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THE DEVELOPMENT OF COMPLEX ABDOMINAL SPOT PATTERNS IN THREE DROSOPHILA SPECIES

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THE DEVELOPMENT OF COMPLEX ABDOMINAL SPOT PATTERNS IN THREE *DROSOPHILA* SPECIES

By

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Abstract

Complex color patterns like the spots on leopards and butterfly wings beg the question – how did these traits evolve? To understand the evolution of novel morphologies, we need to study the differences in morphogenesis between closely related species. Here, I examine and compare the development of complex abdominal spot patterns among three species of *Drosophila* closely related in evolutionary time. Through *in situ* hybridization, I have characterized the expression patterns of three pigmentation genes among the fruit fly species. Additionally, I have built upon previous work in our lab regarding the regulation of pigmentation by beginning to develop an assay to examine interactions between DNA and protein during pupal development. These data have progressed our knowledge of animal pattern development and will facilitate further study of how novel morphologies emerge in nature.

1 Evolution of complex spot patterns in the quinaria group of *Drosophila*

1.1 Abstract

To understand how novel animal patterning emerged, one needs to ask how the development of color patterns changed between diverging species. Here, we examine three species of fruit flies – *Drosophila guttifera* (*D. guttifera*), *Drosophila palustris* (*D. palustris*), and *Drosophila subpalustris* (*D. subpalustris*) – displaying a varying number of abdominal spot rows that were either gained or lost throughout evolutionary time. Through *in situ* hybridization, we examine the mRNA expression patterns for *Dopa decarboxylase* (*Ddc*), *tan* (*t*), and *yellow* (*y*) – three genes that are known to play a role in *Drosophila* pigmentation – during pupal development. Our results show that *Ddc*, *t*, and *y* expression each prefigure the adult abdominal spot patterns in *D. guttifera*, *D. palustris*, and *D. subpalustris*. These data show that these three genes are co-expressed, and may be co-regulated during pupal development and that changes to gene regulation over evolutionary time underlies the variation of abdominal spot rows seen between these three species of *Drosophila*.

1.2 Introduction and Background

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 over evolutionary time occurred (Raff, 2000). This question gave rise to the discipline of Evolutionary Developmental Biology (EvoDevo). EvoDevo provides the questions and tools to compare morphogenesis between different species and facilitates the investigation of how novel structures – like color patterns – emerged. For example, by asking how mutations changed molecular mechanisms over evolutionary time, we can start to learn how the cheetah got its spots. However, mammals like cheetahs and zebras would not make a good model to study color pattern development, given their relatively long gestation periods, low numbers of offspring, and the vast resources and space that would be needed to maintain them. Investigating organismal development requires a model that can be easily cultured in a lab, but still presents morphological diversity. Hence, insects have emerged as a practical system to understand the emergence of novel traits. Butterfly wings have served as a system to better understand the molecular mechanisms underlying complex pattern development (Carroll et al., 1994; Matsuoka and Monteiro, 2018; Monteiro et al., 2013; Zhang et al., 2017; Zhang and Reed, 2016), and examination of cockroaches, 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). However, these findings were built upon the robust knowledge of pattern and pigmentation development gained through the study of fruit flies, in particular, *Drosophila melanogaster* (*D. melanogaster*).

D. melanogaster has served as a model organism to the fields of developmental biology and genetics for over a century, with no foreseeable expiration date for its value (Tolwinski, 2017). This species of *Drosophila* can be inexpensively and quickly cultured in a lab, comes with a well-established genetic toolkit, and has a reliable and annotated genome actively used for nearly two decades (Adams et al., 2000). Our initial understanding of the genetic mechanisms responsible for adult fruit fly pigmentation began with the study of *D. melanogaster* (Walter et al., 1996; Wright, 1987; Wright et al., 1976). These findings built the foundation to understand the processes of insect body pigmentation that have been applied broadly to the *Drosophila* genus and even other insect orders.

To understand how pigmentation patterns develop in adult fruit flies, it is imperative to know their lifecycle. *Drosophila* are holometabolous organisms like butterflies, meaning there will be a complete transformation during their lifetime. Fruit fly larvae will hatch from eggs laid by females. The newly-hatched first-instar larvae will grow in size to progress to the second- and third-instar larvae stages. The third-instar larvae cuticle will darken and toughen to become a puparium. The complete transformation that holometabolous organisms undergo, referred to as metamorphosis,

occurs in the pupa which resides in the puparium. During this pupal development the genes required to form the adult fly – including the genes responsible for creating color patterns – are expressed. The adult fly eventually breaks out, or ecloses, from the puparium ready to begin the *Drosophila* lifecycle again (Flagg, 1988).

In addition to some knowledge of the *Drosophila* lifecycle, a basic comprehension of the biochemical process of pigmentation is needed to understand color pattern development. The *Drosophila* pigmentation pathway outlines the enzymes and reactions necessary to produce black, brown, and yellow coloration seen on the bodies of fruit flies. This biochemical process is shown in Figure 1.1.

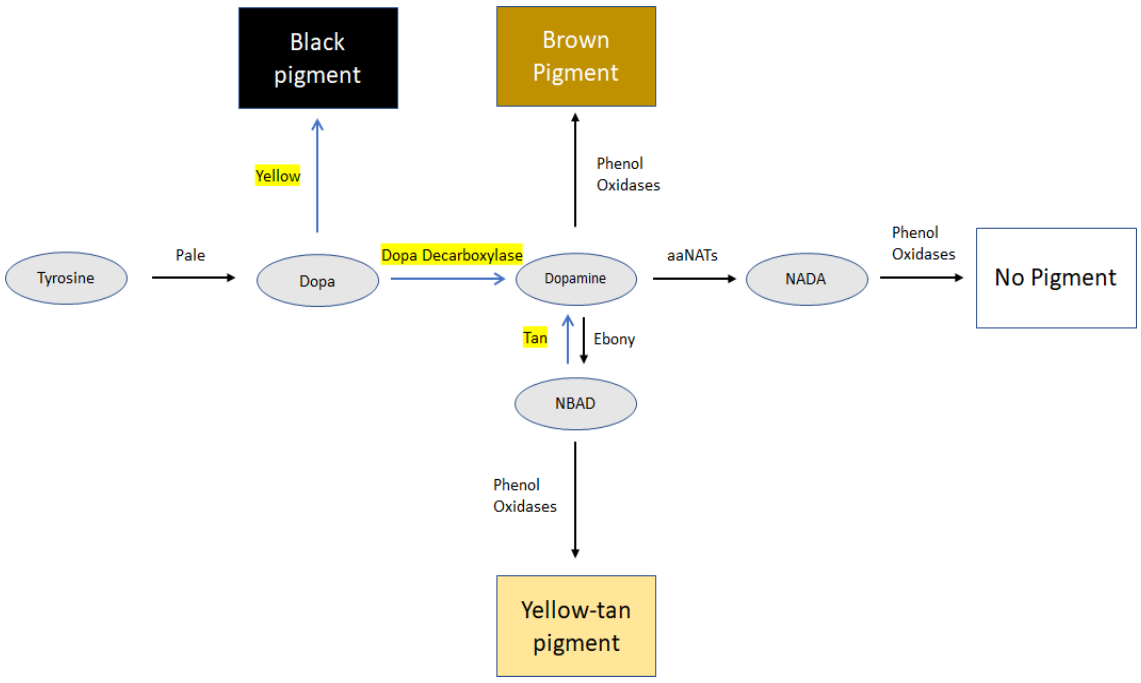


Figure 1.1: The pigmentation pathway of *Drosophila*.

This illustration of the pigmentation pathway is adopted from (Rebeiz and Williams, 2017; True et al., 2005; Wittkopp et al., 2003). Tyrosine is converted to Dopa by Pale. Dopa can be shunted into black

pigmentation by Yellow (encoded by *y*) or further converted into Dopamine by Dopa decarboxylase (encoded by *Ddc*). Dopamine can go one of three ways: 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 then result in a lack of pigmentation through 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*) works opposite of Ebony by converting NBAD into Dopamine, which may result in brown pigment. This figure highlights the gene products for *Ddc*, *t*, and *y*, which this study examines in closer detail.

While the process of *Drosophila* pigmentation patterning involves many genes, our study focuses on three: *Ddc*, *t*, and *y*, which are all essential to the production of black and brown coloration. *Ddc* is integral to the development of *Drosophila* body 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 color pattern development, with *t* mutants expressing less-intense body pigmentation compared to the wild type, and mutants of *y* expressing brown pigmentation as opposed to the standard black color (Biessmann, 1985; Hotta and Benzer, 1969; Kornezos and Chia, 1992; True et al., 2005).

Our understanding of pigmentation development in *D. melanogaster* is invaluable. However, the pigmentation patterning of *D. melanogaster* is relatively simple compared to other fruit flies. In order to comprehend the evolution of complex patterns, other species must be studied. The established genetic toolkit is readily translated to other species of *Drosophila.* This situation allows the robust exploration of genetic mechanisms underlying the complex pattern diversity seen in non-model species of the genus *Drosophila*. The quinaria group, an adaptive radiation of non-model fruit flies, displays a great variety of abdominal and wing pigmentation patterns (Figure

1.2). This abundant morphological diversity and the recent divergence of the lineage (20 million years ago (Scott Chialvo et al., 2019)), position the quinaria group as an excellent system to study the evolution of pigmentation patterning. *D. guttifera* is already becoming established as a model to study complex pattern development (Bollepogu Raja, 2017; Fukutomi et al., 2020; Koshikawa et al., 2017; Werner et al., 2010).

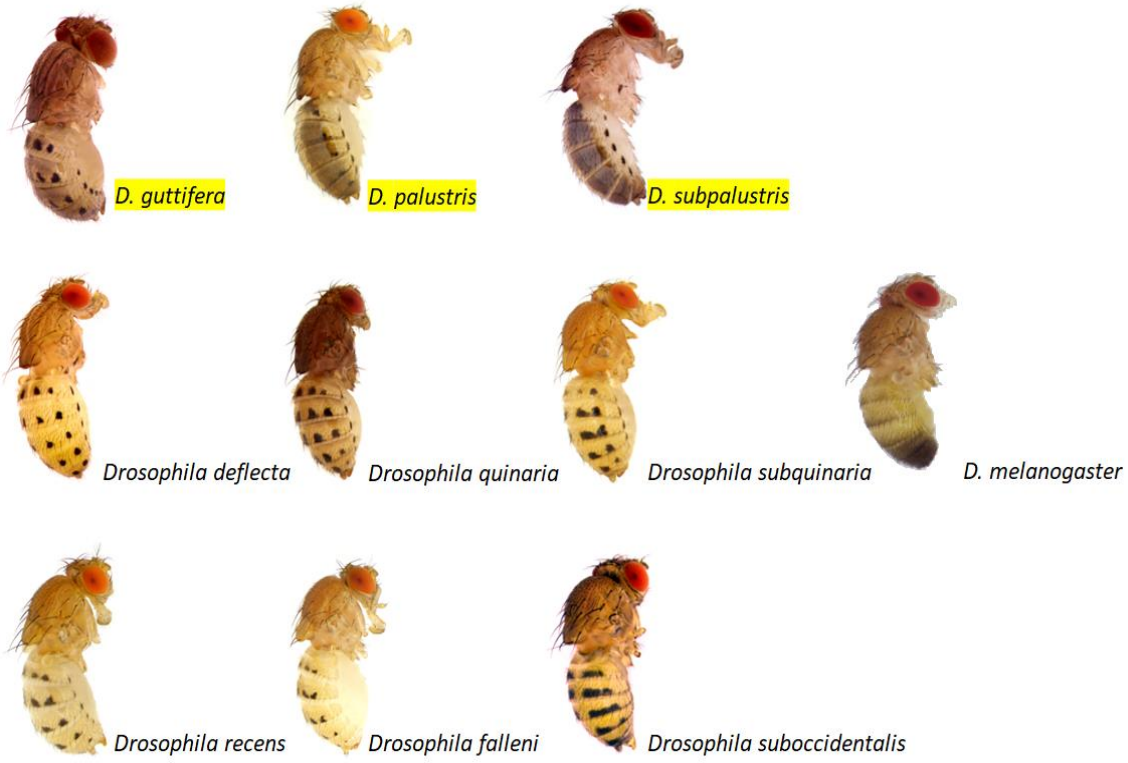


Figure 1.2: The quinaria group of *Drosophila* and *D. melanogaster*.

The diversity of abdominal patterning can be seen throughout several members of the quinaria group. The patterns of spots, single and fused, appear to be gained or lost between the closely related species. *D. melanogaster* is also presented for a comparison of abdominal pigmentation pattern complexity. *D. guttifera*, *D. palustris*, and *D. subpalustris* are highlighted. Figure 3 is adopted from a figure courtesy of Dr. Thomas Werner, with images from (Werner et al., 2018).

Our research continues to study body color patterning in *D. guttifera*, in addition to two other members of the quinaria group – *D. palustris* and *D. subpalustris*. The abdominal spot pattern of *D. guttifera* consists of six rows of spots, three on each side. The pattern of *D. palustris* has four rows of spots (two per side) and that of *D. subpalustris* has two rows (one per side). The spot rows – dorsal, median, and lateral – are outlined in Figure 1.3.

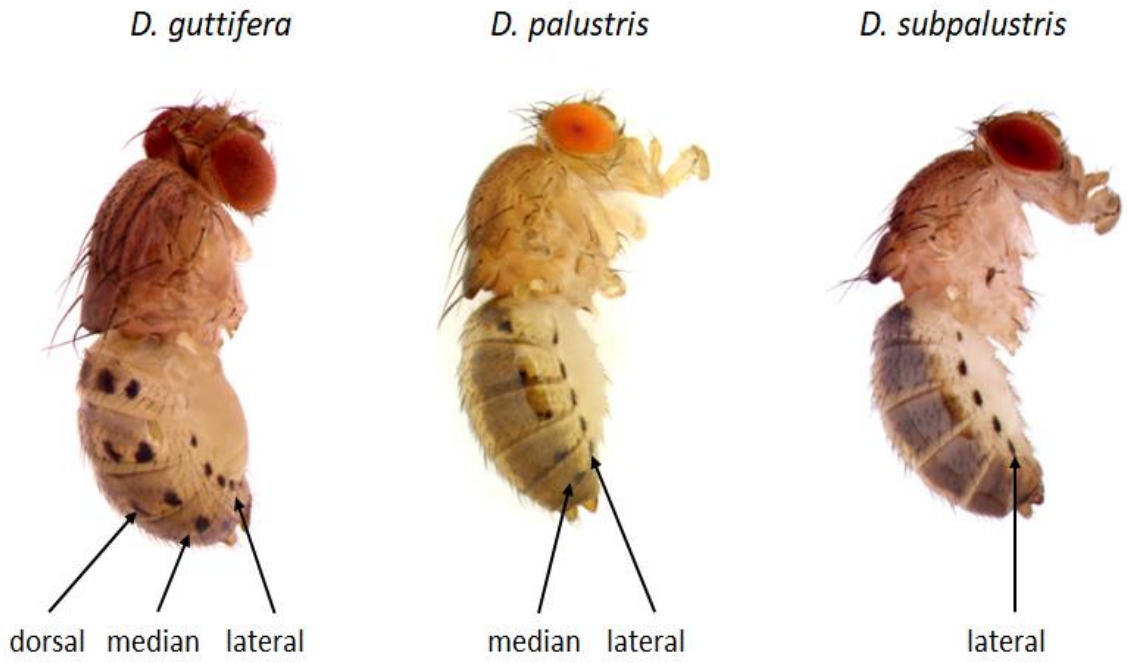


Figure 1.3: Three members of the quinaria group: *D. guttifera*, *D. palustris*, and *D. subpalustris* (lateral view) (Werner et al., 2018)

The evolution of complex abdominal spot patterns between these three species appears to occur through the gain or loss of spot rows. Our hypothesis was that the expression patterns of three genes shown to be involved in pigmentation – *Ddc*, *t*, and *y* – would be expressed differently during pupal development among these three species.

I aimed to characterize these three genes through *in situ* hybridization during the course of pupal development in *D. guttifera*, *D. palustris*, and *D. subpalustris.*

1.3 Materials and Methods

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

The three *Drosophila* species used in this study were *D. guttifera* (*Drosophila* Species Stock Center, currently at Cornell University, stock number 15130 – 1971.10), *D. palustris* (collected by Tessa Steenwinkel (Michigan Technological University) in Waunakee, Wisconsin), and *D. subpalustris* (*Drosophila* Species Stock Center, stock number 15130 – 2071.00). All fly stocks were maintained at room temperature on cornmeal-sucrose-yeast medium (Werner et al., 2018).

1.3.2 Artificial selection experiments

D. palustris exhibits variation in the intensity of its pair of median spot rows. We artificially selected flies with prominent, weak, and non-existent median spot rows. We then created a separate stock for each phenotype.

1.3.3 Identification of pupal stages

The pupal developmental stages for *D. guttifera* have been established, and the characteristics used to describe each pupal stage were recognizable in *D. palustris* and *D. subpalustris* and determined accordingly (Fukutomi et al., 2017).

1.3.4 Design and production of anti-sense RNA *in situ* hybridization probes for *Ddc*, *t*, and *y*

RNA *in situ* hybridization was used to determine the spatial and temporal expression patterns of the pigmentation genes *Ddc*, *t*, and *y* in *D. guttifera*, *D. palustris*, and *D. subpalustris.* Probes were 200 to 500 bases in length, and GenePalette was used for bioinformatics work (Rebeiz and Posakony, 2004). Selected coding regions for *Ddc*, *t*, and *y* were amplified from both *D. guttifera* and *D. palustris* genomic DNA. The *t* probe used for all three species was constructed starting with *D. guttifera* DNA, and the *Ddc* and *y* probes for both *D. palustris* and *D. subpalustris* were derived from *D. palustris* DNA. Due to the close proximity of each species in evolutionary time, the probes were used interchangeably. Mean Green PCR Master Mix was used to amplify the partial coding regions with forward and reverse primers (Table 1). This reagent puts A-tails on the PCR products. After the PCR products were extracted and purified with a Thermo Scientific GeneJET Gel Extraction Kit, the A-tailed, purified PCR products were ligated into the pGEM-TEasy vector. The ligation products were transformed into chemically-competent *E. coli* DH5α cells. The resulting colonies were screened through PCR, using the M13 forward and reverse universal primer pair. Positive colonies were chosen to make minipreps, and the plasmid was then purified with a Thermo Scientific GeneJET Plasmid Miniprep Kit. The insertion direction into the vector was determined through PCR amplification with the M13 forward universal primer and either the internal forward or internal reverse primer (Table 1.1). Depending on the insertion direction,

either SP6 or T7 RNA polymerase was used during the *in vitro* transcription reaction to produce a DIG-labeled RNA anti-sense probe (Roche DIG RNA Labelling Kit (SP6/T7)).

Table 1.1: Primers used to produce *in situ* hybridization probes

Primer Name	**Primer Sequence**
D. guttifera Ddc exon 3 forward	AGCCATTGATTCCGGATGCGG
D. guttifera Ddc exon 3 reverse	AATCGTGTGCTCATCCCACTCG
D. guttifera Ddc exon 3 internal forward	ACTGGCACAGTCCCAAGTTCC
D. guttifera Ddc exon 3 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. 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

1.3.5 **Template sequences for the RNA *in situ* hybridization anti-sense probes**

The sequences of the DNA templates were determined through Eurofins Genomics Tube Sequencing. See "Appendix of *in situ* hybridization probe sequences" for the full sequence data.

1.3.6 **Preparation of the pupae for RNA *in situ* hybridization**

When pupae had developed to the correct pupal stage, they were cut along the anterior-posterior axis either between the eyes or on their side through the eyes. These cut pupae were fixed with a 4% solution of paraformaldehyde (Electron Microscopy Sciences) and kept at -20°C in pure ethanol.

1.3.7 ***in situ* hybridization of the pupae**

The *in situ* hybridization procedures (adopted from (Jeong et al., 2008)) were performed over the course of three to five days, using the anti-sense RNA *in situ* hybridization probe(s) and pupal tissue samples of different developmental stages. The

tissue was washed between each step with PBST. On the first day, pupae were treated with a 1:1 xylenes : ethanol mixture to remove residual fat tissue. The pupal tissue was then fixed (4% paraformaldehyde), treated with Proteinase K (1:25,000 dilution), fixed again (4% paraformaldehyde), and then incubated with the anti-sense RNA probe (1:500) for 18 to 72 hours at 64°C to 65°C. The reaction was gently agitated throughout the incubation to ensure that the hybridization reaction stayed at equilibrium. After the 18 to 72 hours of incubation was completed the pupae were incubated in Roche α-DIG AP Fab Fragments (1:6000) at 4°C overnight. This reaction attached the Fab Fragments bound to the alkaline phosphatase enzyme to the α-DIG present on the anti-sense RNA probes. On the final day, the tissue was incubated with a staining solution of Promega BCIP/NBT in the dark. The alkaline phosphatase-catalyzed reaction converted this staining solution to a purple-stain at the point of mRNA and anti-sense probe hybridization. This staining allows the mRNA expression patterns of the gene of interest to be visualized on the pupal tissue. Staining would become apparent from two to 12+ hours.

1.3.8 **Imaging of *Ddc*, *t*, and *y* expression patterns after *in situ* hybridization**

Pupae were manipulated to remove the head, legs, and wings either before or after staining. The pupal abdomens were then imaged with an Olympus SZX16 microscope and an Olympus DP72 camera. The images were digitally captured with Olympus cellSens software, and the digital images were stacked with Helicon Focus.

1.4 Results

Our group had shown that both *t* and *y* expression during pupal development prefigures the abdominal spot pattern of *D. guttifera* (Bollepogu Raja, 2017). Intrigued by the fact that these two genes are regulated in the exact same complex expression pattern, I wanted to determine if more genes could show such a phenomenon – given the rarity of two genes being expressed in identical complex patterns. I tested for a third known pigmentation gene, *Ddc*, through *in situ* hybridization to determine if it was also expressed in the same spot pattern, and found that this was the case. These data show the co-expression and suggest the co-regulation of three genes into the same expression pattern during pupal development in *D. guttifera*.

To investigate this co-expression further, I chose to study species with subsets of the *D. guttifera* abdominal pattern. I wanted to determine if the expression patterns of *Ddc*, *t*, and *y* changed to prefigure the adult spots in *D. palustris* and *D. subpalustris*. I used *in situ* hybridization to characterize *Ddc*, *t*, and *y* mRNA during pupal development and found that all three genes are expressed to foreshadow the adult abdominal spot patterns of *D. palustris* and *D. subpalustris.* In some instances, there was very weak or no signal for gene expression correlating to the median spot row of *D. palustris.* This faint signal was expected, given the variation of spot intensity seen in the adult fruit flies.

These data show that *Ddc*, *t*, and *y* expression foreshadows the adult spot patterning of *D. guttifera*, *D. palustris*, and *D. subpalustris*. *Ddc* mRNA is present in all three species during the pupal stages P11 and P12, in addition to later stages in *D. guttifera* and *D. palustris*. The expression of *t* is seen in *D. guttifera* at P11 and at P12 in *D. palustris* and *D. subpalustris*. Signal for *y* is seen at P10 in all three species in addition to P12 in *D. palustris*. Additionally, I showed the expression of *t* and *Ddc* prefiguring the abdominal shading seen in *D. palustris* and *D. subpalustris*, respectively. Thus, the mRNA expression amongst species correlate precisely with the presence of black spots on the abdomen. Although correlative in nature, my observations suggest that color pattern diversity within the quinaria group is driven by the deployment of upstream factors that collectively co-regulate these three downstream target genes.

1.4.1 ***t* and *y* expression prefigure the adult *D. guttifera* spot pattern**

Past *in situ* hybridization experiments in our lab show that *t* and *y* foreshadow the adult abdominal spot patterns of *D. guttifera* (Bollepogu Raja, 2017).

1.4.2 *Ddc* expression also foreshadows the adult *D. guttifera* spot pattern

Ddc mRNA correlates with the spot pattern on *D. guttifera* (Figure 1.4).

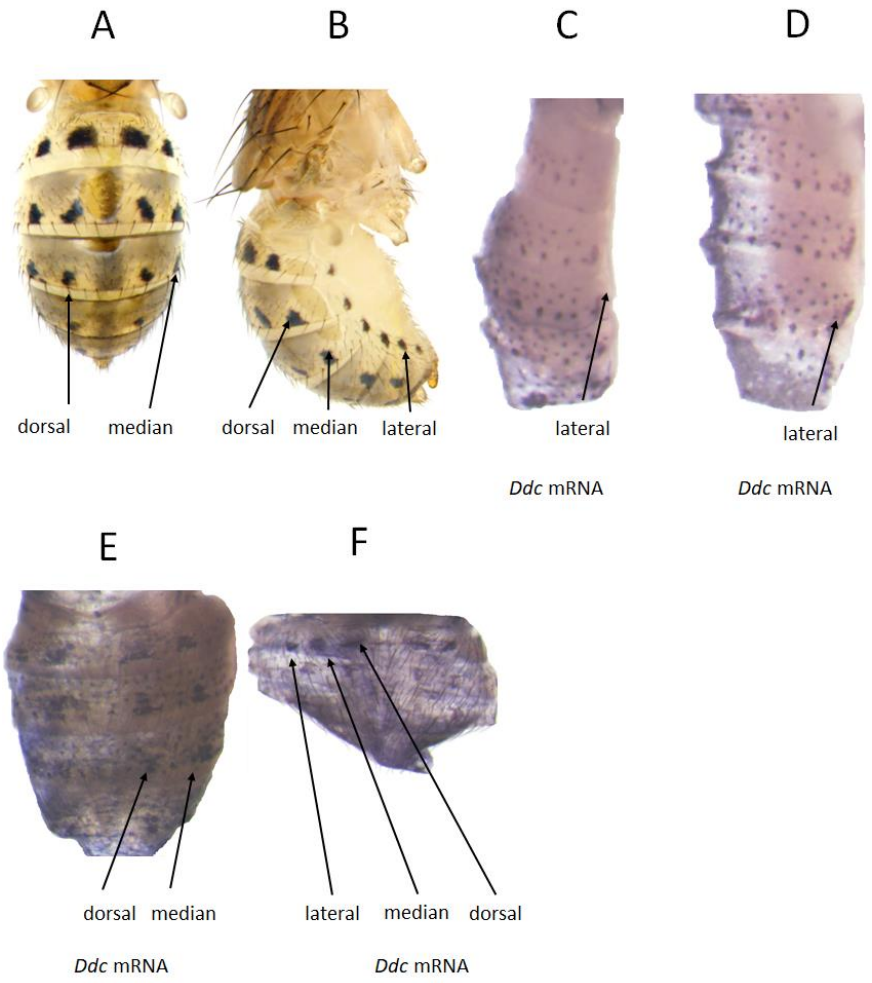


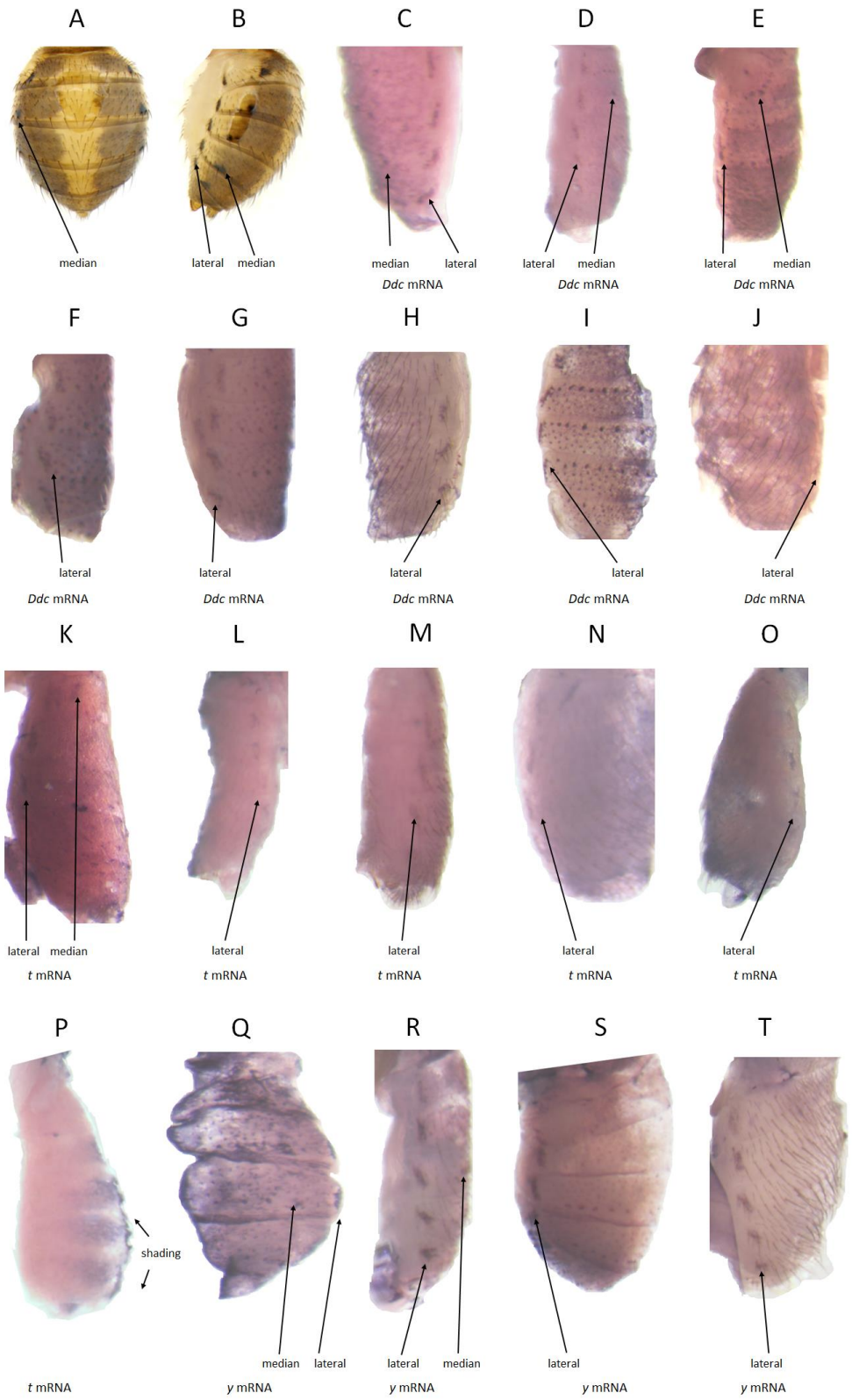
Figure 1.4: *in situ* hybridization of *Ddc* during *D. guttifera* pupal development

(A) Dorsal and (B) lateral view of adult *D. guttifera* (Werner et al., 2018). (C – F) *Ddc* mRNA expression at stages P11 (C, D), P12 (E), and P13 (F).

1.4.3 *Ddc*, *t*, and *y* expression correlates with the adult *D. palustris* spot pattern

The spot pattern of *D. palustris* lacks the dorsal row of spots seen on *D. guttifera*; however, the median and lateral rows are present (with variation to the intensity of

pigmentation seen in the median row). The mRNA expression patterns of *Ddc*, *t*, and *y* prefigure the adult abdominal pattern of *D. palustris.* Expression of *t* is also shown to foreshadow the abdominal shading seen at the median row of spots (Figure 1.5).


A
B
C
D
E
median
lateral median
median lateral
lateral median
lateral median
Ddc mRNA
Ddc mRNA
Ddc mRNA
F
G
H
I
J
lateral
lateral
lateral
lateral
lateral
Ddc mRNA
Ddc mRNA
Ddc mRNA
Ddc mRNA
Ddc mRNA
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lateral median
lateral
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lateral
t mRNA
t mRNA
t mRNA
t mRNA
t mRNA
P
Q
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shading
median lateral
lateral median
lateral
lateral
t mRNA
y mRNA
y mRNA
y mRNA
y mRNA

Figure 1.5: *in situ* hybridization of *Ddc*, *t*, and *y* during *D. palustris* pupal development

(A) Dorsal and (B) lateral view of adult *D. palustris* (Werner et al., 2018). (C – J) *Ddc* mRNA expression at stage P11 (C – F), P12 (G – I), and P12-P15 (J). (K – P) *t* gene expression at stage P11 – P12 (K) and P12 (L – P). (Q – T) *y* mRNA at stages P10 (Q, S) and P12 (R, T).

1.4.4 *Ddc*, *t*, and *y* mRNA also foreshadows the adult *D. subpalustris* spot pattern

D. subpalustris lacks both a dorsal and median row of spots, making its abdominal pattern the "simplest" of the three species of interest. The *Ddc*, *t*, and *y* expression patterns during pupal development prefigure the adult abdominal spot pattern – just as in *D. guttifera* and *D. palustris* – in addition to *Ddc* prefiguring the adult shading pattern (Figure 1.6).

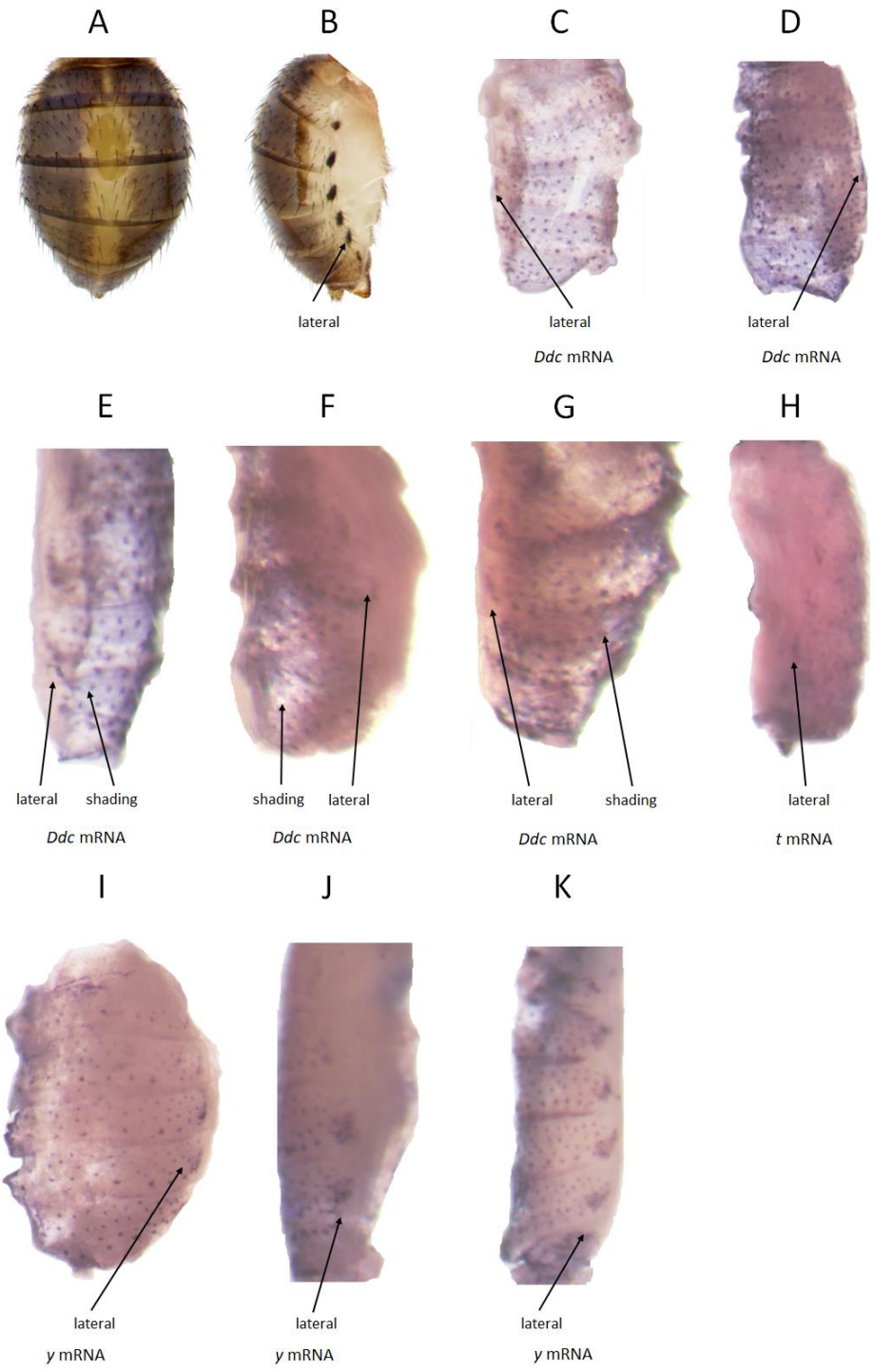

Figure 1.6: *in situ* hybridization of *Ddc*, *t*, and *y* during *D. subpalustris* pupal development

(A) Dorsal and (B) lateral view of adult *D. subpalustris* (Werner et al., 2018). (C – G) *Ddc* mRNA expression at stage P11 (C – D) and P11 – P12 (F, G). (H) *t* gene expression at stage P12. (I – K) *y* mRNA at stage P10.

1.5 Discussion

The adult *D. guttifera*, *D. palustris*, and *D. subpalustris* have different abdominal pigmentation patterns. Among the three species, these spot-patterns appear to be assembled by the gain or loss of spot rows. Here, we show that *Ddc*, *t*, and *y* are uniquely co-expressed to foreshadow these different patterns. Our data support the initial hypothesis that these genes' co-expression correlates with the abdominal spots seen on all three species. These observations suggest that changes to the co-regulation of *Ddc*, *t*, and *y* underlie some of the morphological diversity seen within the quinaria group.

There is not a straightforward method to determine if co-regulation exists between multiple genes (Allocco et al., 2004). Gene co-expression – a phenomenon seen among genes with a related purpose – suggests their co-regulation, and this allows us to hypothesize possible gene co-expression networks (Brown et al., 2000; Eisen et al., 1998; Garg and Achenie, 2002; Spellman and Rubin, 2002; Villa-Vialaneix et al., 2013; Wu et al., 2002; Yu et al., 2003; Zhang and Horvath, 2005). Therefore, we can begin to predict how genes with both similar functions and expression patterns may be co-regulated and then investigate their positions within gene regulatory networks (GRNs). Studying the evolution of GRNs responsible for *Drosophila* pigmentation continues to clarify the morphological diversity seen within the genus and allows us to better understand the evolution of complex structures (Camino et al., 2015; Carroll, 2000; Davidson and Levin, 2005; Grover et al., 2018; Jeong et al., 2008; Koshikawa et al., 2015;

Ordway et al., 2014; Roeske et al., 2018). From the information presented in this study, we can continue to elucidate the GRN(s) responsible for creating complex spot patterns on the abdomen of *D. guttifera*, and start to understand this same process in two untouched species – *D. palustris* and *D. subpalustris*.

The spots seen in *D. guttifera*, *D. palustris*, and *D. subpalustris* provide more than just a model to understand complex color patterns; they are serial homologous structures, just like the rows of spots on butterfly wings (Monteiro, 2008). Studying the GRNs responsible for generating these repeated patterns will progress our understanding of how serial structures evolved in nature. This further justifies the value of the quinaria group as a system to investigate morphogenesis.

The results of *in situ* hybridization shown in this study provide sufficient evidence of co-expression of three pigmentation genes seen among three species of *Drosophila.* The next step to determine possible co-regulation would be cluster analysis of RNA-seq data during stages of pupal development in all three species (Tomancak et al., 2007). This information would provide temporal data of gene expression that could be compared to the temporal and spatial data provided by our *in situ* hybridization results. Correlation among the expression of *Ddc*, *t*, *y*, and transcription factors would provide possible regulatory candidates. We could then use *in situ* hybridization of these candidate transcription factors to provide further spatial and temporal data to determine the GRN(s) dictating the expression of *Ddc*, *t*, and *y* in these three species.

The implications to unraveling the GRN(s) controlling *Ddc* and *y* apply to more than just color pattern development. *Ddc* and *y* are vital to the lifecycles of agricultural pests and disease vectors, such as the Asian tiger mosquito, black cutworm, brown planthopper, and kissing bug (Chen et al., 2018; Lu et al., 2019; Sterkel et al., 2019). We have demonstrated the potential of these three species of *Drosophila* as a robust system to understand the regulation of these pigmentation genes, which could have application to both agriculture and human health.

Previous work in our laboratory investigated the regulation of *t* and *y* in *D. guttifera* to understand the development of this fruit fly's complex abdominal spot pattern. However, there are very few genetic tools available in *D. guttifera* and none are readily available for *D. palustris* and *D. subpalustris*. Despite the difficulties working with non-model organisms, I chose to investigate the development of abdominal pigmentation in these two species. Along with *D. guttifera*, these three members of the quinaria group are an excellent system to study the evolution of pattern development due to the gain or loss of spot rows seen among the species. Establishing methods to produce transgenic *D. palustris* and *D. subpalustris* will facilitate the identification of regulatory elements responsible for the development of their spot patterns. If we elucidate the evolutionary changes to regulatory networks underlying the gain or loss of spot rows among these three species, then we can better understand how complex patterns emerged in nature and begin to ask how the leopard got its spots.

1.6 Conclusion

Here we show that three genes known to be involved in *Drosophila* pigmentation – *Ddc*, *t*, and *y* – have expression patterns during pupal development that precisely foreshadow the abdominal spot patterns of *D. guttifera*, *D. palustris*, and *D. subpalustris.* The co-expression of three genes into the exact same complex pattern is rarely seen, and suggests that the co-regulation of *Ddc*, *t*, and *y* shapes the morphology of these color patterns. This potential co-regulation provides a starting point to not only study pattern development but also how multiple genes are regulated into the same expression pattern. Through cluster analysis or similar approaches, we can understand the GRNs that underlie the evolution of spot patterns in these species and progress our knowledge of how novel traits emerge.

2 Fixation and fragmentation of chromatin from *D. guttifera* pupae for further processing in Chromatin Immunoprecipitation assays

2.1 Abstract

Chromatin immunoprecipitation (ChIP) is a useful tool to examine DNA-protein interactions. However, a ChIP protocol is not readily available for the non-model fruit fly *D. guttifera*. Here we outline a procedure to produce 100-300 base pair fragments of chromatin from *D. guttifera* pupae for further ChIP processing. This information provides the foundation to develop a ChIP-seq or ChIP-PCR assay for *D. guttifera* pupae. Such a protocol will allow us to examine interactions between previously identified *cis*-regulatory elements (CREs) and transcription factors (TFs) in *D. guttifera*.

2.2 Introduction

Novel morphologies arise primarily due to changes in gene expression patterns, which can result from alterations in non-coding DNA (changes in *cis*) or the presence of new TFs, morphogens, or entire gene networks (changes in *trans*), or both (Gompel et al., 2005). Previous work in our lab identified three possible regions of non-coding DNA – or CREs – which may regulate the gene *yellow* (*y*) in *D. guttifera* (Bollepogu Raja, 2017). These putative CREs were labeled as "Spot" (Sp), "Stripe" (S), and "Wing-body" (WB). Sp, S, and WB are suggested to serve as binding sites for TFs to regulate the expression of *y* (Mora et al., 2015; Rojano et al., 2018).

Through *in situ* hybridization, mRNA from both the Hox TF *abd-A* and the pigmentation gene *y* were shown to prefigure the lateral row of spots in *D. guttifera* (Bollepogu Raja, 2017). This correlation prompted the question – is Abd-A binding to any of the CREs (Sp, S, or WB) that possibly regulate *y*? To address this question, we began to develop a ChIP protocol to investigate DNA-protein interactions during *D. guttifera* pupal development (Nelson et al., 2006). ChIP is completed by fixing TFs to chromatin with formaldehyde and then fracturing the chromatin through sonication. Antibodies specific to TFs of interest are used to precipitate the bound chromatin, which can then be either amplified or sequenced. Though no ChIP experiments were run successfully, I determined a method of isolating, fixing, and fragmenting to a set size the chromatin from *D. guttifera* pupae (referred to as a "chromatin preparation"). Here, I outline the steps to produce a chromatin preparation from *D. guttifera* pupae for ChIP experiments.

2.3 Materials and Methods

2.3.1 **Producing and determining the fragment sizes of a chromatin preparation**

- Add 1 mL of 1xPBS to a 1.5 mL-microcentrifuge tube and then add 0.1 g of *D. guttifera* pupae at the developmental stage(s) of interest
- Crack the pupae with a sterile 7 cm polypropylene pellet pestle, blue
- Spin the sample down for 5 minutes at maximum speed and remove the supernatant
- Rinse in 500 µL **ChIP Fix Solution** and vortex quickly
- Spin the sample down for 5 minutes at maximum speed and remove the supernatant
- Crack the floating pupae with a sterile 7 cm polypropylene pellet pestle, blue
- Replace the 500 µL **ChIP Fix Solution** with 1 mL **ChIP Fix Solution** and resuspend the pupae
- Nutate the samples at room temperature for 20 minutes with a Fisher Scientific Nutating Rotator
- Turn on the Bioruptor® Plus sonication device (diagenode) and set the water bath temperature to 4°C
- Add 100 µL of 125 mM Glycine to neutralize the fixation reaction and continue to nutate the samples for five minutes at room temperature
- Spin the sample down for 5 minutes at maximum speed and remove the supernatant

- Add 1 mL of 1xPBS
- Spin the sample down for 5 minutes at maximum speed and remove the supernatant
- Add 350 µL of **ChIP Lysis Buffer**
- Homogenize the pupae with a sterile 7 cm polypropylene pellet pestle, blue
- Quickly spin down the samples
- Sonicate the samples for 40 rounds with a Bioruptor® Plus sonication device (diagenode). One round is 30 seconds of maximum sonication (1 mA) and 60 seconds of rest
- Add 350 µL of phenol chloroform to each sample, vortex, and let the samples sit at room temperature for five minutes
- Centrifuge the samples at maximum speed for 10 minutes at room temperature and then transfer 80 µL of the top layer to a 1.5 mL-microcentrifuge tube
- Add 8 µL 3M Sodium Acetate pH 5.5 and 240 µL 100% ethanol to the microcentrifuge tube
- Store at -70°C to -80°C for at least one hour
- Turn on the refrigerated centrifuge and set the temperature to 4°C
- Spin down at 4°C at maximum speed for 10 minutes
- Take off the supernatant, do not agitate the pellet
- Wash the pellet with 1 mL 70% ethanol
- Spin at maximum speed for five minutes at room temperature

- Take off the supernatant and air dry the samples for five minutes to remove residual ethanol
- Dissolve the pellet in 50 µL of EB Buffer and let samples sit at 4°C overnight
- Run 30 µL of the sample and 15 µL of loading buffer on a 1% agarose gel

2.3.2 **Solution list**

1.8% formaldehyde ChIP Fix Solution (100 mL)**:**

- 4.9 mL 37% formaldehyde
- 10 mL 0.5 M HEPES
- 200 µL 0.5 M EDTA
- 500 µL 100 mM EGTA
- 2 mL 5 M NaCl
- Fill to 100 mL with DI water

ChIP Lysis Buffer (250 mL)**:**

- 5 mL 0.5 M EDTA
- 12.5 mL 1 M Tris-HCl pH 8.0
- 1.25 mL EMPIGEN BB
- 2.5 g SDS
- Fill to 250 mL with DI water

2.4 Results

The chromatin preparation procedure was completed with *D. guttifera* pupae in duplicate and the results are shown in Figure 2.1. The chromatin was fragmented into sizes ranging, primarily, from 100 to 300 base pairs (bp). Lesser amounts of chromatin produced from the protocol range from 300 to 3000 bp.

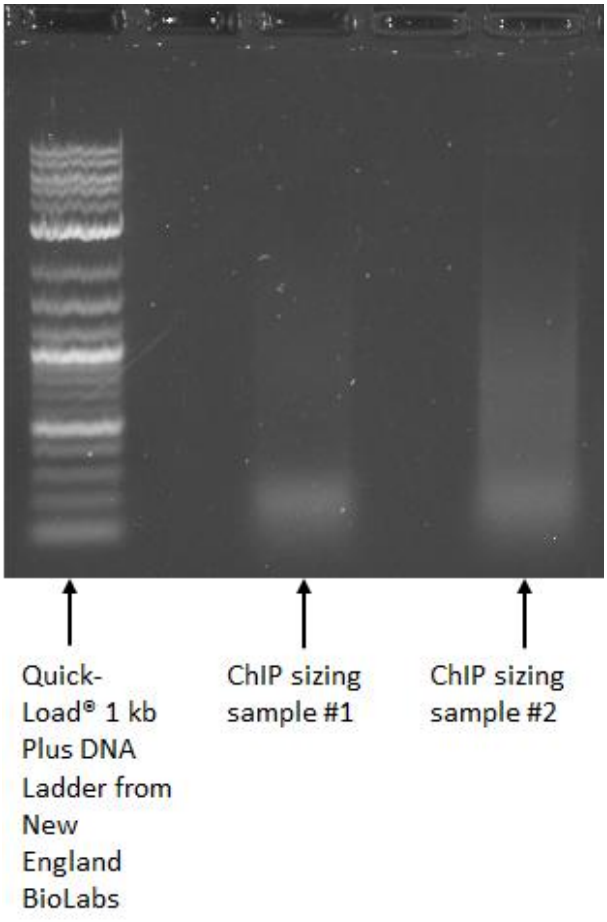

Figure 2.1: Gel electrophoresis of ChIP chromatin preparation

2.5 Discussion and Conclusion

Though no successful ChIP experiments in *D. guttifera* pupae were completed, this protocol provides a starting point to develop and execute future ChIP experiments. Chromatin fragments should be between 150 and 300 bp for ChIP assays (Kidder et al., 2011). In order to possibly increase the DNA yield, pupae can be frozen and homogenized before starting the chromatin preparation. However, this change in protocol may affect the efficacy of the sonication; thus, the procedure would need to be re-validated to achieve the correct chromatin fragmentation.

Before using ChIP to examine potential TF interactions with the putative *y* CREs, validation of a protocol should be done. Histone acetylation is frequently seen at transcriptionally active sites in the genome, meaning that a ChIP assay using an antibody for acetyl groups and the CREs could prove useful in developing a protocol (Creyghton et al., 2010).

It should also be noted that TFs interacting with the identified CREs in *D. guttifera* may only be occurring in cells that will become incorporated into the spot pattern. This means that only small patches of epithelial cells on the developing pupa may be relevant to the study. ChIP requires large amounts of cells, anywhere from 10^4 to over 10^7 (MilliporeSigma, 2020). Producing this large of a sample of tissue providing the applicable environment of TFs may not be technically feasible. Other applications requiring smaller samples, such as MicroChIP and CUT&RUN, may be better suited to

understand DNA-protein interactions in this situation (Acevedo et al., 2007; Skene and Henikoff, 2017).

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4 Appendix of *in situ* hybridization probe sequences

4.1 *D. guttifera Ddc* exon 3 probe sequence

NNNNNNNNNNNNNNGAANNNNNGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGC

GGGAATTCGATTAGCCATTGATTCCGGATGCGGCGCCCGAAAAGCCCGAGGATTGGCAGGATG

TGATGAAGGACATCGAACGGGTCATCATGCCGGGCGTCACACACTGGTACAGTCCCAAGTTCC

ATGCGTACTTCCCCACGGCCAACTCGTATCCGGCCATTGTGGCGGACATGTTGAGCGGAGCGAT

TGCCTGCATTGGATTCACTTGGATTGCGAGTCCGGCGTGCACTGAACTCGAGGTGGCCATGCTG

GATTGGCTGGGCAAGATGTTGGATCTGCCTGCCGAGTTTTTGGCCTGCTCGGGCGGCAAGGGC

GGCGGCGTCATCCAAGGAACGGCCAGTGAATCCACATTGGTGGCATTACTGGGCGCCAAGGCG

AAGAAGCTGCAGGAGGTGAAGGCCAAGCATCCCGAGTGGGATGAGCACACGATTAATCACTA

GTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAG

CTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGT

GAGATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTANAGCCTG

GGGTGCCTAATGAGTGAGCTAACTCACGTTAATTGCATTGCGCTCACTGCCCGCTTGCCATACG

GGAGACCTGTCGTGACCACTGCGTTAATGAATCGNNCANCGCGCGTGGNNAAGCGNGNNNG

CGNAGTGTGGGCACGTCGCGNNNNTTCANGCAATGACNCTNNACTCAATNAAATTAAGATGG

TNNNNNNATCTGAGCTGCTGCACGNCCGNATCNNGTANNCGAGACTTGCAGTTGAGATGGAA

NAGAGTAAAGNGTTTACNNTGCNGNNNNTTTGNNGGTCTGGGCGTCGGCNNGNGGANGGN

NCAAGTGCCAGGANNNGGTNNGATTTGGNGGTANGGGNAGGNNTNNAANNTAANANANN

GNNGGAAANCTGGNNNNNNCCNNNACCNNCNNTTGCTGCTTTTTTTGGANNCNNNNNNANCA

ANNNNNNCNNGNNNNTATNNNNTNNNNG

4.2 *D. guttifera t* exon 5 probe sequence

NNNNNNNNNNNAGGGCGNTTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCG

GGAATTCGATTCAGCGTCTGCTTGGCCACACGGAGGACGCACTGACGGAGACGCTGAATCATT

ACTACTTTGTGGTCGCTCACATCATCAACGACAAGCCGCAGGGCAAGTACAATGTGCGGGAGG

AGCACTTCATGTCCCTCTGCTACGCTGGCCACTTGCCCGGCTACACAATGAGCCACAATCGCCAT

GGACTCGTGTTCAGCATCAACACCATTAGCGCCGAATTGTTGCGCAGCGGCAAAATCACTAGTG

AATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTT

GAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAA

ATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGG

TGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAA

ACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTG

GGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGT

ATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAA

CATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTT

CCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAA

CCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTC

CGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT

AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACG

AACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTA

AGANNNNN

4.3 *D. palustris Ddc* exon 3 probe sequence

NNNNNNNNNNNNGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCG

GGAATTCGATTTATCGTCATCACATGAAGGGCATCGAGACCGCCGACTCCTTCAACTTTAATCCA

CACAAATGGATGCTGGTCAACTTTGACTGCTCGGCCATGTGGCTCAAGGATCCCAGTTGGGTGG

TGAATGCCTTCAATGTGGATCCTCTTTATTTGAAGCACGATATGCAGGGATCTGCTCCCGACTAT

CGCCACTGGCAAATCCCGCTGGGCAGACGTTTCCGTGCTCTCAAACTCTGGTTTGTGCTGCGTCT

TTACGGTGTAGAGAATCTCCAGGCTCACATCCGACGTCATTGCGGATTTGCCAAGCAGTTTAGT

GAGCTCTGTGTGGCGGATAAACGTTTCGAGCTGGCTGCTGAGGTGAACATGGGTCTGGTCTGC

TTCCGCCTCAAGGGAACTAATGAAAGGAACGAGGCGCTACTGAAGCGCATCAATGGACGCGGC

AAGATTCACATGGTGCCGGCCAAGATCCGGGATGTCTACTTCTTGCGCATGAATCACTAGTGAA

TTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGA

GTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAAT

TGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTG

CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAAC

CTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGG

CGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATC

AGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACAT

GTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCAT

AGGCTCCGCCCCCCTGACNAANATCACAAAAATCGACCCTCAAGTCAAAGNTGGNGAANCCCG

ACGGGACNNTAANNNNNCAAGGGGTNTNN

4.4 D. palustris y exon 2 probe sequence

NNNNNNNNNNNNNGGGCGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGC

GGGAATTCGATTCGATGCCATGGAATTGCGGAGTGTACAGCATCGACGAATGCCAGCAGCCAA

TGGCATTCTGATCAATCAGATTGAACAGCTCGACGCCATCGTCGCTCATCACTCGGGCCGTGGT

GTGAGCATTTCCTCCACGCTCGTCCAAAGCCACAAACTCATGGTAACTGTCCTCGGTGCGGGAT

TCGTCTCTCAAGATTCGCGTGGACACCGCGAACTGGCGATGACTGGCCAGGGGACTGAAGTAC

AGGGTGCGATAGCCATCGGAGCGAATGGGCGACAAGGCGATGCCAAAGATGCCCTCCTCAATC

ACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGC

ATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCT

GTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAG

CCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAG

TCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTG

CGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCG

AGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGG

AAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGG

CGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTG

GCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCT

CCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCNCCTTTCTCCCTTCGGGAAGCGTGGCGCT

TTCTCTAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAGCTGGGCTGNNT

GCNGAACCCCCGTT