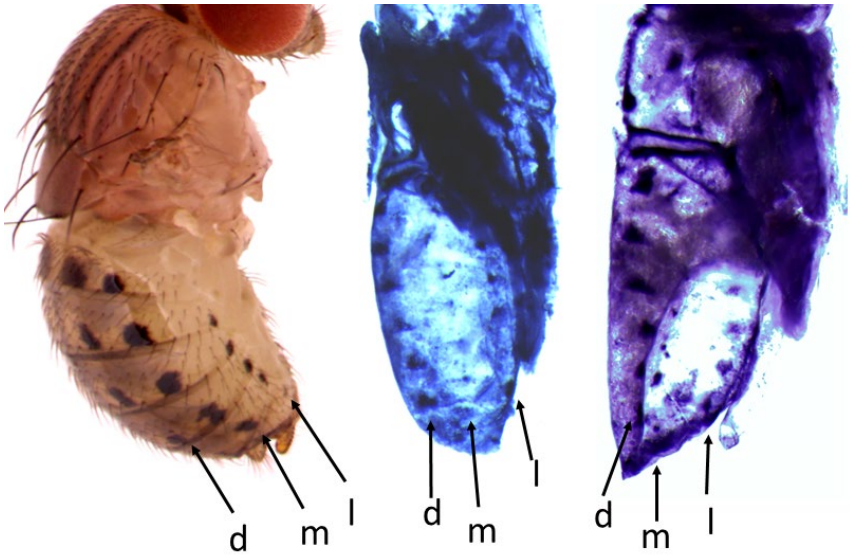

Figure 2.4. The mRNA expression pattern of *wingless* (*wg*) in the early pupal stage (P7) of *D. guttifera* foreshadowing the adult abdominal spot pattern. The spot rows are labeled as lateral (l), median (m), and dorsal (d).

Figure 2.5. *wingless* mRNA expression foreshadows the adult spot pattern on the wing of *D. guttifera*. This image has been previously published in Werner *et al.*[6].

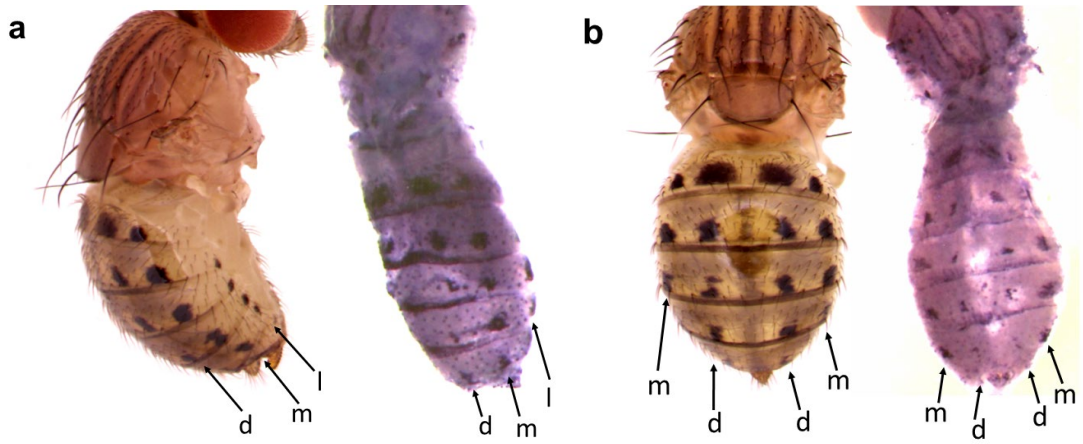

Figure 2.6. *in situ* Hybridization signals detecting *yellow* mRNA during pupal stage P10 of *D. guttifera* foreshadow the abdominal spot pattern of the adult. (a) Dorsal cut showing the lateral pattern of *yellow* mRNA expression. **(b)** Lateral cut showing the dorsal pattern of *yellow* mRNA expression. The spot rows are designated as lateral (l), median (m), and dorsal (d).

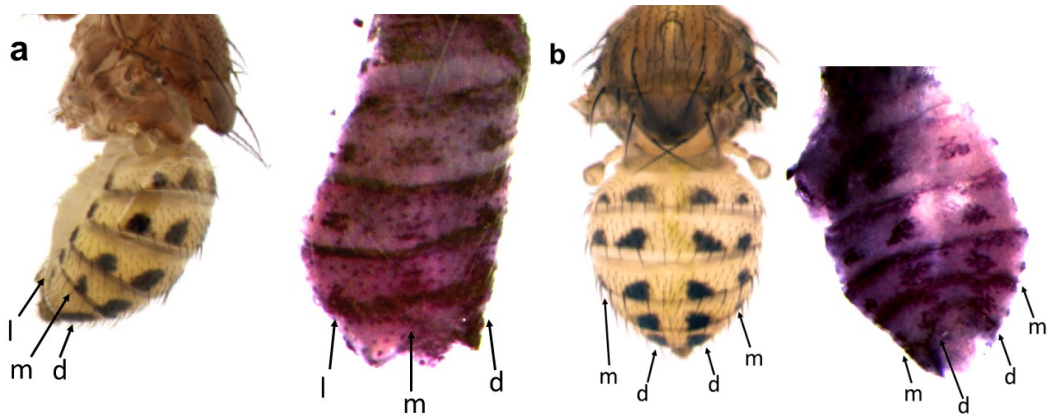

Figure 2.7. The *in situ* hybridization signals of the *yellow* gene expression pattern during *D. quinaria* pupal development (P10) foreshadowing the adult abdominal spot pattern. The spot rows are designated as lateral (l), median (m), and dorsal (d).

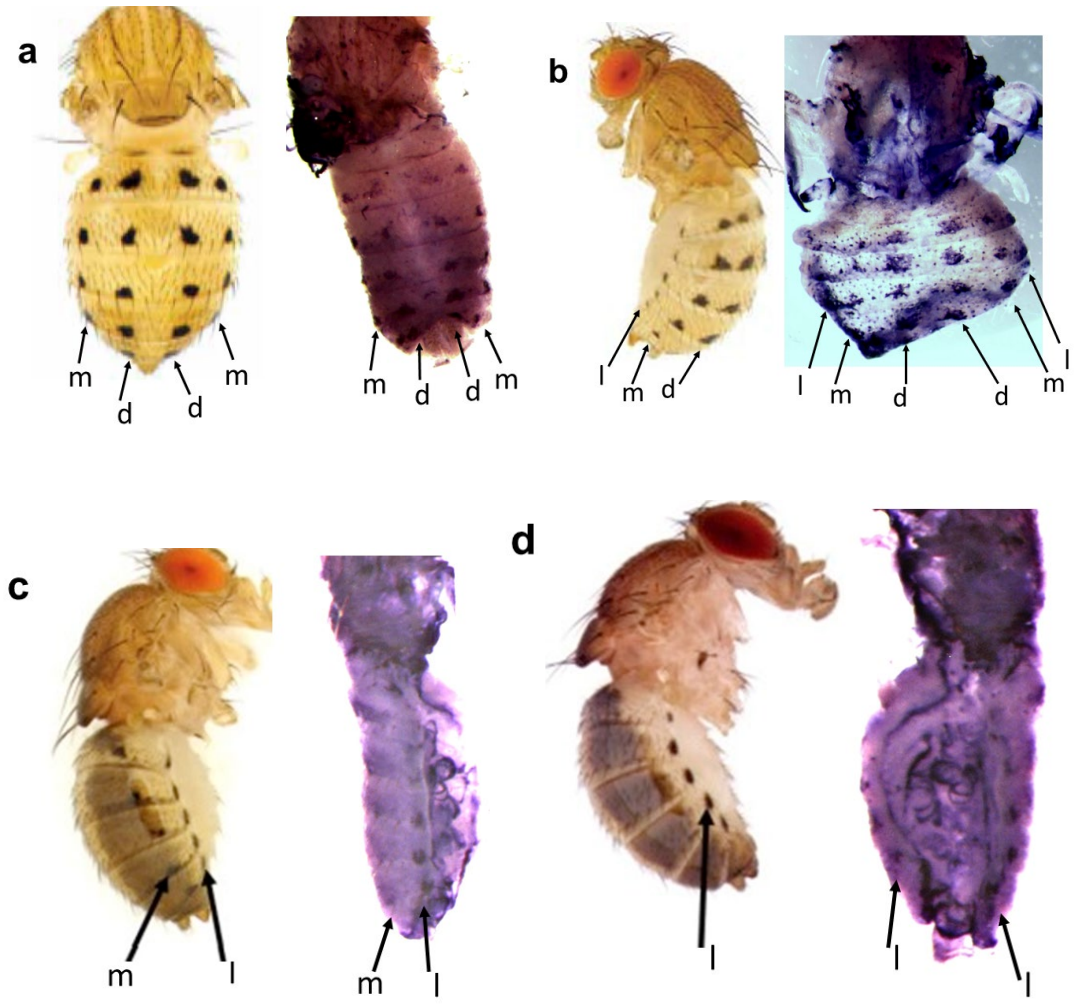

Figure 2.8. The *in situ* hybridization signals of the *yellow* (*y*) and *tan* (*t*) transcripts during *D. deflecta, D. recens, D. palustris,* and *D. subpalustris* pupal development prefigure the adult abdominal spot pattern. The images of the adult flies have been

previously published in Werner *et al.*[22] (**a, b**) *yellow* mRNA expression in *D. deflecta* and *D. recens* respectively, during pupal stage P10. (**c, d**) *tan* mRNA expression in *D. palustris* and *D. subpalustris*, respectively, during pupal stage P11, as shown in Dion *et al.*[5].

2.8 Supplementary information

Table S1: List of primers used to prepare probes for ISH.

The *D. guttifera t* exon 5 forward and reverse primer pair was used to amplify *D. guttifera* genomic DNA to produce the probe used to perform the *tan* ISH in *D. palustris* and *D. subpalustris.* The forward and reverse primer pair for *D. guttifera y* exon 2 was used to amplify *D. guttifera* genomic DNA to develop the probe to determine *yellow* gene expression in *D. guttifera* and *D. deflecta*. The *D. palustris* forward and reverse primer pair for *y* exon 2 was used to amplify *D. quinaria* and *D. recens* genomic DNA to make the probes used to determine *yellow* gene expression patterns in both *D. recens* and *D. quinaria.* We used the probes generated from a different species' DNA to perform ISH due to the close evolutionary relationships of species within the *quinaria* species group. All internal forward and internal reverse primer pairs were used for verification of the gene identity during the probe-making process.

Primer Name	**Primer Sequence**
D. guttifera t exon 5 forward	CAGCGTCTGCTTGGCCACACG
D. guttifera t exon 5 reverse	TTGCCGCTGCGCAACAATTCGG
D. guttifera t exon 5 internal forward	GCTGAATCATTACTACTTTGTGG
D. guttifera t exon 5 internal reverse	AATGGTGTTGATGCTGAACACG
D. palustris y exon 2 forward	GAGGAGGGCATCTTTGGC
D. palustris y exon 2 reverse	CGATGCCATGGAATTGCGG
D. palustris y exon 2 internal forward	TCTCGCACCGAGGACAGC
D. palustris y exon 2 internal reverse	CGATCAGATTGAACAGCTCG
D. melanogaster wg exon 4 forward	CACGTCCAAGCGGAGATGCG
D. melanogaster wg exon 4 reverse	GGCGACGGCATGTTCGGGTG
D. melanogaster wg exon 4 internal forward	TGCCATGGCATGTCCGGATCG
D. melanogaster wg exon 4 internal reverse	GTTCAGCATACGCTCCTCCTCC
pGEM®-T Easy M13F	GTAAAACGACGGCCAGT
pGEM®-T Easy M13R	CAGGAAACAGCTATGAC

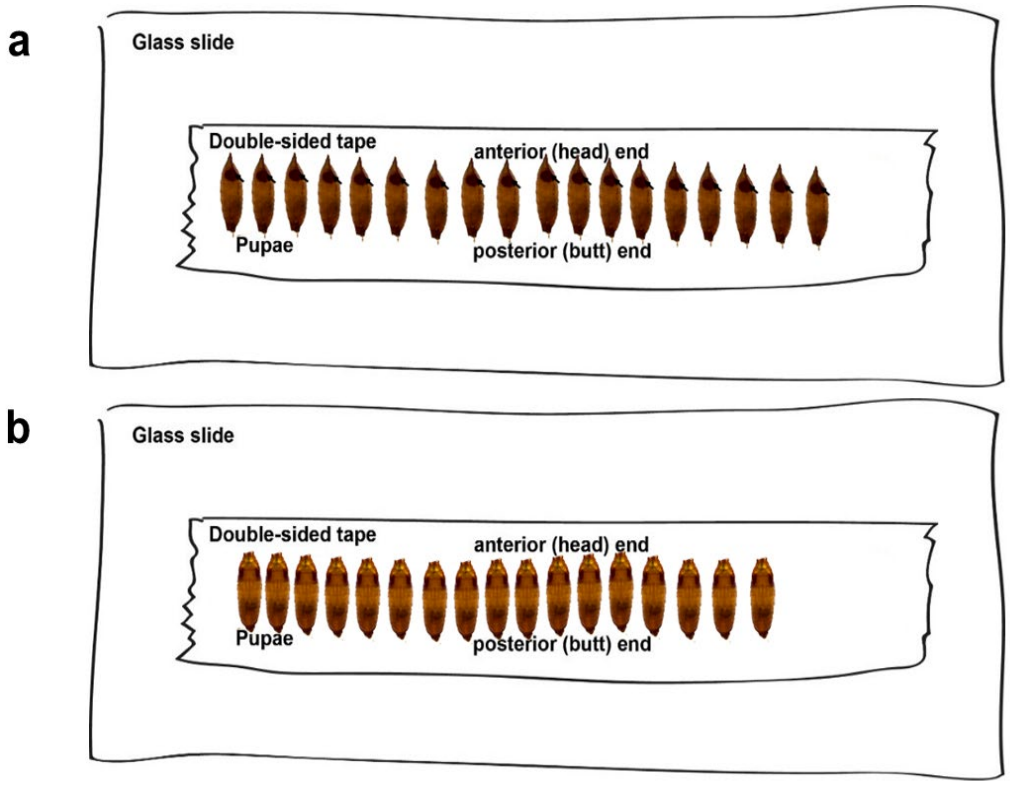

Figure S2.1. *D. guttifera* pupae lined up for lateral and dorsal cuts. (**a**) The pupae were positioned on the side to perform a cut that separates the dorsal from the ventral half (lateral cut) (**b**) the pupae were placed with their ventral side facing the tape to make a cut between both eyes (dorsal cut).

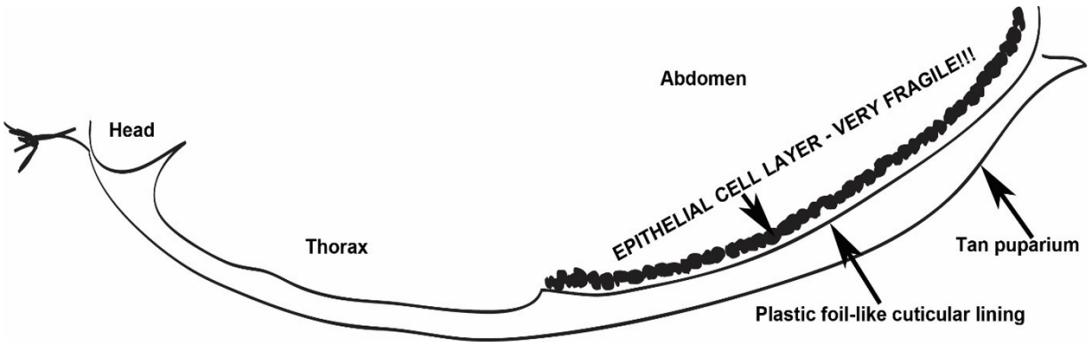

Figure S2.2. A sketch of a *Drosophila* pupal abdomen showing the internal epithelial cell layer and the cuticular lining holding the cells.

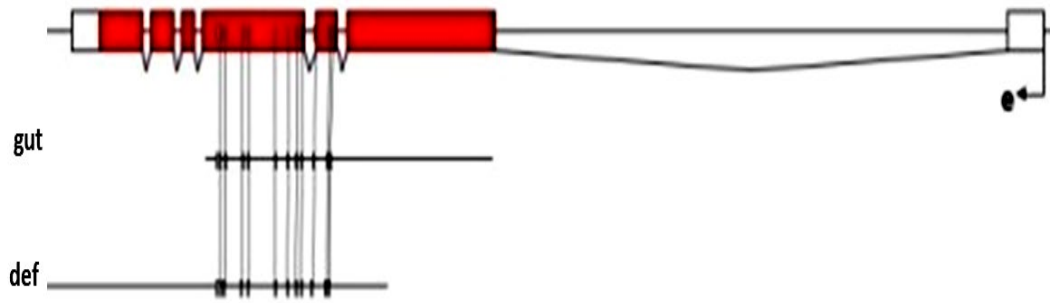

Figure S2.3. A graphical representation of the conserved region on the *ebony* gene of *D. melanogaster* (top row), *D. guttifera* (gut), *and D. deflecta* (def).

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3 Chapter 3A

THE REGULATION OF A PIGMENTATION GENE IN THE FORMATION OF COMPLEX COLOR PATTERNS IN *DROSOPHILA* ABDOMENS

The material presented in this chapter is under review in Nature Communications

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3.1 Abstract

Changes in *cis*-regulatory modules (CRMs) that control developmental gene expression patterns have been implicated in the evolution of animal morphology[1-6]. However, the genetic mechanisms underlying complex morphological traits remain largely unknown. Here we investigated the molecular mechanisms that induce the pigmentation gene *yellow* (*y*) in a complex spot and shade pattern on the abdomen of the quinaria group species *Drosophila guttifera*. We show that the *y* expression pattern is controlled by only one CRM, which contains a stripe-inducing CRM at its core. We identified several developmental genes that may collectively interact with the CRM to orchestrate the patterning in the pupal abdomen of *D. guttifera*. We further show that the core CRM is conserved among *D. guttifera* and the closely related quinaria group species *Drosophila deflecta*, which displays a similarly spotted abdominal pigment pattern. Our data suggest that besides direct activation of patterns in distinct spots, abdominal spot pattern in *Drosophila* species may have evolved through partial repression of an ancestral stripe pattern, leaving isolated spots behind. Abdominal pigment patterns of extant quinaria group species support the partial repression hypothesis and further emphasize the modularity of the *D. guttifera* pattern.

3.2 Introduction and results

How complex morphological features develop and evolve is a question of foremost importance in biology. To address this question, we identified genes underlying abdominal pigmentation pattern development in *Drosophila guttifera* (*D. guttifera*). The abdomen is decorated with six rows of black spots that run along the anterior-posterior axis, divided by a dark dorsal midline shade. This color pattern shows four sub-patterns: a dorsal, median, and lateral pair of spot rows, plus the dorsal midline shade (**Fig. 3a, b**). *D. guttifera* belongs to the quinaria species group, whose members display highly diverse abdominal pigmentation patterns[7,8]. While *D. guttifera* shows the most complex pattern of this group, most other quinaria group species lack at least one of the four sub-patterns, illustrating the pattern modularity among species. Interestingly, the stripe patterns of certain species often separate into spots[7,8]. In this study, we show that the abdominal pigment patterns of quinaria group members may be formed by a combination of localized spot induction and partial stripe repression

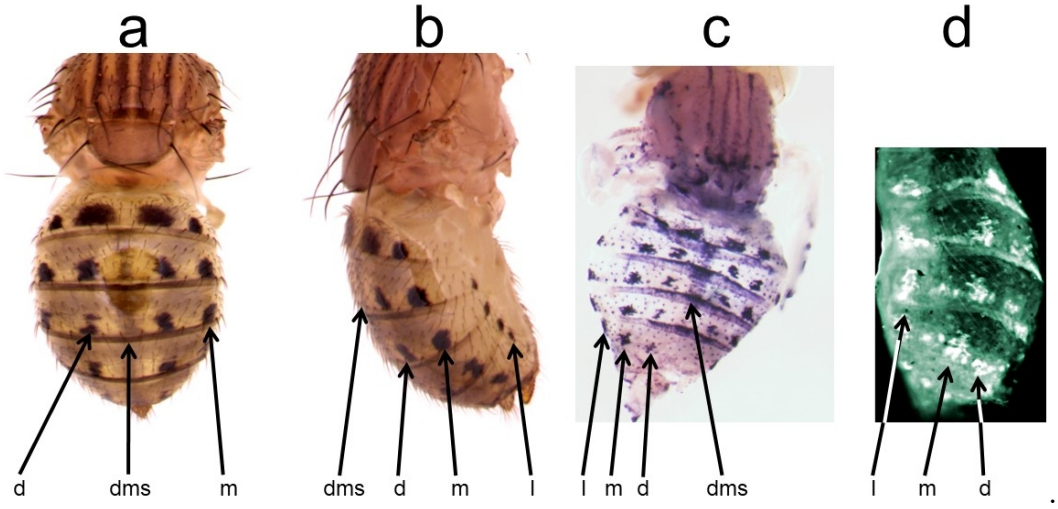

Figure 3.1: The *D. guttifera* abdominal color pattern is modular. a, Adult, dorsal view. b, Adult, lateral view. c, *yellow* mRNA expression pattern in a pupal abdomen. d, Yellow protein expression pattern in a pupal abdomen. dms = dorsal midline shade, d = dorsal, m = median, l = lateral spot rows.

We focused on the regulation of the *yellow* (*y*) gene, which is required for the formation of black melanin in insects[8-14]. Several *y* gene CRMs have been identified in various *Drosophila* species, and changes in these CRMs and/or in the deployment of *trans*-factors that regulate *y* gene expression have been implicated in the diversification of wing and body pigment patterns[12,15-19]. In *D. guttifera* pupae, *y* gene expression and the location of the Y protein accurately prefigured the complex adult abdominal pigment pattern (**Fig. 3.1c, d**). In order to identify putative upstream activators of *y*, we performed an *in situ* hybridization screen for genes expressed in ways prefiguring the *y* gene expression pattern. We found that *wingless* (*wg*) expression precisely foreshadowed the six rows of black spots (**Fig. 3.2b**). Additionally, *decapentaplegic* (*dpp*) expression foreshadowed the dorsal and median pairs of spot rows (**Fig. 3.2c**), while *abdominal-A* (*abd-A*) expression correlated with the lateral pair of spot rows and the dorsal midline shade (**Fig. 3.2d, e**). *hedgehog* (*hh*) and *zerknullt* (*zen*) were additionally expressed along the dorsal midline of the abdomen (**Fig. 3.2f, g**). Thus, the activation of the *D. guttifera* color pattern appears to be induced in a modular fashion, which is in agreement with our observation that abdominal pigmentation patterns within the quinaria group are variations of the *D. guttifera* pattern ground plan (**Fig. 3.3**). This situation is reminiscent of the wing pattern ground plan in nymphalid butterflies[20,21].

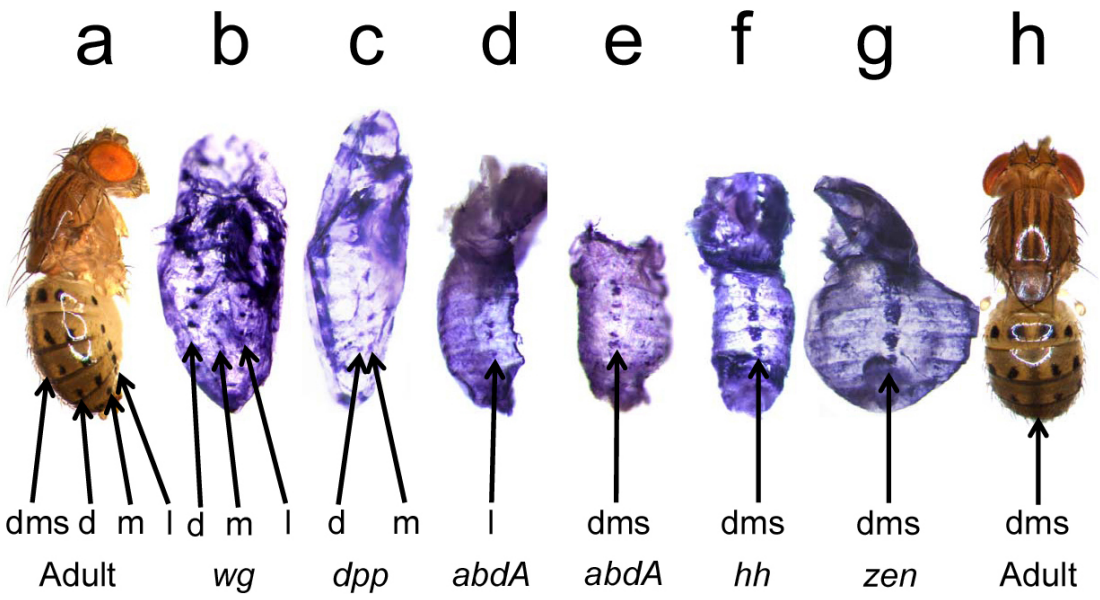

Figure 3.2: The mRNA expression patterns of five developmental genes foreshadow the *yellow* expression pattern. a, Adult, lateral view. b-g, *in situ* hybridizations in pupal abdomens. h, Adult, dorsal view. dms = dorsal midline shade, d = dorsal, m = median, l = lateral spot rows (Image by Dr. Raja).

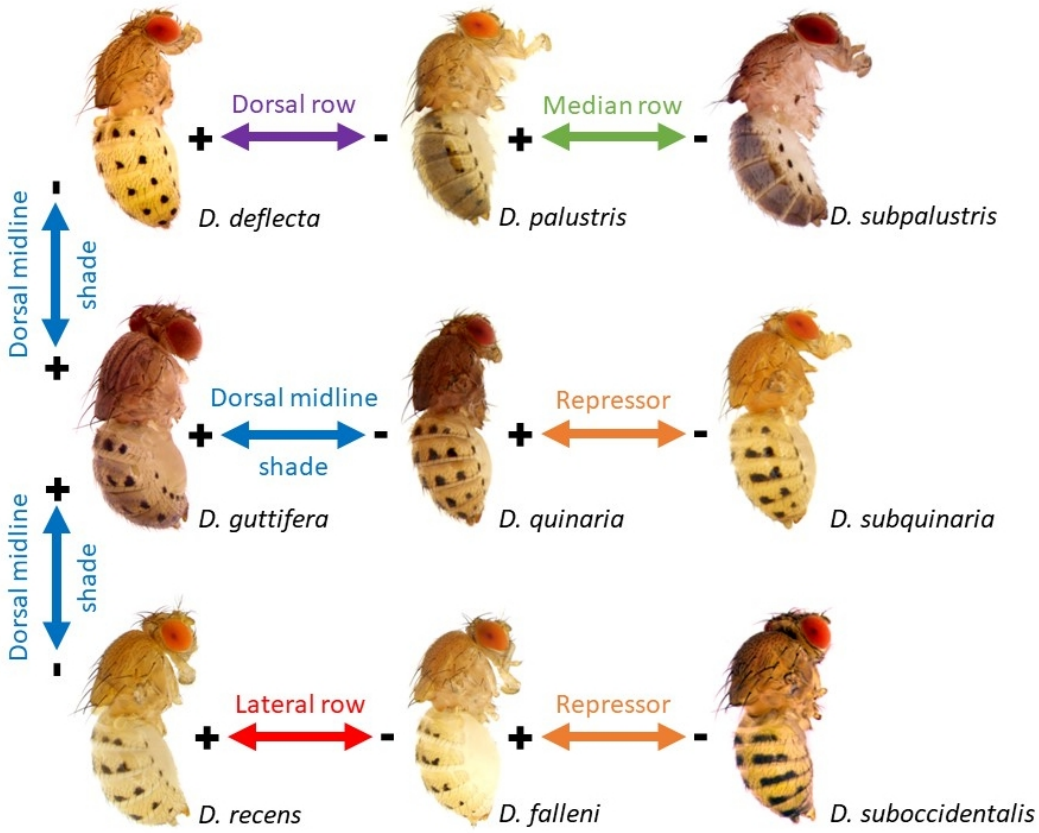

Figure 3.3: Deviations from the *D. guttifera* ground plan create the diversity of quinaria species' abdominal color patterns. + = gain, - = loss of a pattern element. "Repressor" suggests stripes may be broken into spots by repressors of pigmentation and vice versa. The illustration does not imply any evolutionary direction; it solely illustrates the modularity of these complex patterns.

We hypothesized that the developmental candidate genes may activate the *y* gene through four CRMs, each controlling one sub-pattern to assemble the complete melanin pattern. We searched for these CRMs by transforming *D. guttifera* with *DsRed* reporter constructs containing non-coding fragments of the 42 kb *D. guttifera y* gene locus[12] (**Fig. 3.4**). Surprisingly, only one 953 bp fragment from the *y* intron, the *gut y spot* CRM, drove

expression closely resembling all six spot rows on the developing abdomen (**Fig. 3.5**). To isolate possible sub-pattern-inducing CRMs, we subdivided the *gut y spot* CRM into 8 partially overlapping sub-fragments. Unexpectedly, the 636 bp left sub-fragment displayed horizontal stripe expression along the posterior edges of each abdominal segment, while the 570 bp right fragment was inactive (#1 & #2, **Fig. 3.5**). Further dissection of this CRM revealed a 259 bp sub-fragment, which contained the minimal *gut y core stripe* CRM with some additional dorsal midline shade activity (#7, **Fig. 3.5**). These results suggest that the *D. guttifera* spots may have evolved from an ancestral stripe pattern that became partially repressed to isolate the spots. Currently, we cannot offer any direct evidence for specific candidate repressor genes. Neither the *in situ* hybridization experiments nor the bioinformatics analyses, using Jaspar, resulted in putative pigment stripe repressors. Although we identified 24 Engrailed (En)-binding sites and 19 Homothorax (Hth)-binding sites in the *gut y spot* CRM (both are known repressors of pigmentation in *Drosophila*[15,22]), these sites were not enriched in the right half of the CRM, as we would have expected. However, our transcription factor binding site analysis of the *gut y spot* CRM sequence revealed putative transcription factor binding sites for most of the developmental genes that we identified as potential activators in our *in situ* hybridization screen, except for *hh*. This suggests that localized spot activation by these developmental factors contributes to the formation of the pattern.

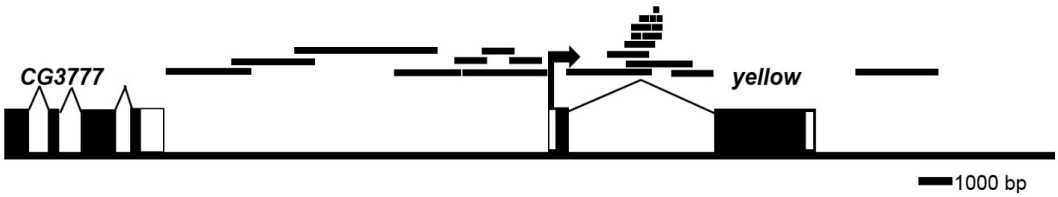

Figure 3.4: The *y* gene locus. The horizontal bars indicate the DNA fragments of the *D. guttifera y* gene that were tested in transgenic *D. guttifera* for regulatory activity (Image by Dr. Raja).

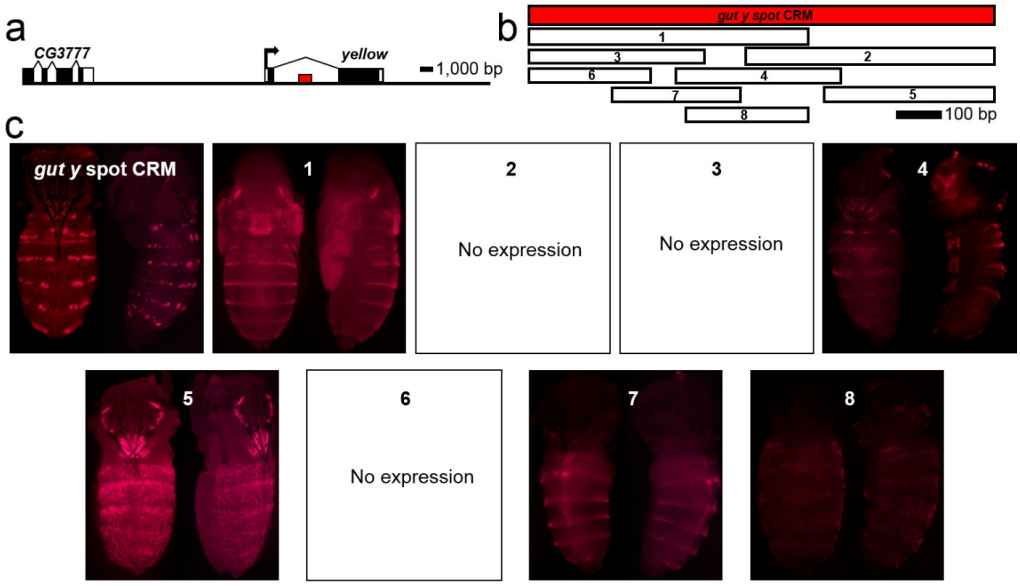

Figure 3.5: The *gut y spot* CRM is harbored within the *y* intron. a, The *y* gene locus. The red bar indicates the relative position of the *gut y spot* CRM. b, Sub-dividing the *gut y spot* CRM revealed horizontal stripes on each abdominal segment. The white bars (1-8) represent sub-fragments of the *gut y spot* CRM, and the corresponding pupal *DsRed* expression patterns in transgenic *D. guttifera* are shown (Image by Dr. Raja).

Next, we asked whether the abdominal pigment spot pattern of a species closely related to *D. guttifera* shares a similar developmental basis. We thus performed *in situ* hybridization experiments in *Drosophila deflecta* (*D. deflecta)*. This species displays six longitudinal spot rows on its abdomen, but lacks the dorsal midline shade (**Fig. 3.6a, b**). As in *D. guttifera*, *y* mRNA in *D. deflecta* pupal abdomens was expressed in six rows of spots, except along the dorsal midline (**Fig. 3.6c**). Similarly, *wg* foreshadowed all six rows of spots, while *dpp* expression matched all but the lateral spot rows (**Fig. 3.7b, c**). In contrast to the *D. guttifera* results, *abd-A*, *hh*, and *zen* were absent along the dorsal midline, which is in agreement with the lack of pigment in *D. deflecta* adults (**Fig. 3.7d, e, f, g**). However, *abd-A* expression was not detectable where the lateral spot rows will form (**Fig. 3.7d**), suggesting that these particular spots are controlled differently in *D. deflecta*. We next cloned the 938 bp orthologous *def y spot* CRM and transformed it into *D. guttifera*, using the *DsRed* reporter assay. The *def y spot* CRM drove faint dorsal spot row and stripe expression, especially along the dorsal spots (**Fig. 3.8**). We further subdivided the *def y spot* CRM into 8 sub-fragments and identified a minimal *def y core stripe* CRM (288 bp) (#7, **Fig. 3.8**). This sub-fragment drove a striped pattern, but without the dorsal midline shade activity seen in the *D. guttifera* minimal *gut y core stripe* CRM (#7, **Fig. 3.8**). We further transformed the *gut y spot* and *def y spot* CRMs including all sub-fragments into *D. melanogaster* to test if *D. melanogaster trans*-factors can bind to and activate these two quinaria group species' spot CRMs. As a result, none of the reporter constructs showed any expression (data not shown). This suggests that the hypothetical ancestral stripe pattern of the quinaria group and the pigment stripes found on the *D. melanogaster* abdominal tergites[23] have evolved independently by changes in

trans. As the spot CRMs from *D. guttifera* and *D. deflecta* are not orthologous to any sequences within the *D. melanogaster y* locus, changes in *cis* have also contributed to the diversification of pigment patterns between *D. melanogaster* and the quinaria species group.

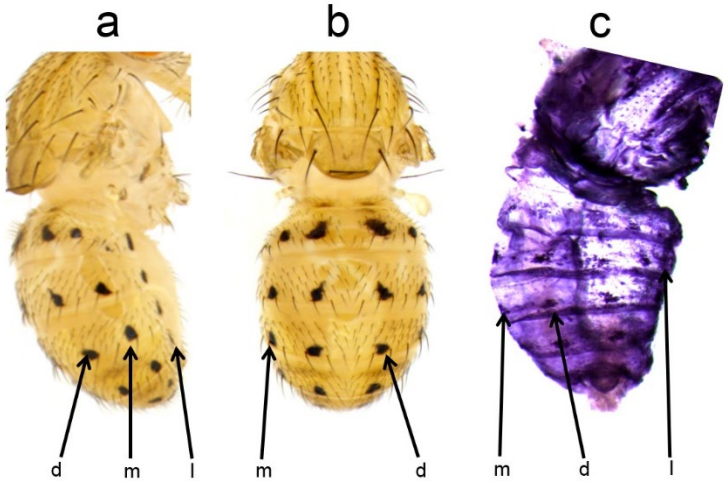

Figure 3.6: The *y* gene expression pattern in *D. deflecta* foreshadows the black spot pattern on the adult abdomen. a, Adult lateral view. b, Adult dorsal view. c, *y* mRNA in the pupal epidermis. d = dorsal, m = median, l = lateral (Image by Dr. Raja).

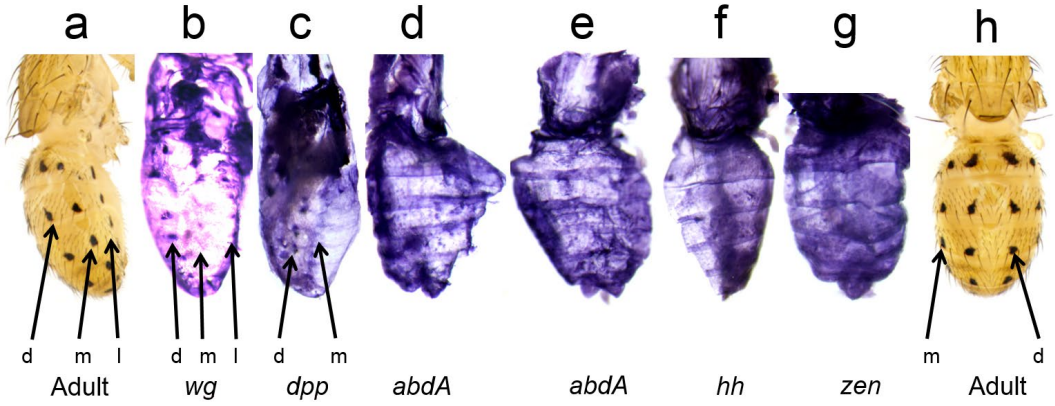

Figure 3.7: Developmental gene expression patterns in *D. deflecta* foreshadow distinct subsets of the adult abdominal color pattern. a, h, Adult, lateral view. b-g, Pupal *in situ* hybridizations. d = dorsal, m = median, l = lateral (Image by Dr. Raja).

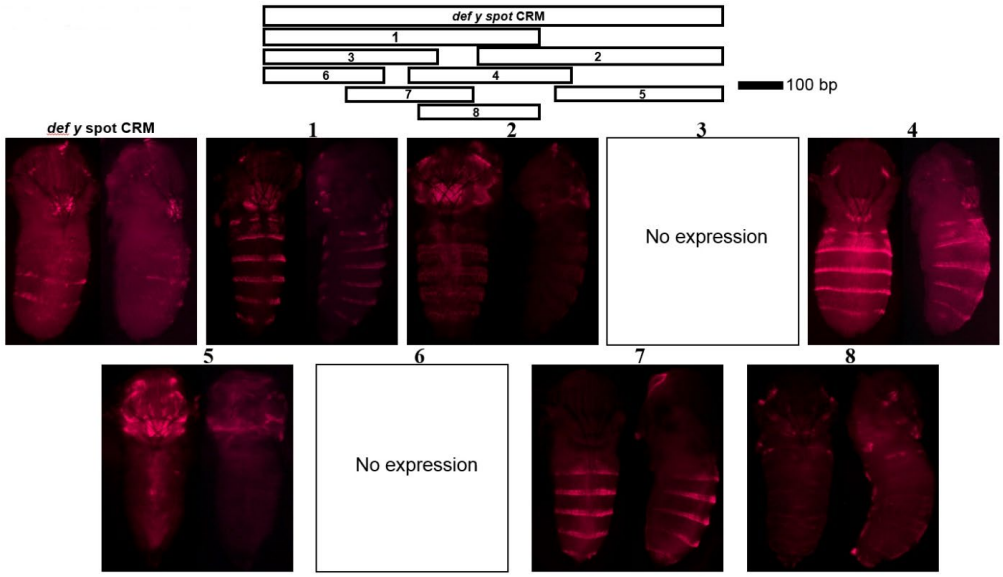

Figure 3.8: The orthologous *D. deflecta* region (*def y spot* CRM) analyzed in transgenic *D. guttifera.* The white bars (1-8) indicate sub-fragments of the *def y spot* CRM that were tested for reporter activity. The corresponding DsRed reporter expression patterns in developing pupae are shown (Image by Dr. Raja).

In contrast to the *D. guttifera* wing spot pattern[12], the abdominal pigment pattern develops in the absence of visible physical landmarks. *wg*, *dpp*, and *hh* are homologous to known proto-oncogenes in humans[24], while *zen* and *abdA* are Hox genes. The abdominal color pattern of *D. guttifera* appears to be regulated by multiple developmental pathways consisting of activators and repressors acting in parallel, possibly targeting pigmentation genes other than *y* as well[18,19,25,26]. Further evidence for

the repression of stripes can be seen in *Drosophila falleni*'s intraspecific pigment variation, another member of the quinaria species group (**Fig. 3.9**). Our multi-pathway model fits well with the observation that the abdominal pattern variation presented by quinaria group members is largely due to modular derivations from the *D. guttifera* ground plan (**Fig. 3.3**). This scenario is reminiscent of the modularity found in butterfly wing patterns. Because insects use similar genes for color pattern development[21,27-30], the quinaria group may serve as a valuable model to understand insect color pattern evolution. Future work should aim to manipulate the genes involved in pigmentation to test if they interact according to the reaction-diffusion model, as predicted by Alan Turing[31].

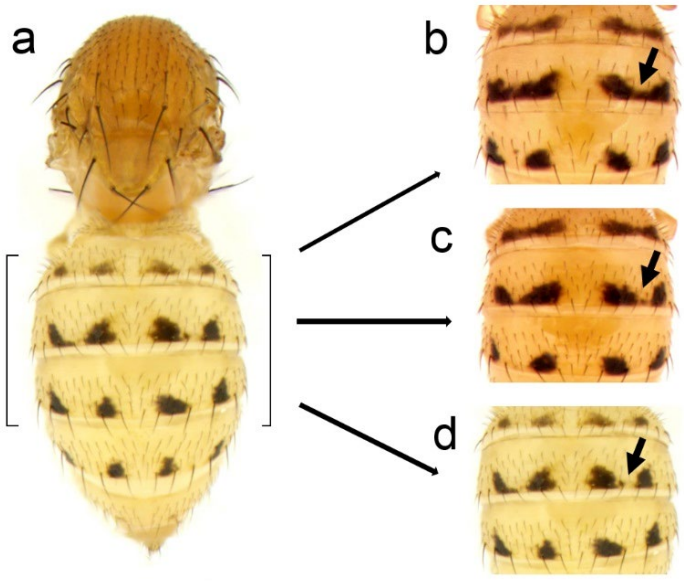

Figure 3.9: The abdominal pigment stripes of *D. falleni* break down into pigment spots. Intraspecific variation, as illustrated here, is very common in *D. falleni*. a, Adult

abdomen. b-d, Adult abdominal pigment spots developing from stripe repression (arrows) (Image by Dr. Raja).

3.3 Materials and Methods

3.3.1 Molecular procedures

In situ hybridizations were carried out with species-specific RNA probes, as described in[12], but with abdomens cut into halves and cleaned from the internal organs. At least three positive pupae were observed for each result shown. Additional images for verification purposes are provided in Extended Data Figs. 6-16. Immunohistochemistry for the Y protein in abdomens was performed according to[15], with abdomens cut in half and cleaned with 1X PBS. *D*. *guttifera* CRMs were identified and tested in *D. guttifera* according to[12] and in *D. melanogaster* as described in[23]. Transgenic experiments were performed as outlined in[32]. Pupal stages were identified according to[33].

3.3.2 Drosophila stocks

All fly stocks were a kind gift from the Sean B. Carroll Laboratory (University of Wisconsin - Madison) and were cultured at room temperature. We used the *D. melanogaster* fly strain VK00006 (cytogenic location 19E7), the *D. guttifera* stock no.15130–1971.10, and the *D. deflecta* stock no. 15130-2018.00 for gene expression and transgenic analyses.

3.3.3 PCR primer sequences

We used the following primers to amplify the CRM sequences:

(iii) *gut y spot* CRM: Fwd: 5'-CAGCTGCGGTTGAGTACGAC-3'and Rvs: 5'-GCCAACTCGACGGGAATTC-3'. Restriction sites: KpnI and SacII.

(iv) *def y spot* CRM: Fwd: 5'-CAGCTGCTGCGGTTCAGTAG-3' and Rvs: 5'-GCTAGACACACGTTGGTTTGCT-3'. Restriction sites: KpnI and SacII.

(v) *gut y spot* CRM sub-fragment #1: Fwd: 5'-CAGCTGCGGTTGAGTACGAC-3' and Rvs: 5'-ACTGAATCTGATTTCGGCTCG-3'. Restriction sites: KpnI and SacII.

(vi) *gut y spot* CRM sub-fragment #2: Fwd: 5'-AGTTAATCGCCAGTCAATAATGGC-3' and Rvs: 5'- GAATTCCCGTCGAGTTGGC-3'. Restriction sites: KpnI and SacII.

(vii) *gut y spot* CRM sub-fragment #3: Fwd: 5'-CAGCTGCGGTTGAGTACGAC-3' and Rvs: 5'-GCCATTATTGACTGGCGATTAAC-3'. Restriction sites: KpnI and SacII.

(viii) *gut y spot* CRM sub-fragment #4: Fwd: 5'-AAATGAAGCTCAGTGAGCCGC-3' and Rvs: 5'-ACTGAATCTGATTTCGGCTCG-3'. Restriction sites: KpnI and SacII.

(ix) *gut y spot* CRM sub-fragment #5: Fwd: 5'-AGCATCTGAAACTTAAACGCCG-3' and Rvs: 5'-GAATTCCCGTCGAGTTGGC-3'. Restriction sites: KpnI and SacII.

(x) *gut y spot* CRM sub-fragment #6: Fwd: 5'-CAGCTGCGGTTGAGTACGAC-3' and Rvs: 5'-CAGCGATATTAATTTTTTATTCAATGG-3'. Restriction sites: KpnI and SacII.

(xi) *gut y spot* CRM sub-fragment #7(*gut y core stripe* CRM): Fwd: 5'-AAATGAAGCTCAGTGAGCCGC-3' and Rvs: 5'-GCGATTTGTTTGTCAAGTCAAC-3'. Restriction sites: KpnI and SacII.

(xii) *gut y spot* CRM sub-fragment #8: Fwd: 5'-AAATGAAGCTCAGTGAGCCGC-3' and Rvs: 5'-GTTGACTTGACAAACAAATCGC-3'. Restriction sites: KpnI and SacII.

(xiii) *def y spot* CRM sub-fragment #1: Fwd: 5'-CAGCTGCTGCGGTTCAGTAG-3' and Rvs: 5'-ATTGTCGCAGCTGCCTAACG-3'. Restriction sites: KpnI and SacII.

(xiv) *def y spot* CRM sub-fragment #2: Fwd: 5'-AACGAAGCTCACTGAGCTGC-3' and Rvs: 5'-AGCAAACCAACGTGTGTCTAGC-3'. Restriction sites: KpnI and SacII.

(xv) *def y spot* CRM sub-fragment #3: Fwd: 5'-CAGCTGCTGCGGTTCAGTAG-3' and Rvs: 5'-GTTAAAAGCAGCCAGTTGGCC-3'. Restriction sites: KpnI and SacII.

(xvi) *def y spot* CRM sub-fragment #4: Fwd: 5'-CAAAGAATCGAATTCGGAGACAG-3' and Rvs: 5'-ATTGTCGCAGCTGCCTAACG-3'. Restriction sites: KpnI and SacII. (Clone name: *def y* 1.1C2)

(xvii) *def y spot* CRM sub-fragment #5: Fwd: 5'-GAATGAGATTCGTTAGGCAGC-3' and Rvs: 5'-AGCAAACCAACGTGTGTCTAGC-3'. Restriction sites: KpnI and SacII.

(xviii) *def y spot* CRM sub-fragment #6: Fwd: 5'-CAGCTGCTGCGGTTCAGTAG-3' and Rvs: 5'-TTCAACGGATATTCGTTCAATTTC-3'. Restriction sites: KpnI and SacII.

(xix) *def y spot* CRM sub-fragment #7 (*def y core stripe* CRM): Fwd: 5'-CAAAGAATCGAATTCGGAGACAG-3' and Rvs: 5'-GTCAGGCAATGTAAATGTTGTCG-3'. Restriction sites: KpnI and SacII.

(xx) *def y spot* CRM sub-fragment #8: Fwd: 5'-AACGAAGCTCACTGAGCTGC-3' and Rvs: 5'-ATTGTCGCAGCTGCCTAACG-3'. Restriction sites: KpnI and SacII.

These forward and reverse primer sequences do not include restriction sites.

We used the following primers to amplify the coding region of the genes to synthesize *in situ* hybridization probes:

gut def y ex2 Fwd: 5'-CCAACATCGCCGTGGACATTG

gut def y ex2 Rvs: 3'-AATTGCGGAGTGTACGGCATCG

mel y ex2 Fwd: 5'-CTAACATTGCCGTGGATATAGGC

mel y ex2 Rvs: 3'-AATTGCGGTGAGTACGGCATTG

gut hh ex3 Fwd: 5'-GTGAGCAGTGTTCAGAGTCG

gut def hh ex3 Rvs: 3'-TACATATTGTATAGGGTATCTGTCTG

def hh ex3 Fwd: 5'-GTAAGCAGTGTCCAAAGACGC

mel wg ex4 Fwd: 5'-CACGTCCAAGCGGAGATGCG

mel wg ex4 Rvs: 3'- GGCGACGGCATGTTCGGGTG

gut def abd-A ex1 Fwd: 5’-TTCGCTGCTCGTAACGCCACAAC

gut abd-A ex1 Rvs: 3’-TTTGCTGTCGACGCTGGCAGCG

def abd-A ex1 Rvs: 3’-TTTGCTGTCGACGTTGGCAGCG

gut def dpp ex3 Fwd: 5’-CATCGGGAGCCGAGCTATTGGC

gut def dpp ex3 Rvs: 3’-GCATCATAGCCCGCTGGCGC

gut def zen ex2 Fwd: 5’-CAGTTGGTGGAACTGGAGCAGG

gut zen ex1 Rvs: 3’-TGCTACTGGACACTGCCACTGG

3.4 Acknowledgments

We thank Ryan Bensen, Abigail Meisel, Bridgette Rebbeck, and Jason Hu for technical assistance. This research was funded by NIH grant #1R15GM107801-01A1 to T.W.

3.5 Author Contributions

K.K.B.R., M.S. and T.W. conceived and designed the experiments; K.K.B.R, P.M.E.N., T.E.S, E.B., P.P.K, A.McQ., E.M., A.A., A.N. and T.W. performed the experiments; K.K.B.R., S.M. and T.W. analyzed the data; T.W. obtained funding; K.K.B.R and T.W. wrote the paper; K.K.B.R, S.M., P.M.E.N., T.E.S., E.B., P.P.K., A.McQ., E.M., A.A., A.N. and T.W. edited the paper.

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Chapter 3B

GENETIC MANIPULATION OF DEVELOPMENTAL GENES TO PROVIDE EVIDENCE FOR THEIR INVOLVEMENT IN *D. GUTTIFERA* ABDOMINAL PIGMENTATION

The data presented here are based on the comments received from the reviewers of our article in *Nature Communications* (see chapter 3 of this dissertation for details)

3.1 Introduction and Results

The abdominal color pattern of *D. guttifera* may be regulated by multiple developmental pathways involving the actions of activators and repressors acting independently, possibly interacting with downstream pigmentation genes other than *y* [1, 2]**.** Previously in our lab, through *in situ* hybridization, we found the expression of five toolkit genes, *wg, dpp, hh, abd-A,* and *zen*, prefiguring the *D. guttifera* adult abdominal spot pattern in distinct subsets of the full pigmentation pattern. Interestingly, these toolkit genes are expressed during the early stage of pupal development, suggesting that they encode upstream activators of downstream pigmentation genes. In order to gain insights into the possible role of toolkit genes in pigmentation, we analyzed the pupal expression patterns of the *wg* gene to detect changes among species that show differences in their adult pigmentation patterns. As we have shown, *wg* foreshadows the six rows of spots in the *D. guttifera* abdomen. We decided to detect the *wg* mRNA expression patterns in species that have lost one or two pairs of

spot rows, *D. palustris* and *D. subpalustris,* respectively. Our results show that *wg* expression prefigures the lateral and median spot rows in the *D. palustris* abdomen, while *D. subpalustris* shows *wg* expression foreshadowing the lateral row of spots. Both expression patterns are seen at the pupal stage P7, which is the same time at which *wg* foreshadows the abdominal spot pattern in D. *guttifera* (**Fig. 3.10**). These findings suggest that the *wg* gene may be the activator of the patterns in *D. palustris* and *D. subpalustris* because its expression is prefiguring the exact spot rows where future adult spots will appear. The correlation between the appearance of *wg* expression spots and the adult pattern of black pigment spots suggests that the abdominal color patterns of *D. palustris* and *D. subpalustris* have evolved due to changes in *trans*.

Our results provide correlative evidence that *wg* may be an upstream regulator of pigmentation, likely of the *y* gene. The results also suggest that the differential deployment of the Wg morphogen (a *trans*-regulatory change) may be responsible for the differences in the adult spot patterns among the three species, similar to the evolution of the *D. guttifera* wing spots. However, functional genetic studies are necessary to provide evidence for the roles of toolkit genes, such as *wg*, *hh, abd-A,* and *dpp*, in pigmentation.

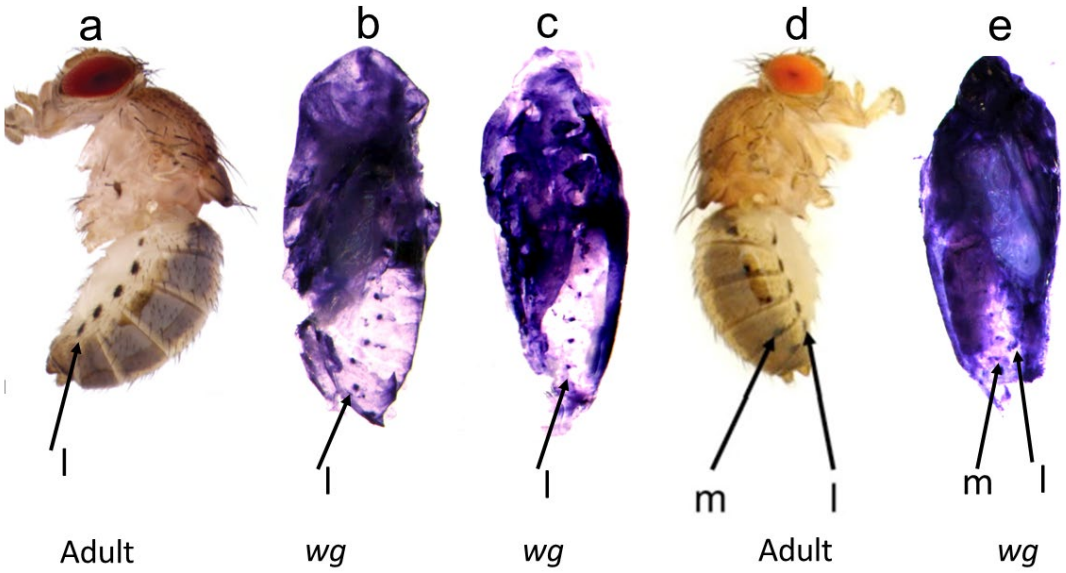

Figure 3.10: The pupal expression patterns of *wg* mRNA during pupal development of the *D. palustris* and *D. subpalustris* precisely foreshadow the adult abdominal spot patterns. (a) Lateral view of an adult *D. subpalustris.* (b,c) *wg* mRNA expression during stage P7 pupal development of *D. subpalustris.* (d) Lateral view of an adult *D. palustris* (e) *wg* mRNA expression during stage P7 pupal development of *D. palustris.* The spot rows are designated as lateral (l) and median (m).

Until recently, genetic manipulation of non-model *Drosophila* species has been very difficult. However, the successful manipulation of the *D. guttifera*, a non-model species, by Werner *et al.*, gave us an insight into how to ectopically express genes in the *D. guttifera* [3]. Therefore, we ectopically expressed the cDNAs of three out of the five toolkit genes (*wg*, *hh*, and *abd-A*), which were earlier identified as putative upstream activators of downstream pigmentation genes. We used the *gut y* stripe CRE region reported by Raja *et al*. to drive the expression of *wg, hh,* and *abd-A* cDNAs in stripes around the intersegmental

regions of the *D. guttifera* abdomen [4]. We intended to change the spot pattern on the *D. guttifera* abdomen into a stripe pattern. Successful overexpression of these toolkit cDNAs will provide further evidence for the involvement of the toolkit genes in orchestrating the complex abdominal color pattern in *D. guttifera*.

We transformed *D. guttifera* by injecting the embryos' syncytium blastoderm stage with a modified *piggy*Bac plasmid containing the *gut y* stripe CRE, heat shock promoter (hsp), and each cDNA of the toolkit genes *wg, hh,* and *abd-A* (**Fig. 3.11**), thus creating germline transformations. We identified the transgenic *D. guttifera* during the larval stage by screening for an enhanced green fluorescent protein (EGFP) expression patterns in the larval eye disks and brains [5]. As a result of this transformation, we successfully created several transgenic lines of *D. guttifera* containing the *gut y* stripe CRE-hsp-*wg* cDNA, *gut y* stripe CRE-hsp-*hh* cDNA, and *gut y* stripe CRE-hsp-*abd-A* cDNA. Note that all the toolkit cDNAs used in this experiment were synthesized from *D. melanogaster* mRNAs.

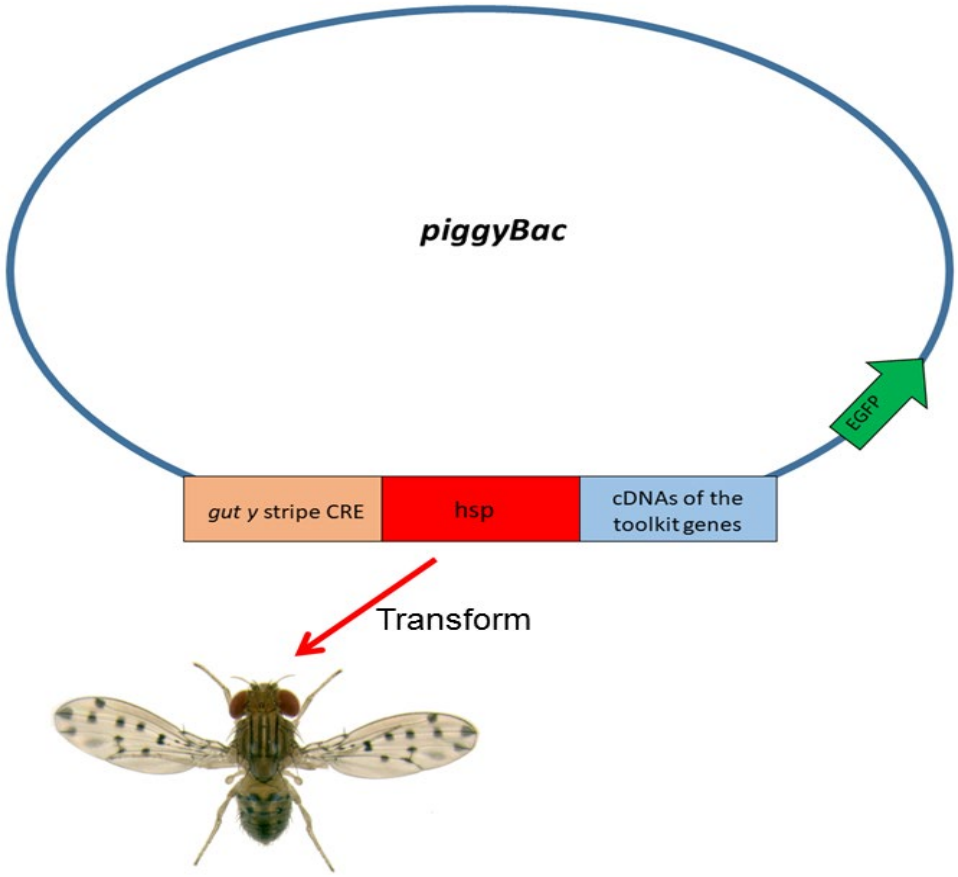

Figure 3.11: A sketch of a construct assembled in the *piggyBac* plasmid that was used to transform *D. guttifera*.

Unfortunately, the *gut y* stripe CRE could not drive the expression of the cDNAs of the three toolkit genes in stripes along the intersegmental region of *D. guttifera's* abdomen because the *gut y* stripe CRE is a late-acting driver. As shown earlier, the *gut y* stripe CRE drove the DsRed expression at the pupal stages P10 through P15; this is when the *gut y* stripe CRE is active, while the toolkit genes were expressed in the early pupal stages P7 and P8. Unfortunately, no earlier acting abdominal CRE is available for this species to ectopically express our toolkit genes.

To confirm whether the *gut y* stripe CRE drove the cDNAs of the toolkit genes later on, i.e., during pupal stages P10-P15, we performed an abdominal *in situ* hybridization experiment on the transgenic *D. guttifera* during the pupal stages P10 to P12. Our *in situ* hybridization data show the ectopic expression of *wg* and *hh* cDNAs in stripes around the intersegmental regions of P10 pupae, which correlates with the time the *gut y* stripe CRE is active, as shown by the transgenic reporter assay result (**Fig. 3.12**). Thus, this further confirms that our overexpression experiment worked. However, we could not change the spot pattern on the *D. guttifera* abdomen into a stripe pattern because the driver (*gut y* stripe CRE) did not act at the time it was needed to drive the cDNAs of the toolkit genes.

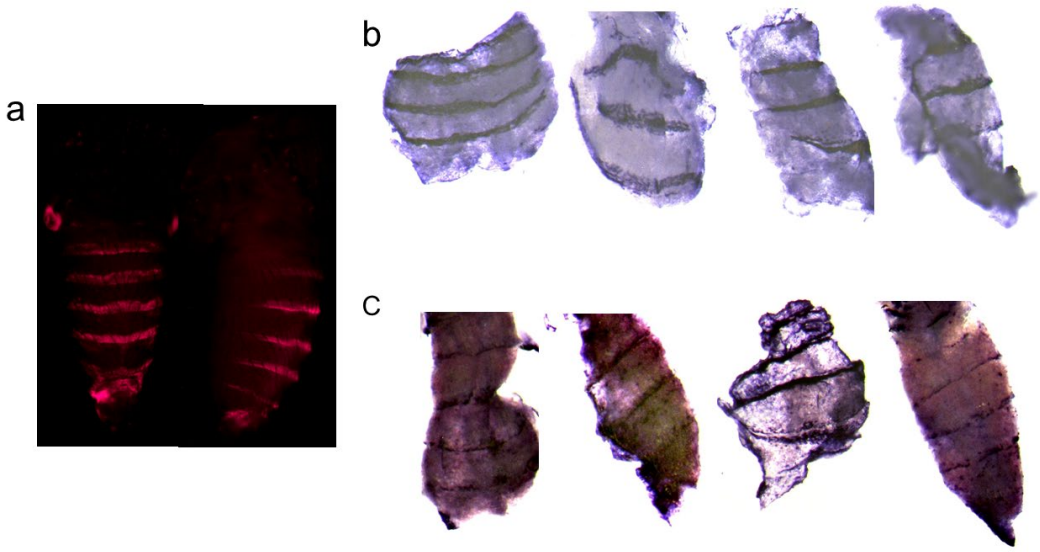

Figure 3.12: The *in situ* hybridization signals showing that the *hh* cDNA and *wg* cDNA ectopic expression patterns correlate with the *gut y* stripe CRE reporter assay. (a) DsRed reporter assay showing the activity of the *gut y* stripe CRE at stage P10. (b) *in situ* Hybridization signal indicating the ectopic expression of *hh* cDNA along the tergites of the

P10 pupae of *D. guttifera*. (c) *in situ* Hybridization signal showing the ectopic expression of *wg* cDNA along the tergites of the P10 pupae of *D. guttifera*.

Surprisingly, two transgenic lines among the lines injected with the *gut y* stripe CRE-hsp-*wg* cDNA construct produced adult D. guttifera flies with dark stripes along the longitudinal veins of the wings. The dark stripes are on the longitudinal veins L2, L3, L4, and L5 of *D. guttifera*'s wings, as shown in **Fig. 3.13**. This phenotype was most likely independent of the *gut y* stripe CRE activity. We suspect that our construct might have landed in the vicinity of an unknown CRE in the *D. guttifera* genome that drove the *wg* cDNA's expression in a stripe pattern. This "accidental" ectopic expression result corroborates the finding by Werner *et al.* that *wg* gene is sufficient and necessary to induce wing pigmentation [3].

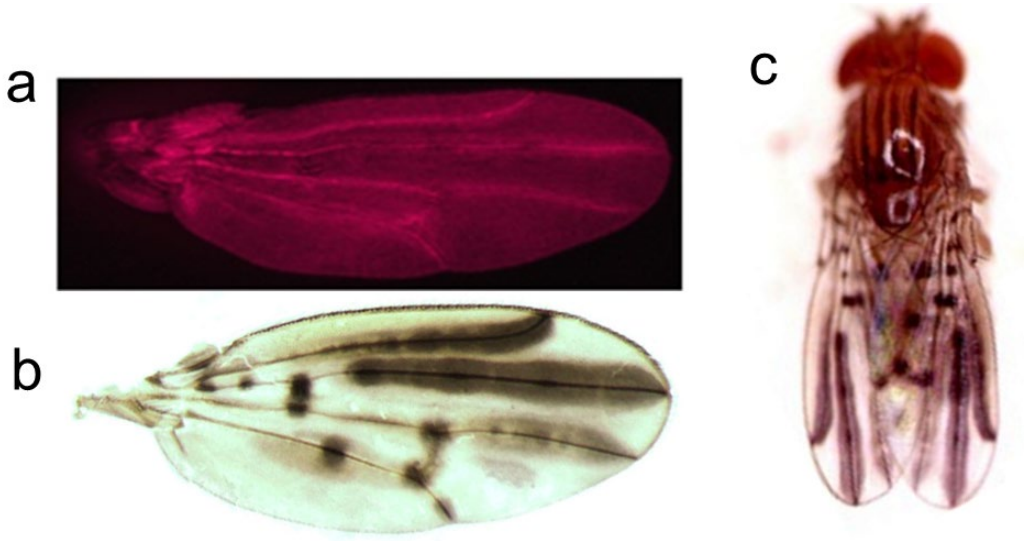

Figure 3.13: An unknown CRE may have driven the *wg* cDNA. (a) The wing of an adult *D. guttifera* wild-type. (b, c) Ectopic wing pigmentation of an adult *D. guttifera* expressing the *wg* cDNA construct.

Furthermore, we knocked down the toolkit mRNAs by the RNA interference technique, through a ubiquitous heat shock driver. RNA-mediated interference (RNAi) has been used for gene expression studies in insects [6, 7, 8]. Injection of double-stranded RNA into an organism silences the target gene's expression by rapid degradation of the mRNA. Until recently, the targeted expression of RNAi constructs in non-model organisms has been challenging. However, Chen *et al*. have developed a *piggyBac*-based vector called *Pogostick*, which has two multiple cloning sites for the reverse-orientation cloning of identical DNA. This technique relies on a heat shock promoter to synthesize the interference RNA [9]. We utilized this approach to downregulate the expression of *wg, hh, abd-A, and dpp*, in all cells of *D. guttifera*. We aimed to selectively reduce the modular components of the *D. guttifera* abdominal spot pattern.

After injecting each RNAi construct into the *D. guttifera* embryos, we generated stable transgenic lines. We picked up the transgenic larvae and allowed them to develop into pupae. Then we performed heat shock on the transgenic pupae at stages P7 and P8, which correspond to the stages where the toolkit genes' expression patterns are shown by *in situ* hybridization (**Fig. 3.2**, in Chapter 3A). As a result of knocking down the mRNAs of the toolkit genes, the transgenic fruit flies suffered developmental abnormalities, causing them to die before we could observe the pigmentation patterns. We suggest that this outcome is due to the vital roles that toolkit genes play at every stage of *Drosophila* development.

3.2 Future Directions

1. The *gut y* stripe CRE failed to ectopically express the cDNAs of the five developmental genes (*wg*, *hh*, *dpp*, *abd-A*, and *zen*) at the right time in the *D. guttifera* abdomen, which is the likely reason for why we were unable to manipulate the adult pigment pattern. It is therefore imperative to identify an appropriate enhancer to ectopically express these genes in a tissue-specific manner, at the right time. One way to find suitable enhancers is by performing an enhancer trap experiment. Once the toolkit genes can be expressed with the help of enhancers that drive earlier pupal expression, we expect that the ectopic expression of our genes will lead to changes in the color pattern of adult flies. Another strategy such as CRISPR activation (CRISPRa) can be used to overexpress the toolkit genes. The CRISPRa system uses the nuclease-deactivated Cas9 (dCas9) that cannot produce a double-stranded break, but can be directed by special guide RNAs (gRNA) to the transcription start sites to induce upregulation of the target genes. Such experiments will provide conclusive genetic evidence that the five developmental genes are sufficient for establishing the abdominal color pattern in *D. guttifera*.
2. Our attempt to knockdown the toolkit genes by RNAi technique resulted into developmental abnormalities for *D. guttifera*. This outcome might be due to knocking down the toolkit genes in all cells using the whole organismal heat shock method. Therefore, an RNAi technique that can be controlled to knockdown mRNA in certain cells will be helpful to mitigate this problem. The precise spatial and temporal control offered by the laser-mediated heat shock will enable us to induce mRNA knockdown in groups of cells of the pupal abdomens

of *D. guttifera*. Details on how to design and set up the heat shock laser are described by Ramos *et al* [10]. It is important to detect the binding sites of the five putative activators on the *gut y* spot CRE to further provide a proof for their involvements during pigmentation. In order to identify individual binding sites of the putative upstream activators within the isolated *gut y* spot CRE, the Chromatin Precipitation (ChIP) and DNA Affinity Purification Sequencing (DAP-seq) techniques can be helpful. ChIP is an *in vivo* process that can be used to investigate the interaction between proteins and DNA in the cell, while DAP-seq is an *in vitro* technique that can identify several possible transcription factors binding motifs. Although no ChIP or DAP-seq experiments have been reported for *D. guttifera*, the application of these techniques to our research will help us to provide direct evidence for the activities of the putative upstream activators during pigmentation.

3. Our research is the first to report the co-expression of pigmentation genes in *D. guttifera, D. palustris,* and *D. subpalustris*. This finding provides a starting point to understand how multiple genes are regulated and co-expressed in the same pattern at the same point. The simple spot patterns on the abdomens of *D. palustris* and *D. subpalustris* also offer us the opportunity to explore how complex patterns on the abdomen of other quinaria species have evolved. To fully understand the role of each pigmentation gene in color pattern development in these species, we must utilize RNA interference to down regulate the pigmentation genes or use CRISPR/Cas 9 technique to knockout the pigmentation genes, in addition to overexpression of the pigmentation genes. Although

transgenic methods are established in *D. guttifera*, this tool is not available for *D. palustris* and *D. subpalustris*. Pursuing the development of transgenic technique for *D. palustris* and *D. subpalustris* will facilitate a robust investigation of the mechanisms underlying these three species' morphological diversity, and will help facilitate access to study the complex patterning of the 26 species of the quinaria species group. Understanding of color pattern development is far from complete. The continuation of the study of these three fruit flies, and the quinaria group as a whole, will help us to understand how complex color patterns evolved in animals.

3.3 Acknowledgments

We would like to thank Dr. Frasch Manfred for sending us the *D. melanogaster hh* cDNA from Germany, and Dr. Antonia Monteiro for giving us the *Pogostick* plasmid.

3.4 Author Contributions

Conceived and designed the experiments: Mujeeb Shittu and Thomas Werner. Performed the experiments: Mujeeb Shittu, Tessa Steenwinkel, Lucinda Hall, and David Trine. Analyzed the data: Mujeeb Shittu and Thomas Werner. Contributed reagents/materials: Thomas Werner. Wrote the article: Mujeeb Shittu. Edited the article: Mujeeb Shittu and Thomas Werner.

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4 Chapter 4

THE MODULAR EXPRESSION PATTERNS OF THREE PIGMENTATION GENES PREFIGURE UNIQUE ABDOMINAL MORPHOLOGIES SEEN AMONG THREE *DROSOPHILA* SPECIES

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4.1 Abstract

To understand how novel animal body colorations emerged, one needs to ask how the development of color patterns differs among closely related species. Here we examine three species of fruit flies – *Drosophila guttifera* (*D. guttifera*), *D. palustris*, and *D. subpalustris* – displaying a varying number of abdominal spot rows. Through *in situ* hybridization experiments, we examine the mRNA expression patterns for the pigmentation genes *Dopa decarboxylase* (*Ddc*), *tan* (*t*), and *yellow* (*y*) during pupal development. Our results show that *Ddc*, *t*, and *y* are co-expressed in modular, identical patterns, each foreshadowing the adult abdominal spots in *D. guttifera*, *D. palustris*, and *D. subpalustris*. We suggest that differences in the expression patterns of these three genes partially underlie the morphological diversity of the *quinaria* species group.

4.2 Introduction

The complexity and diversity of animal body coloration in the natural world are astounding. Unique patterns like cheetah spots and zebra stripes beg the question – how did these traits evolve? To understand how novel morphologies arose, one needs to ask how alterations to organismal development occurred over evolutionary time (Raff, 2000). Butterfly wings have served as a system to unravel the molecular mechanisms underlying complex pattern development (Carroll et al., 1994; Matsuoka and Monteiro, 2018; Monteiro et al., 2013; Zhang and Reed, 2016; Zhang et al., 2017), and the examination of American cockroaches, large milkweed bugs, and twin-spotted assassin bugs progressed the knowledge of the process of body coloration (Lemonds et al., 2016, Liu et al., 2014; Zhang et al., 2019). Moreover, pigmentation has been shown to be vital to the lifecycles of agricultural pests and human disease vectors, such as the Asian tiger mosquito, black cutworm, brown planthopper, and kissing bug (Berni et al., 2020; Chen et al., 2018; Liu et al., 2019; Lu et al., 2019; Noh et al., 2020; Sterkel et al., 2019). However, these studies were built upon the robust knowledge of pattern and pigmentation development gained through the study of fruit flies, in particular, *D. melanogaster*.

The role of *D. melanogaster* as a model to understand fruit fly pigmentation spans decades (Brehme 1941; Wright 1987). Recent studies have examined the relationship between pigmentation and thermal plasticity (De Castro et al., 2018; Gibert et al., 2017), and how pigmentation of the male sex comb contributes to *Drosophila* mating success (Massey et al., 2019b). Investigating how pigmentation develops in *D. melanogaster* provided the foundation to understand the same processes in other fruit flies. This

knowledge, in turn, has facilitated studies of species divergence (Lamb et al., 2020) and positioned *Drosophila* pigmentation as a model to study how gene-regulatory networks – the regulatory mechanisms responsible for organismal development (Davidson and Levin, 2005) – evolved (Camino et al., 2015; Gibert et al., 2018; Grover et al., 2018; Ordway et al., 2014; Rebeiz and Williams, 2017; Roeske et al., 2018). The *Drosophila* pigmentation pathway with the enzymes and reactions necessary to produce black, brown, and yellow coloration seen on the bodies of fruit flies, is shown in **Figure 4.1** (Gibert et al., 2017; Massey et al., 2019a; Rebeiz and Williams, 2017; True et al., 2005; Wittkopp et al., 2003).

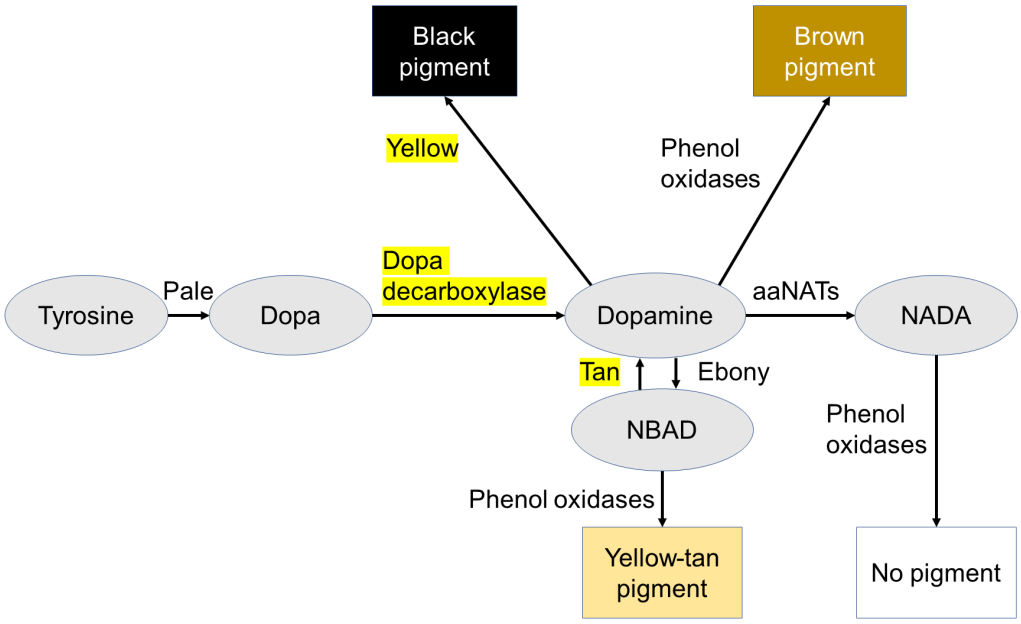

Figure 4.1. The pigmentation pathway of *Drosophila*. This illustration of the pigmentation pathway is adopted from (Gibert et al., 2017; Massey et al., 2019a; Rebeiz and Williams, 2017; True et al., 2005; Wittkopp et al., 2003) (Image by Will Dion).

Tyrosine is converted to dopa by Pale, which is then converted into dopamine by Dopa decarboxylase (encoded by *Ddc*). Dopamine proceeds one of four ways: Yellow (encoded by *y*) can convert it into black melanin; it can become brown pigment through the activity of phenol oxidases; it can be converted into *N*–acetyl dopamine (NADA) through arylalkylamine *N*-acetyl transferases (aaNATs) and thus result in a lack of pigmentation through the activity of phenol oxidases; or it may become *N*-β-alanyl dopamine (NBAD) through the activity of Ebony, followed by a transition to a yellow-tan pigment by phenol oxidases. The protein Tan (encoded by *t*) functions opposite of Ebony by converting NBAD into dopamine. The gene products for *Ddc*, *t*, and *y* are highlighted.

While the process of *Drosophila* pigmentation patterning involves many genes, our study focuses on three: *Ddc*, *t*, and *y*, which are all essential for the production of black and brown coloration. *Ddc* is integral to the development of *Drosophila* pigmentation, with the mutant phenotype lacking the dark coloration seen on the wild type fly (Walter et al., 1996; Wright et al., 1976). The genes *t* and *y* are also required for melanization. Mutants of the *t* gene exhibit a tan as opposed to a black body pigmentation (Hotta and Benzer, 1969; McEwen, 1918; True et al., 2005), while *y* mutants display a yellow body color (Biessmann, 1985; Brehme, 1941).

D. melanogaster has a relatively simple abdominal pigmentation pattern, as compared to other *Drosophila* species. The *quinaria* group, an adaptive radiation of non-model fruit flies, displays a great variety of abdominal and wing pigmentation patterns (Bray et al., 2014; Werner et al., 2018). This abundant morphological diversity and the recent divergence of the lineage (approximately 10 to 20 million years ago (Izumitani et al.,

2016; Spicer and Jaenike, 1996)) will help facilitate the identification of molecular mechanisms underlying differences in species morphology. One member of the *quinaria* group, *D. guttifera*, has emerged as a model to study complex pattern development (Fukutomi et al., 2020; Koshikawa et al., 2015; Koshikawa et al., 2017; Raja et al., 2020; Shittu et al., 2020; Werner et al., 2010).

The abdominal spot pattern of *D. guttifera* consists of six rows of spots: three rows on the left side (dorsal, median, and lateral row), which are mirrored on the right side of the abdomen (**Fig. 4.2**). *D. palustris* lacks a pattern module (and sometimes two) of those seen in *D. guttifera*: the dorsal pair of spot rows is always missing; while the median spots display varying intensity and can even be completely absent (Werner et al., 2018). The most extreme reduction of this patterning theme among the three species is evident in *D. subpalustris*, where only the lateral pair of spot rows is present (**Fig. 4.2**). Thus, the interspecific and even intraspecific differences in spot patterns are facilitated by the selective presence or absence of entire spot row pairs (modules) on the adult abdomens.

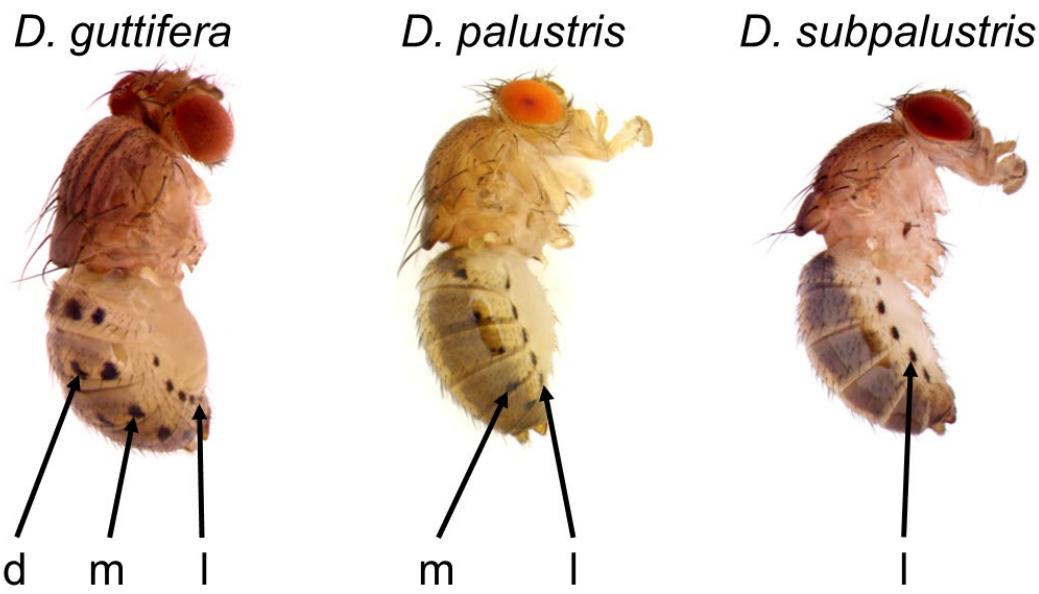

Figure 4.2. Spot pattern complexity in the *quinaria* species group. Three members of the *quinaria* group are shown from a lateral view. The dorsal (d), median (m), and lateral (l) rows of spots are labeled. Images are from (Werner et al., 2018).

In addition to displaying spots, the abdomens of each of the three fruit fly species exhibit wide areas of dark shading. *D. guttifera* shows two somewhat distinct shaded regions: a wide swath that is shared by all three species encompassing the spotted region, plus a specific dorsal midline shade. Furthermore, *D. guttifera* shows blackish stripes along the dorsal segment boundaries, which are absent in the other two species.

In the current study, we show that abdominal color pattern diversity among the *quinaria* species group members *D. guttifera*, *D. palustris*, and *D. subpalustris* is strictly modular and that *Ddc*, *t*, and *y* are co-expressed in identical patterns where dark spots will appear.

4.3 Results

4.3.1 *D. guttifera* pattern development

The gene expression patterns of *Ddc*, *t*, and *y* during pupal development foreshadowed the abdominal adult spots of *D. guttifera*. *Ddc* mRNA was detected at pupal stages P10, P12 and P13, *t* mRNA at P11 and P12, and *y* mRNA at P10 (**Fig. 4.3**) (see section 5.2 for information regarding pupal (P) stages). For the rest of the pattern, only *y* expression correlated with both the dorsal midline shade and intersegment stripes at stage P10 (**Fig. 4.4**). However, we were unable to detect any gene expression foreshadowing the broader shading around the dorsal and median spot rows.

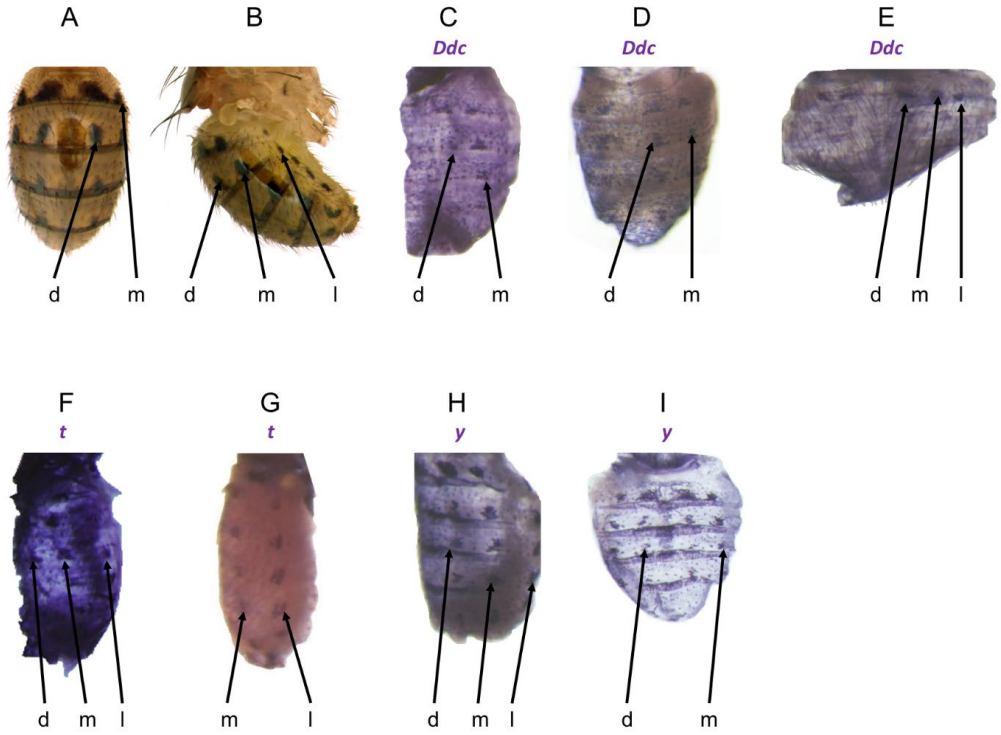

Figure 4.3. The *in situ* hybridization signals of *Ddc*, *t*, and *y* during *D. guttifera* pupal development foreshadowed the adult spot pattern. The spot rows are labeled as dorsal

(d), median (m), and lateral (l). (A, B) Adult *D. guttifera* from a dorsal and lateral view, respectively (Werner et al., 2018). (C, D, E) *Ddc* mRNA expression at stages P10, P12, and P13, respectively. (F, G) *t* mRNA at stages P11 and P12, respectively. (H, I) *y* mRNA expression at stage P10, respectively.

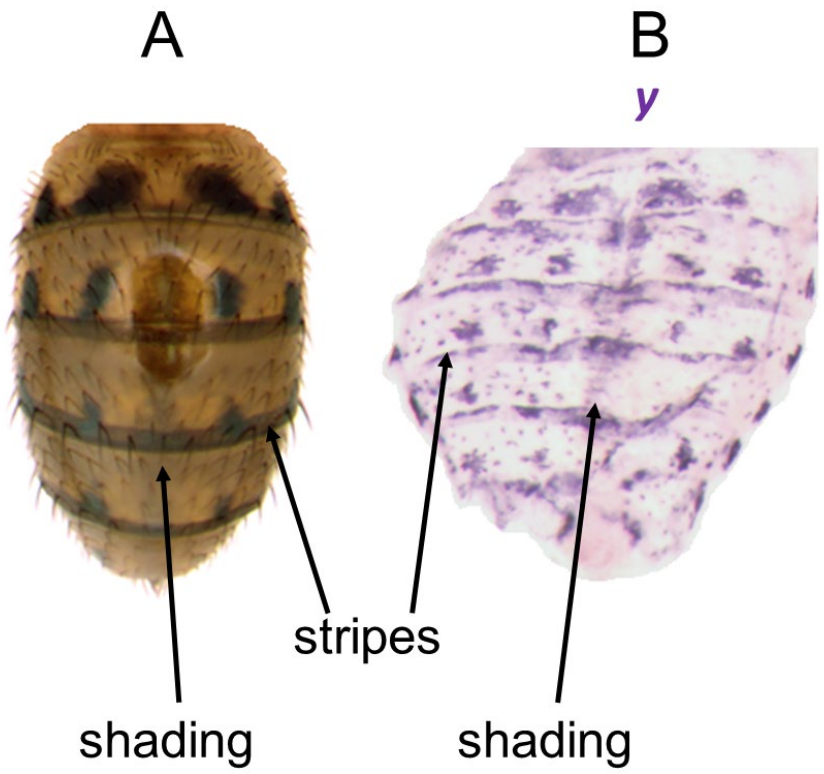

Figure 4.4. The *in situ* hybridization result of *y* during *D. guttifera* pupal development correlated with the adult abdominal dorsal midline shading and the intersegment stripes. (A) Dorsal view of adult *D. guttifera* (Werner et al., 2018). (B) *y* mRNA expression at stage P10 foreshadowing the dorsal midline shading and the intersegment stripes.

4.3.2 *D. palustris* pattern development

D. palustris lacks at least three components of the *D. guttifera* pattern: the dorsal pair of spot rows (sometimes even the median spot row pair), the dorsal midline shade, and the intersegment stripes. Just as in *D. guttifera*, the mRNA expression patterns of *Ddc*, *t*, and *y* prefigured the adult *D. palustris* spot pigmentation. *Ddc* mRNA was present at stages P11 and P12, *t* at P11 and P12, and *y* at P10 and P12 (**Fig. 4.5**). However, only the expression of *t* mRNA at stage P12 correlated with the shading pattern (**Fig. 4.6**).

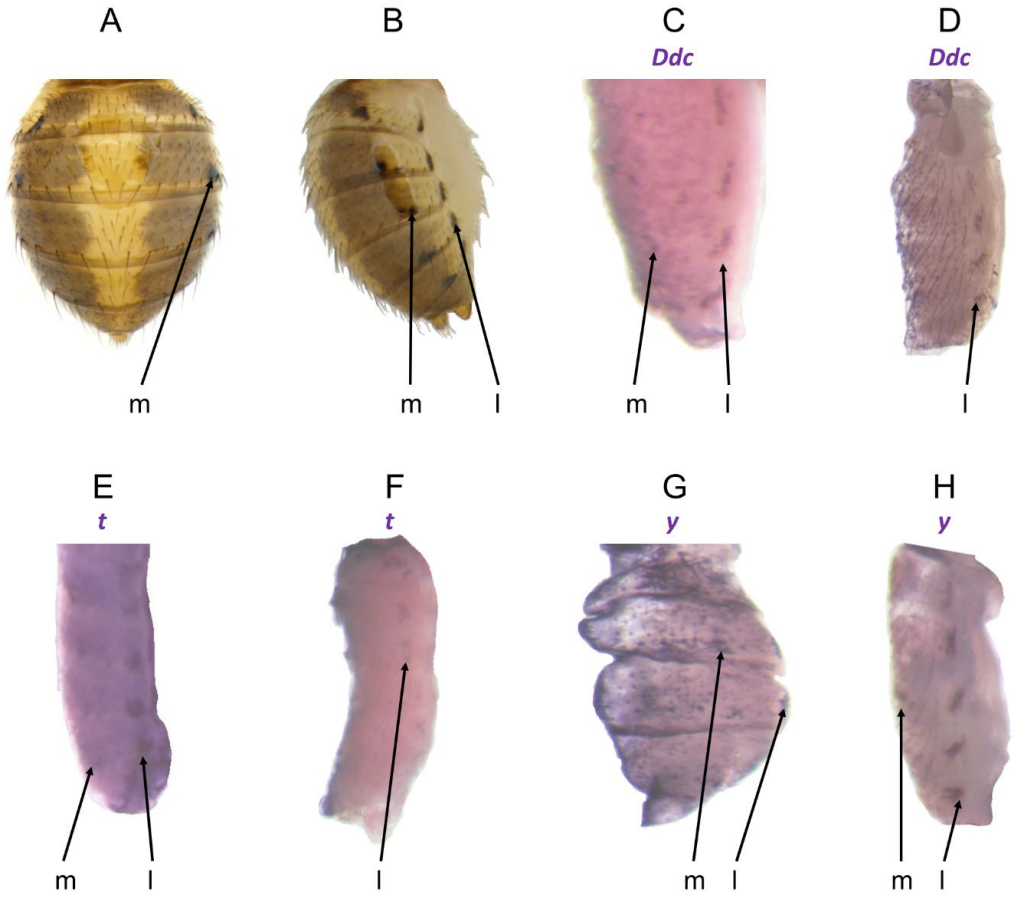

Figure 4.5. The *in situ* hybridization signals of *Ddc*, *t*, and *y* during *D. palustris* pupal development foreshadowed the abdominal spot pattern. The spot rows are labeled as

median (m) and lateral (l). (A, B) Adult *D. palustris* from a dorsal and lateral view, respectively (Werner et al., 2018). (C, D) *Ddc* mRNA expression at stages P11 and P12, respectively. (E, F) *t* gene expression foreshadowing spots at stages P11 and P12, respectively. (G, H) *y* mRNA expression at stages P10 and P12, respectively.

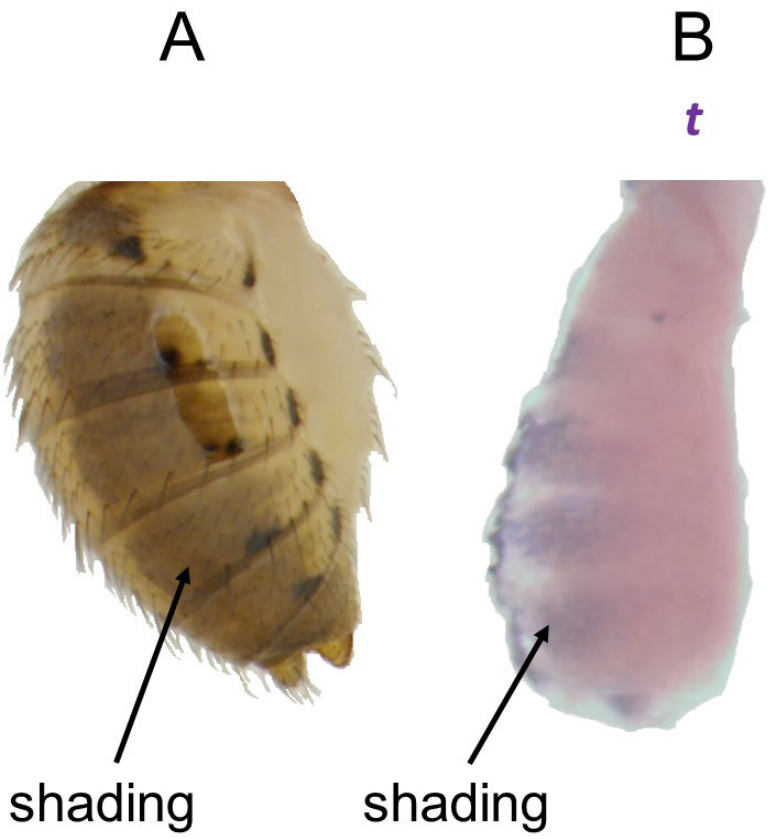

Figure 4.6. The *in situ* hybridization result of *t* during *D. palustris* pupal development correlated with the adult abdominal shading. (A) Lateral view of adult *D. palustris* (Werner et al., 2018). (B) *t* mRNA expression at stage P12 prefiguring the shading.

4.3.3 *D. subpalustris* pattern development

D. subpalustris exhibits the simplest pattern among the three species studied: one pair of lateral spot rows and shading similar to that of *D. palustris*. The *Ddc*, *t*, and *y* expression patterns during pupal development foreshadowed the abdominal spots of *D. subpalustris*; *in situ* hybridization signals were seen for *Ddc* at stage P11 and between stages P11 and P12, *t* at stages P11 and P12, and *y* at stage P10 (**Fig. 4.7**). The shading pattern is prefigured by *Ddc* mRNA at stage P11 (**Fig. 4.8**).

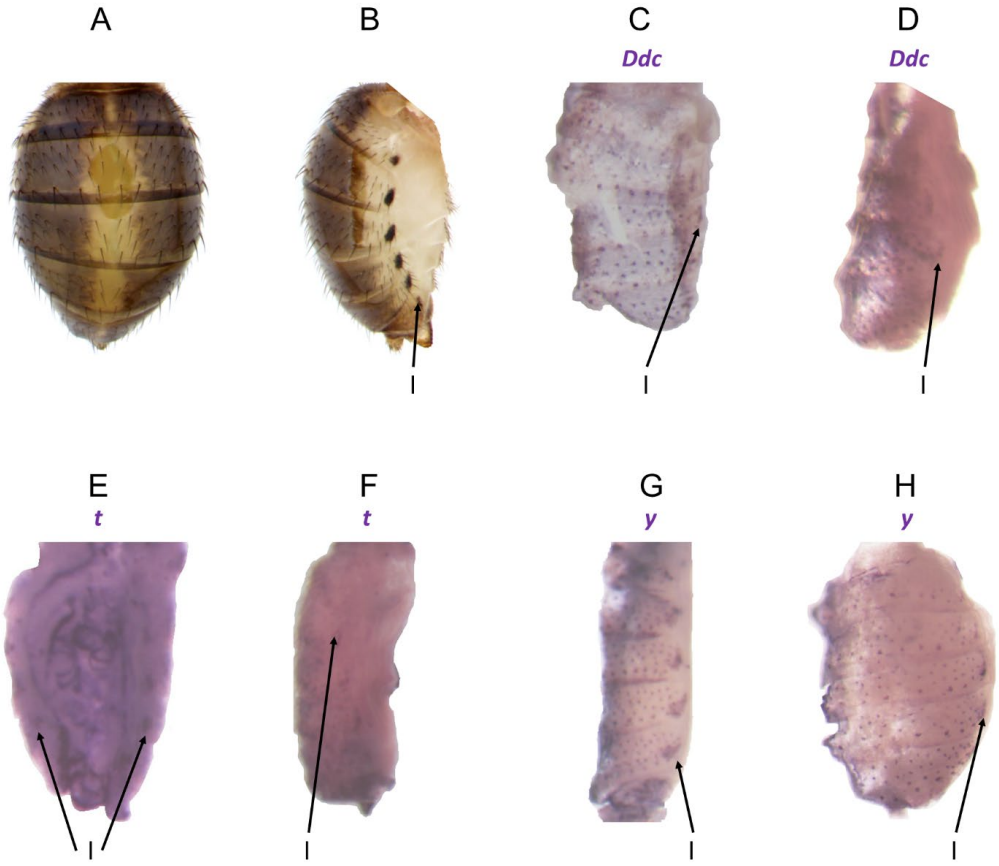

Figure 4.7. The *in situ* hybridization signals for *Ddc*, *t*, and *y* during *D. subpalustris* pupal development prefigured the abdominal spot pattern. The spot rows are labeled

as lateral (l). (A, B) Adult *D. subpalustris* from a dorsal and lateral view, respectively (Werner et al., 2018). (C, D) *Ddc* gene expression foreshadowing spots at stage P11 and between stages P11 and P12, respectively. (E, F) *t* gene expression at stage P11 and P12, respectively. Image (E) is taken from a ventral view. (G, H) *y* mRNA expression at stage P10, respectively.

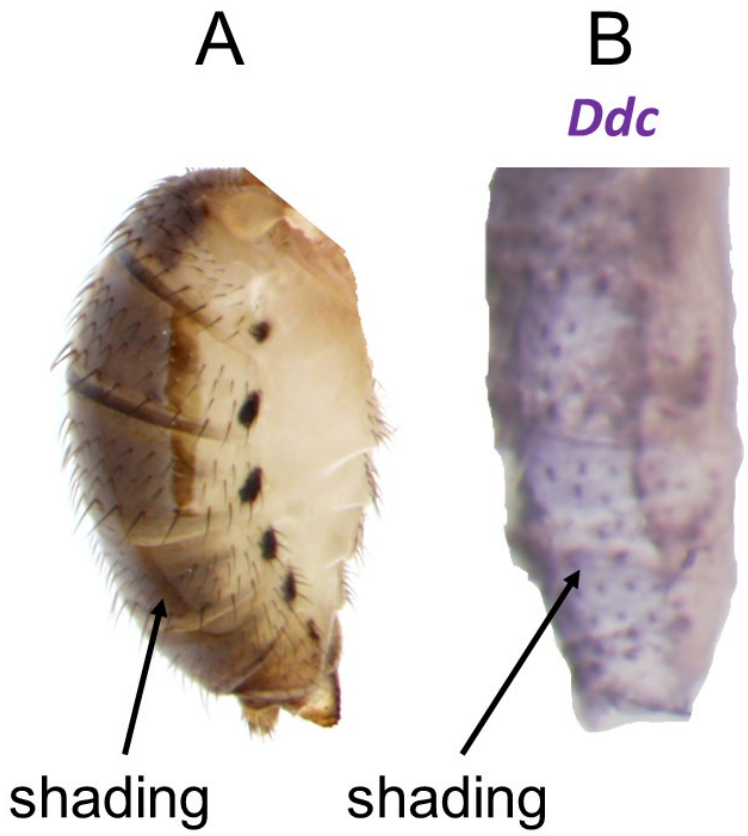

Figure 4.8. The *in situ* hybridization result for *Ddc* during *D. subpalustris* pupal development foreshadowed the adult abdominal shading. (A) Lateral view of adult *D. subpalustris* (Werner et al., 2018). (B) *Ddc* mRNA expression at stage P11.

4.4 Discussion

Here we show the evidence of pigmentation gene expression patterns prefiguring the complex coloration of three *Drosophila* species. *Ddc*, *t*, and *y* are spatially co-expressed in the developing abdomens, precisely foreshadowing the diverse dark spots in three *quinaria* group species. Interestingly, the shades and intersegment stripes are uniquely foreshadowed by only one of the three genes: *Ddc* in *D. subpalustris*, *t* in *D. palustris*, and *y* in *D. guttifera.* These data suggest that the regulation of *Ddc*, *t*, and *y* possibly co-evolved to paint complex abdominal spot patterns in concert, but not to collectively regulate the shading.

The spot pattern diversity seen among the three non-model species alone position them as an emerging system to study color pattern diversity. We show correlative evidence that the co-expression of three pigmentation genes is likely responsible for the spot patterning of these three *quinaria* group species. Intriguingly, each pair of spot rows behaves like a set of independent, serial homologs, similar to the repetitive pattern elements within butterfly wing sections (Monteiro 2008). Thus, these fruit fly abdominal pigmentation patterns may have broader implications to progressing our understanding of color pattern evolution and development across insects.

We show the expression patterns of three genes occurring at different pupal stages, ranging from P10 to P12. However, it has been shown in *D. guttifera* that this developmental timeframe is very short (P10 lasts almost 12 hours, however stages P11 through P13 are completed in less than 10 hours (Fukutomi et al., 2017)). Thus, we cannot state that these genes' activities are restricted to the developmental stages shown

here. It is also important to note that the lack of *in situ* hybridization signal could be a result of gene expression levels below the detection limit. This is likely why there is little to no signal among the pigmentation genes foreshadowing the median rows of spots in *D. palustris*. Additionally, the many tiny dots of *in situ* hybridization signal seen on the abdomens most likely correlate with the bristle sockets of the developing fly.

To fully understand the role of each gene in these three species' color pattern development, we must utilize RNA interference and gene overexpression, as well as CRISPR/Cas9 approaches. Transgenic methods are established in *D. guttifera* (Shittu et al., 2020), and developing similar protocols to produce transgenic *D. palustris* and *D. subpalustris* will facilitate our further understanding of the evolution of color patterning among these three species. Pursuing the development of such approaches will facilitate a robust investigation of the mechanisms underlying these three species' morphological diversity. Furthermore, these advances will facilitate access to study the complex patterning of the 26 members (Scott Chialvo et al., 2019) of the *quinaria* species group, which displays many modular combinations of spots, stripes, and shapes.

4.5 Conclusion

Our research is the first to show the expression patterns of pigmentation genes in *D. palustris* and *D. subpalustris*. Additionally, we provide further data with regards to an emerging model organism to study complex color pattern development, *D. guttifera*. Here, we provide qualitative evidence that the modular activities of *Ddc*, *t*, and *y* prefigure the abdominal spot patterns seen among these three species. These data offer a

starting point for future transgenic studies to better understand the molecular mechanisms that underlie these unique modular morphologies. Our understanding of complex color pattern development is far from complete; however, continuing to study these three fruit flies, and the *quinaria* group as a whole, will help us connect the dots.

4.6 Materials and methods

4.6.1 *Drosophila* stocks – *D. guttifera, D. palustris, and D. subpalustris*

D. guttifera and *D. subpalustris* were purchased from the *Drosophila* Species Stock Center, stock numbers 15130 – 1971.10 and 15130 – 2071.00, respectively. We collected *D. palustris* in Waunakee, Wisconsin. All fly stocks were maintained at room temperature on cornmeal-sucrose-yeast medium (Werner et al., 2018).

4.6.2 Identification of pupal stages

Pupal developmental stages for *D. guttifera* were determined according to (Bainbridge and Bownes, 1981; Fukutomi et al., 2017). The same characteristics used to establish *D. guttifera* pupal stages were seen in *D. palustris* and *D. subpalustris* pupae, and were therefore used to determine the developmental stages of these two fruit flies.

4.6.3 *in situ* hybridization probe design for *Ddc, t,* and *y*

RNA *in situ* hybridization probes were 200 to 500 bases in length. We used Mean Green PCR Master Mix (Syzygy Biotech Solutions) to amplify the partial coding regions with forward and reverse primers (Table 2.5). The PCR products were extracted and purified with a Thermo Scientific GeneJET Gel Extraction Kit and cloned into the pGEM-TEasy

vector, using *E. coli* DH5α cells. Colony PCR with the M13 forward and reverse universal primer pair was used for screening, and the Thermo Scientific GeneJET Plasmid Miniprep Kit was used for plasmid purification. The insertion direction into the pGEM-TEasy vector was determined through PCR with the M13 forward universal primer and either the internal forward or internal reverse primer (Table 2.5). Depending on the insertion direction, either SP6 or T7 RNA polymerase was used to produce a DIG (digoxigenin)-labeled RNA anti-sense probe (Roche DIG RNA Labelling Kit (SP6/T7)). GenePalette was used for computational biology (Rebeiz and Posakony, 2004).

Primer Name	**Primer Sequence**
D. guttifera Ddc exon 3 (a) forward	CACATGAAGGGCATCGAGACCGC
D. guttifera Ddc exon 3 (a) reverse	CATGCGCAAGAAGTAGACATCCCG
D. guttifera Ddc exon 3 (a) internal forward	CAACTTTGACTGCTCGGC
D. guttifera Ddc exon 3 (a) internal reverse	CATGTTCACCTCAGCAGC
D. guttifera Ddc exon 3 (b) forward	AGCCATTGATTCCGGATGCGG
D. guttifera Ddc exon 3 (b) reverse	AATCGTGTGCTCATCCCACTCG
D. guttifera Ddc exon 3 (b) internal forward	ACTGGCACAGTCCCAAGTTCC
D. guttifera Ddc exon 3 (b) internal reverse	CATCTTGCCCAGCCAATCTAGC
D. guttifera t exon 5 forward	CAGCGTCTGCTTGGCCACACG
D. guttifera t exon 5 reverse	TTGCCGCTGCGCAACAATTCGG
D. guttifera t exon 5 internal forward	GCTGAATCATTACTACTTTGTGG
D. guttifera t exon 5 internal reverse	AATGGTGTTGATGCTGAACACG

D. guttifera y exon 2 forward	CCAACATCGCCGTGGACATTG
D. guttifera y exon 2 reverse	AATTGCGGAGTGTACGGCATCG
D. guttifera y exon 2 internal forward	CTCCTACTTCTTCCCGGATCCC
D. guttifera y exon 2 internal reverse	ATCAGATTGAACAGCTCGACGCC
D. palustris Ddc exon 3 forward	TATCGTCATCACATGAAGGGC
D. palustris Ddc exon 3 reverse	GCCATGCGCAAGAAGTAGAC
D. palustris Ddc exon 3 internal forward	TGAAGCACGACATGCAGGG
D. palustris Ddc exon 3 internal reverse	CAGACCCATGTTCACCTC
D. palustris y exon 2 forward	GAGGAGGGCATCTTTGGC
D. palustris y exon 2 reverse	CGATGCCATGGAATTGCGG
D. palustris y exon 2 internal forward	TCTCGCACCGAGGACAGC
D. palustris y exon 2 internal reverse	CGATCAGATTGAACAGCTCG

Table 2.5: Primers used to construct *in situ* hybridization probes. The *D. guttifera Ddc* exon 3 forward and reverse primer pair was used to amplify *D. guttifera* genomic DNA to make the probe to test for *D. guttifera Ddc* expression. Primer set (a) was used to generate **Figure 4.3** (C), while set (b) was used for Figures 3 (D) and (E). The *D. guttifera t* exon 5 forward and reverse primer pair amplified *D. guttifera* genomic DNA to produce the probe used to characterize *t* in all three species. The forward and reverse primer pair for *D. guttifera y* exon 2 was used to amplify *D. guttifera* genomic DNA to develop the probe to determine *y* expression in *D. guttifera*. The *D. palustris* forward and reverse primer pairs for *Ddc* exon 3 and *y* exon 2 were used to amplify *D. palustris* genomic DNA to make the probes used to determine *Ddc* and *y* expression patterns in both *D. palustris* and *D. subpalustris.* Our choice to use probes constructed from a

different species' DNA was based on the close relationship of the *quinaria* species group (Izumitani et al., 2016; Spicer and Jaenike, 1996). All internal forward and internal reverse primer pairs were used for verification of the gene identity during the probe-making process.

4.6.4 *in situ* hybridization of the pupae

The details on how to perform *in situ* hybridization in *Drosophila* species is in Chapter 2 of this dissertation.

4.7 Acknowledgments

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4.8 Author Contributions

William A. Dion: Data curation, Investigation, Project administration, Visualization, Writing - original draft, Writing - review & editing. **Mujeeb O. Shittu:** Supervision, Investigation, Writing - review & editing. **Tessa E. Steenwinkel:** Resources, Writing - review & editing. **Komal K.B. Raja:** Investigation, Writing - review & editing. **Prajakta P. Kokate:** Investigation, Writing - review & editing. **Thomas Werner:** Conceptualization, Funding acquisition, Resources, Validation, Methodology, Writing - review & editing.

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