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The modular expression patterns of three pigmentation genes prefigure unique abdominal morphologies seen among three Drosophila species

William A. Dion
Michigan Technological University, wadion@mtu.edu

Mujeeb O. Shittu
Michigan Technological University, mshittu@mtu.edu

Tessa Steenwinkel
Michigan Technological University, testeenw@mtu.edu

Komal K.B. Raja
Michigan Technological University

Prajakta P. Kokate
Michigan Technological University, pkokate@mtu.edu

See next page for additional authors

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The modular expression patterns of three pigmentation genes prefigure unique abdominal morphologies seen among three *Drosophila* species

William A. Dion, Mujeeb O. Shittu, Tessa E. Steenwinkel, Komal K.B. Raja [1], Prajakta P. Kokate, Thomas Werner [*]

Department of Biological Sciences, Michigan Technological University, Houghton, MI, USA

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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.

1. 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 Fig. 1 (Gibert et al., 2017; Massey et al., 2019a; Rebeiz and Williams, 2017; True et al., 2005; Wittkopp et al., 2003).

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

* Corresponding author.
E-mail address: twerner@mtu.edu (T. Werner).
[1] Current address: Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, United States of America.

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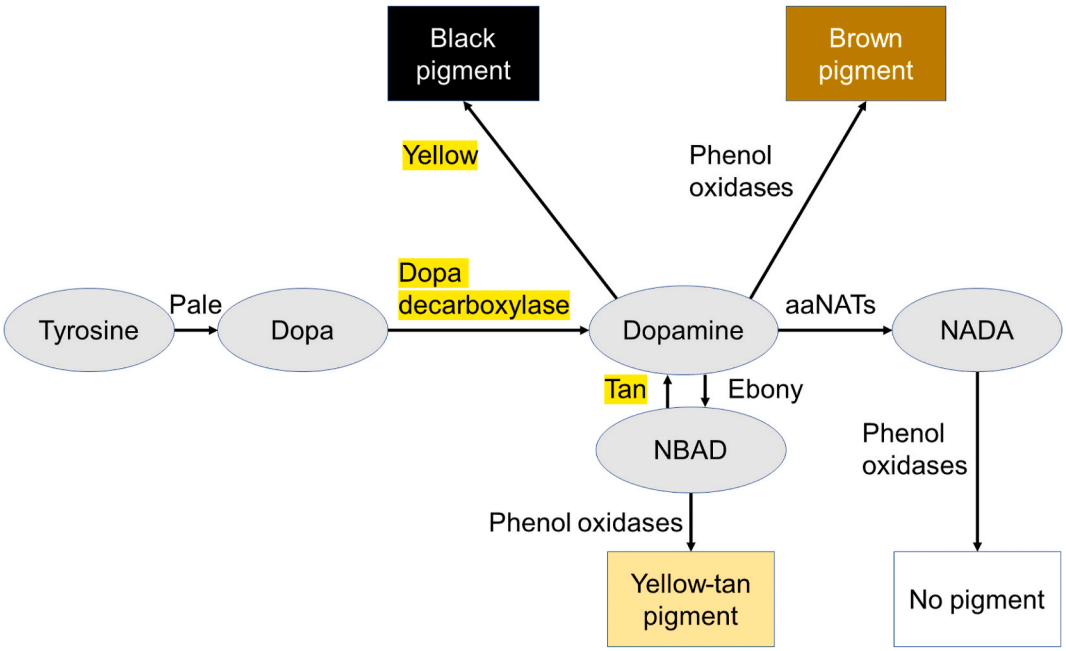

Fig. 1. The pigmentation pathway of *Drosophila*. This illustration of the pigmentation pathway is adopted from (Gibert et al., 2017; Massey et al., 2019a; Rebeiz and Williams, 2017; True et al., 2005; Wittkopp et al., 2003). Tyrosine is converted to dopa by Pale, which is then converted into dopamine 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.

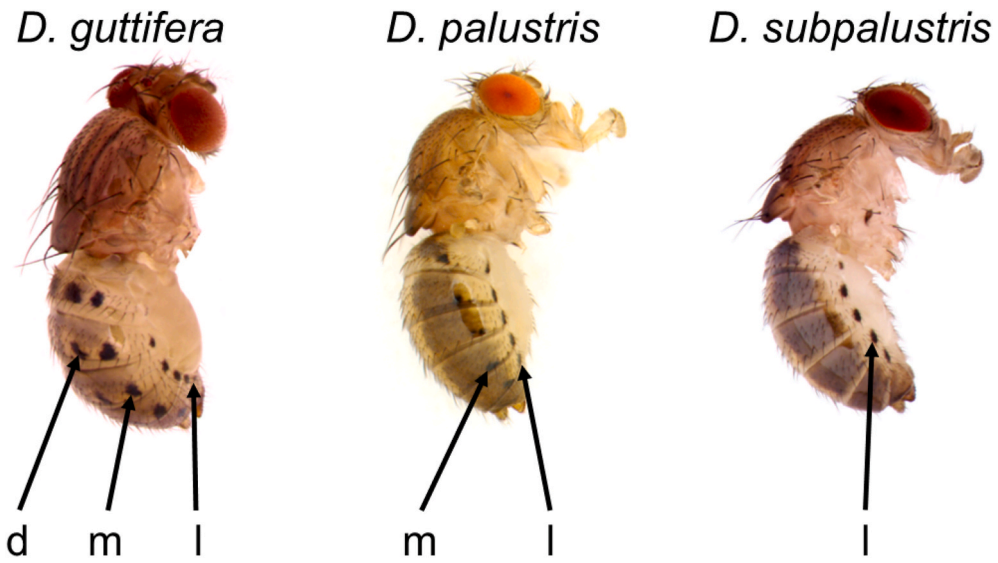

Fig. 2. Spot pattern complexity in the *quinaria* species group. Three members of the *quinaria* group are shown from a lateral view. The dorsal (d), median (m), and lateral (l) rows of spots are labeled. Images are from (Werner et al., 2018).

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 10–20 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, 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. 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

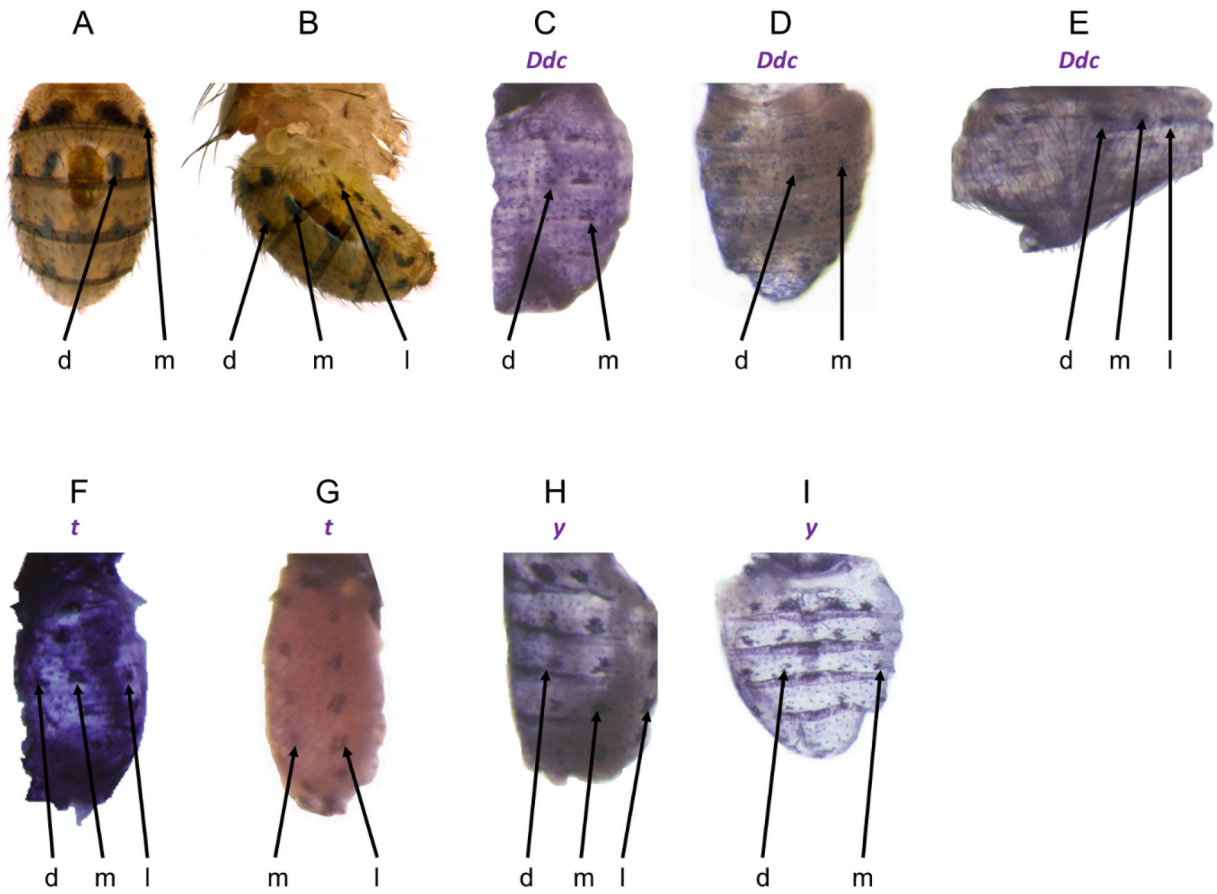

Fig. 3. The *in situ* hybridization signals of *Ddc*, *t*, and *y* during *D. guttifera* pupal development foreshadowed the adult spot pattern. The spot rows are labeled as dorsal (d), median (m), and lateral (l). (A, B) Adult *D. guttifera* from a dorsal and lateral view, respectively (Werner et al., 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.

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. 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.

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.

2. Results

2.1. 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. 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). However, we were unable to detect any gene expression

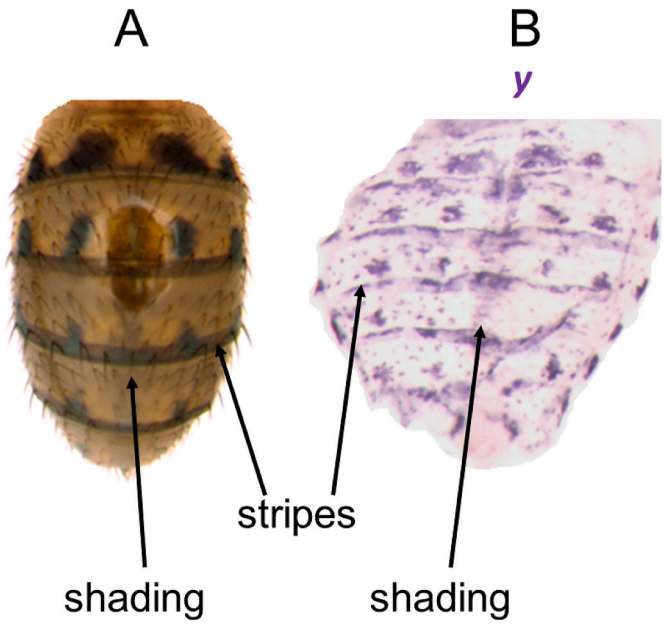

Fig. 4. The *in situ* hybridization result of *y* during *D. guttifera* pupal development correlated with the adult abdominal dorsal midline shading and the intersegment stripes. (A) Dorsal view of adult *D. guttifera* (Werner et al., 2018). (B) *y* mRNA expression at stage P10 foreshadowing the dorsal midline shading and the intersegment stripes.

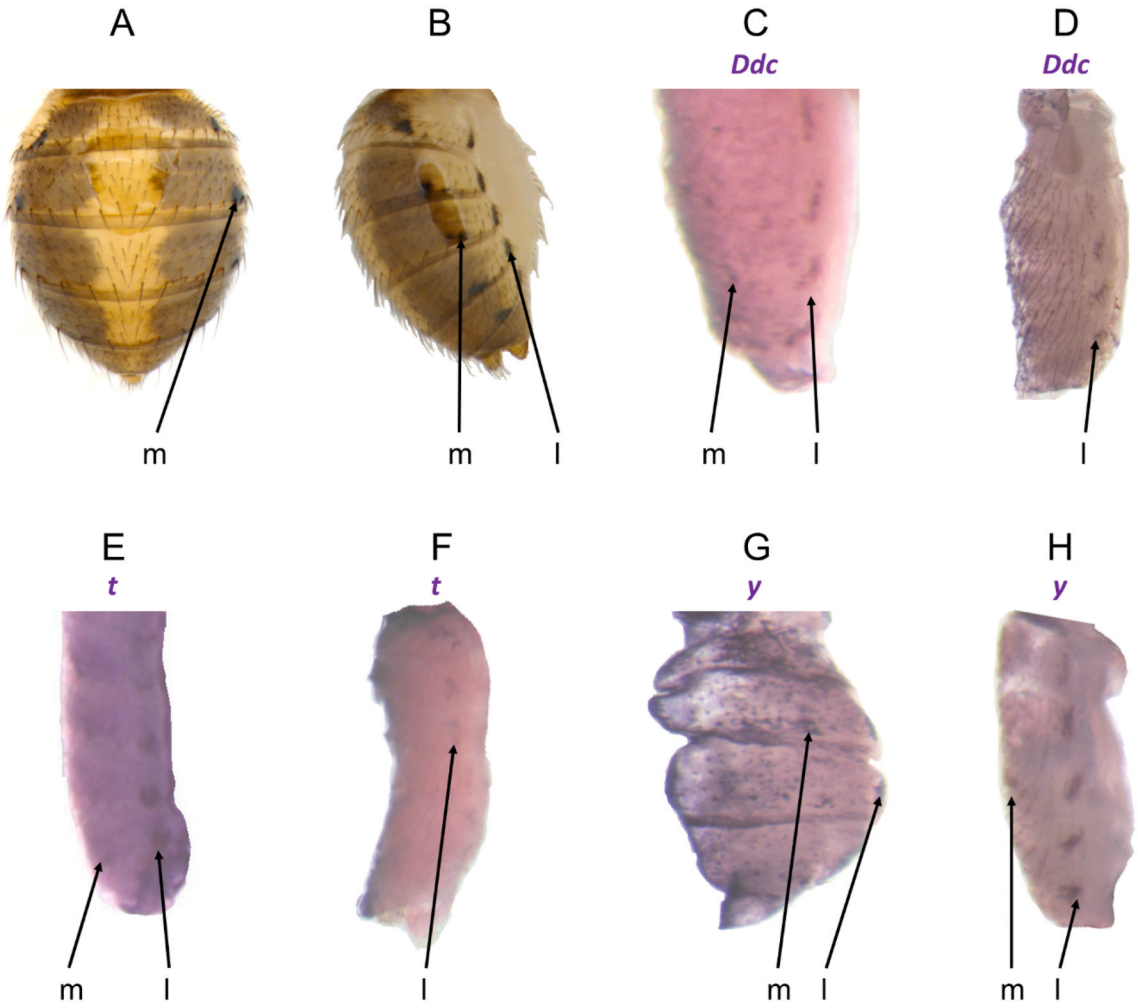

Fig. 5. The *in situ* hybridization signals of *Ddc*, *t*, and *y* during *D. palustris* pupal development foreshadowed the abdominal spot pattern. The spot rows are labeled as median (m) and lateral (l). (A, B) Adult *D. palustris* from a dorsal and lateral view, respectively (Werner et al., 2018). (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.

foreshadowing the broader shading around the dorsal and median spot rows.

2.2. *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. 5). However, only the expression of *t* mRNA at stage P12 correlated with the shading pattern (Fig. 6).

2.3. *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. 7). The shading pattern is prefigured by *Ddc* mRNA at stage P11 (Fig. 8).

3. 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.

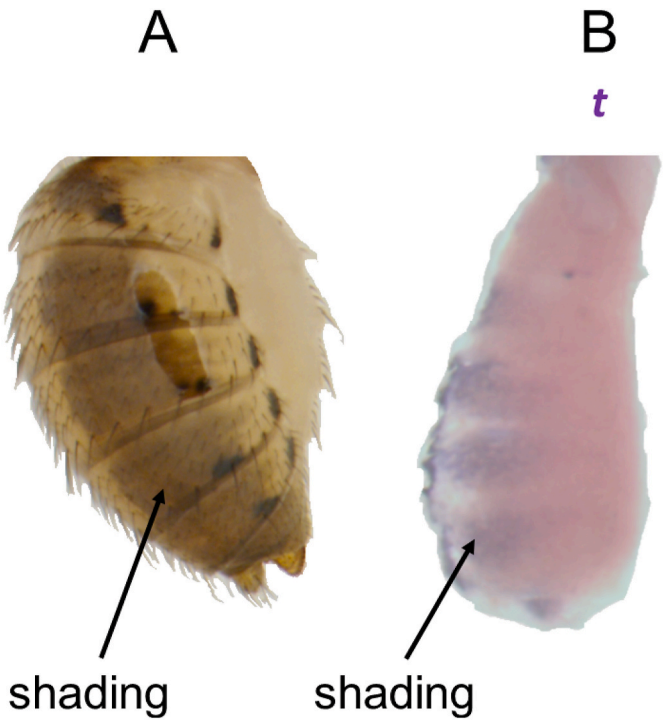

Fig. 6. The *in situ* hybridization result of *t* during *D. palustris* pupal development correlated with the adult abdominal shading. (A) Lateral view of adult *D. palustris* (Werner et al., 2018). (B) *t* mRNA expression at stage P12 prefiguring the shading.

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 h, however stages P11 through P13 are completed in less than 10 h (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 how color pattern development evolved 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. 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 in *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.

5. Experimental procedures

5.1. 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).

5.2. 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.

5.3. In situ hybridization probe design for Ddc, t, and y

RNA *in situ* hybridization probes were 200–500 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 1). 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 1). 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).

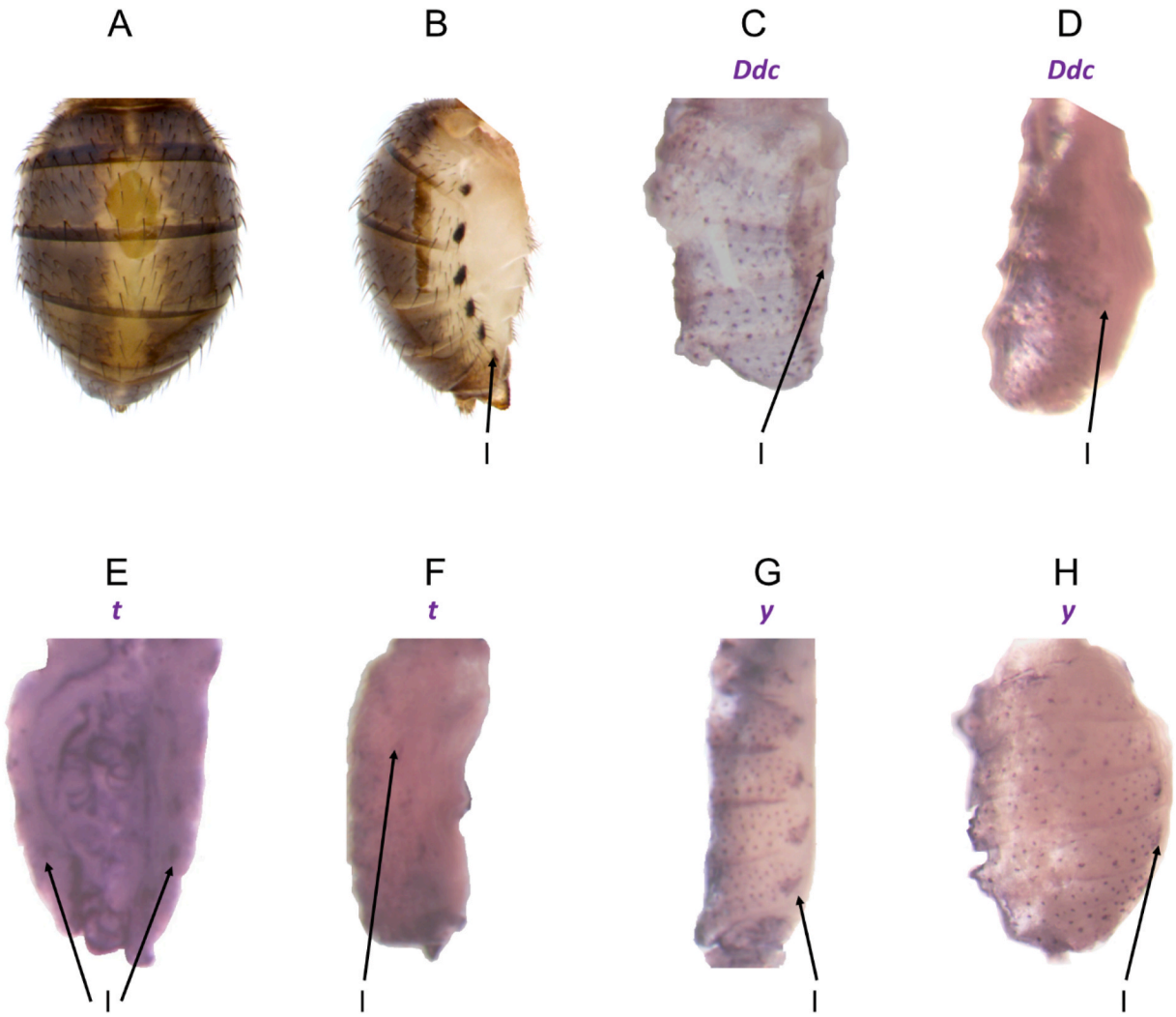

Fig. 7. The *in situ* hybridization signals for *Ddc*, *t*, and *y* during *D. subpalustris* pupal development prefigured the abdominal spot pattern. The spot rows are labeled as lateral (l). (A, B) Adult *D. subpalustris* from a dorsal and lateral view, respectively (Werner et al., 2018). (C, D) *Ddc* 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.

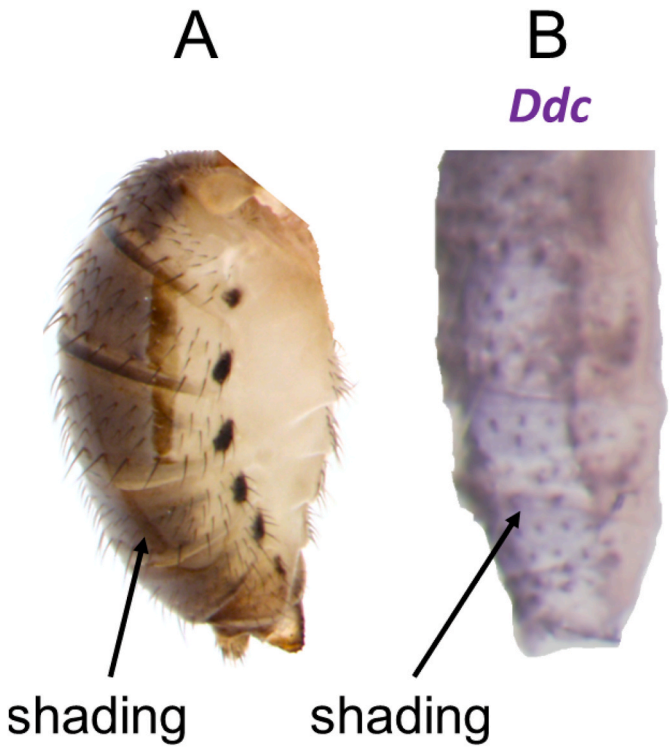

Fig. 8. The *in situ* hybridization result for *Ddc* during *D. subpalustris* pupal development foreshadowed the adult abdominal shading. (A) Lateral view of adult *D. subpalustris* (Werner et al., 2018). (B) *Ddc* mRNA expression at stage P11.

5.4. Preparation of pupae for RNA in situ hybridization

When pupae matured to the desired developmental stage, they were cut along the anterior-posterior axis either between the eyes or on their side through the eyes. The pupal halves were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) and kept at −20 °C in pure ethanol.

5.5. In situ hybridization of the pupae

The *in situ* hybridization procedure was adopted from (Jeong et al., 2008). The tissues were washed between each step with PBST. On the first day, pupae were treated with a 1:1 xylenes to ethanol mixture to remove residual fat tissue. The pupal tissue was then fixed (4% paraformaldehyde), treated with Proteinase K (from *Tritirachium album*, Sigma-Aldrich) for 10–15 min (1:25,000 dilution of a 10 mg/mL stock solution), fixed again (4% paraformaldehyde), and then incubated with the anti-sense RNA probe (1:500 dilution) for 18–72 h at 64 °C–65 °C. Pupae were gently agitated periodically. The pupae were then incubated in Roche α-DIG AP Fab Fragments (1:6000) at 4 °C overnight. On the final day, the tissues were incubated with the BCIP/NBT staining solution (Promega) in the dark until patterns were fully developed (approximately two to 18 h).

5.6. Imaging of Ddc, t, and y expression patterns after in situ hybridization

z-Stacks of images were taken with Olympus cellSens software, using an Olympus SZX16 microscope and an Olympus DP72 camera. The digital images were stacked with Helicon Focus software.

Table 1
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 Fig. 3 (C), while set (b) was used for Fig. 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.

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

5.7. Key Resources Table

For a summary of the resources essential to replicating this study, please see the Key Resources Table.

Reagent or resource	Source	Identifier
Antibodies		
Bacterial and Virus Strains		
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
Critical Commercial Assays		

(*continued on next page*)

(*continued*)

Reagent or resource	Source	Identifier
Deposited Data		
Experimental Models: Cell Lines		
Experimental Models: Organisms/Strains		
Drosophila guttifera	This paper	*Drosophila* Species Stock Center, stock number 15130–1971.10. Note that this stock was not available through the *Drosophila* species stock center as of July 5, 2020 and is available from the laboratory of Dr. Thomas Werner at Michigan Technological University by request.
Drosophila palustris	This paper	N/A – Available from the Laboratory of Dr. Thomas Werner at Michigan Technological University by request.
Drosophila subpalustris	This paper	*Drosophila* Species Stock Center, stock numbers 15130–2071.00. Note that this stock was not available through the *Drosophila* species stock center as of July 5, 2020 and is available from the laboratory of Dr. Thomas Werner at Michigan Technological University by request.
Oligonucleotides		
Primers for *in situ* hybridization probes, see Table 1	This paper	N/A
Recombinant DNA		
Software and Algorithms		
GenePalette Software	Rebeiz and Posakony (2004)	http://www.genepalette.org/
Olympus cellSens	N/A	https://www.olympus-lifescience.com/en/software/cellsens/
Helicon Focus	N/A	https://www.heliconsoft.com/heliconsoft-products/helicon-focus/
Other		
DIG RNA Labelling Kit (SP6/T7)	Roche	https://www.sigmaaldrich.com/catalog/product/roche/11175025910?lang=en&region=US
α-DIG AP Fab Fragments	Roche	https://www.sigmaaldrich.com/catalog/product/roche/11093274910?lang=en&region=US
BCIP/NBT staining solution	Promega	https://www.promega.com/products/biochemicals-and-labware/biochemical-buffers-and-reagents/bcip_nbt-color-development-substrate-_5_bromo_4_chloro_3_indolyl_phosphate_nitro-blue-tetrazolium_/?catNum=S3771
Proteinase K from *Tritirachium album*	Sigma-Aldrich	https://www.sigmaaldrich.com/catalog/product/sigma/p6556?lang=en&region=US

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CRediT authorship contribution statement

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.

Declaration of competing interest

None.

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