DECIPHERING THE *EPARGYREUS CLARUS* (SILVER-SPOTTED SKIPPER) WING PATTERNING GENES

By

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I would like to dedicate this work
to my family and friends for standing by me
and supporting me unconditionally, and
to my mentors for guiding me
throughout the adventure
of fulfilling a master's degree.

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ABBREVIATIONS

Mya	Million years ago
DE	Differential expression
mRNA	messenger RNA
FW0	Larval forewing
FW1	Proximal forewing compartment
FW2	Medial forewing compartment
FW3	Distal forewing compartment
HW0	Larval hindwing
HW1	Proximal hindwing compartment
HW2	Medial hindwing compartment
HW3	Distal hindwing compartment
SE	Single-end sequencing
PE	Paired-end sequencing
Mb	Megabase (1,000,000 base pairs)
SAM	Sequence Alignment Map
BAM	Binary Alignment Map
ISH	*in-situ* hybridization
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
IGV	Interactive Genome Viewer
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
PCA	Principal component analysis

ABSTRACT

Butterfly wing patterns have captivated evolutionary and developmental biologists for decades because of their vast diversity. Studies have focused on the molecular basis of pattern variation in the Nymphalidae, with little effort put toward skippers, family Hesperiidae. This study had two aims: identify the presence of Wnt genes through ISH and pharmacological drug injections, and test differentially expressed genes in three developmental stages, between wing types, and between wing compartments of *E. clarus* using RNA-sequencing. I hypothesized, that a simple yet modular set of genes was involved in wing pattern development of the skipper, similar to that of nymphalid butterflies and that genes such as *WntA* and *Ubx* would be differentially expressed as in nymphalids. ISH confirmed the presence of two Wnt genes and RNA-seq provided evidence of differential expression, suggesting that the genes underlining wing pattern diversity are being shared between these two distantly related families.

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GENERAL INTRODUCTION

How do butterflies make stripes and patterns on their wings? Because of their vast diversity, butterflies have become a classic non-model system for studying the evolution of genes underlying the diversification of phenotypic traits (*e. g.* wing patterns) (Jiggins et al., 2017; Sekimura & Nijhout, 2017). The variety of these patterns are considered crucial adaptations that allow butterflies to rapidly evolve (Deshmukh et al., 2018) and are not limited to simple variation in specific wing areas. In fact, butterflies show sexual dimorphism, polymorphism and season-dependent coloration, and at the same time can develop different ventral/dorsal patterns (Sekimura & Nijhout, 2017). Understanding the origin of these complex patterns is a difficult task. Nonetheless, many resources have been directed towards elucidating the molecular basis of wing pattern variation focusing on the large and diverse Nymphalidae butterfly family (Abbasi & Marcus, 2015; Deshmukh et al., 2018; Hanly et al., 2019; Kodandaramaiah, 2009; Martin et al., 2012; Mazo-Vargas et al., 2017; Van Belleghem et al., 2017). Regardless, efforts towards identifying the molecules controlling wing patterns in other butterfly families are minimal. Recently however, skipper butterflies belonging to the Hesperiidae family have been shown to present a widespread convergence of wing patterns similar to those observed in Nymphalidae (Li et al., 2019), suggesting that similar developmental principles apply across these distantly diverse lineages (~80 Mya) (Figure 1) (Espeland et al., 2018). It is no mystery that in many butterfly species there is a conserved yet flexible assortment of genes that control wing pattern development (Hanly et al., 2019; Huber et al., 2015; Van Belleghem et al., 2017). For this reason, assessing the extent to which a similar developmental genetic toolkit is shared by all butterflies is an important first step toward understanding the mechanisms that promoted the radiation of skippers and butterflies more broadly.

Lepidoptera: a diverse order

The order Lepidoptera, which includes butterflies and moths, originated approximately 300 Mya [according to recent phylogenetic studies (Kawahara et al., 2019)] and consists of more than 160,000 described species. Lepidoptera represents one of the most diverse insect orders in terms of wing shapes and color patterns (Kristensen et al., 2007). Moreover, butterflies and moths account for approximately eighteen percent of all described species (Hanly et al., 2019) and, even though they are considered non-model organisms, their vast range of wing patterns make them the perfect study system for researching the molecular basis of wing pattern and shape diversity. In addition to their wing pattern diversity, Lepidopterans are found in a large range of habitats and provide some level of structural and ecological homology that allows for their study in an evolutionary framework (Kristensen et al., 2007). For these reasons, evolutionary and developmental biologists have studied the convergent evolution and genetic architecture of these adaptive traits in Lepidoptera in an attempt to better understand the molecular basis of these phenotypic variations.

Wing pattern diversity: within and between butterfly species

Broad wing pattern diversity has not only been observed between individuals of different species but also within individuals of a single genus and even of a single species. One of the most prominent cases of extreme wing pattern diversity within a species is represented by *Heliconius* butterflies (Deshmukh et al., 2018; Huber et al., 2015; Kronforst & Papa, 2015). *Heliconius erato*, for example, has over 30 butterfly races with very different wing color patterns – for which the genetic architecture has been well documented (Van Belleghem et al., 2017). *Vanessa* butterflies are a good example of wing pattern variation within a genus. Wing color pattern evolution has been studied for all 22 described *Vanessa* species (Abbasi & Marcus, 2015), revealing a modular

genetic architecture. In the skippers, we observe a similarly remarkable phenomenon of within-genus diversity. Prior to work performed by Li et al (2019), the genera *Ectomis*, *Hypocryptothrix*, and *Heronia* were considered separate because of their vast phenotypic diversity. Using modern phylogenetic methods, the authors discovered that – despite varying greatly in wing coloration, pattern, and shape – all three genera (plus two additional genera) belong to the same genus; *Ectomis*.

Wing coloration: how do butterflies use color?

The colors on a butterfly's wing are determined by two main processes: (a) color pigments are deposited on tiny structures called scales (*e.g.* red, black, and yellow colors) or (b) the physical structure of the scales control how light is reflected off the wing surface (*e.g.* blue, white, and green colors) (Nijhout, 1981). Butterflies use the colors resulting from these processes in many ways, including thermoregulation. Butterflies are heliotherms which means they mainly derive heat from the sun, while moths are myotherms, deriving heat through muscle energy (Clench, 1966). There are three specific types of wing color patterns that have an important effect on heat exchange and thermoregulation (Clench, 1966). The first of these patterns is *border color*, which refers to the presence of a dark (usually black) border on the dorsal surface of some butterfly wings, allowing the ability of exchanging heat without limiting the space needed for other functional pigmentation. The second pattern is *vein coloration*, which refers to similarly dark venation on the wings. In both cases, these darker patterns allow heat exchange without limiting the space needed for other functional pigmentation (*e.g.* warning colors). For example, in the monarch butterfly (*Danaus plexippus)*, *vein coloration* serving for heat exchange (black) is observed in tandem with warning coloration (bright orange). Additionally, butterflies can have *bicolored wing surfaces* where the ventral side is one color and the dorsal side develops a different color. This bicolouration

allows them compromise between environmental or behavioral requirements and survival. Lastly, wing coloration and thermoregulation may be affected by elevation. In *Colias* butterflies, where there is a population living on an elevation gradient, butterfly wing melanization is observed to increase with elevation (Ellers & Boggs, 2003).

Butterflies also use wing colors as a *defense mechanism* against predators (*e.g.* cryptic coloration, aposematism, and mimicry). Cryptic coloration, also known as camouflage, is used by butterflies as a means to decrease predator visibility by "blending" into their background when perched. Aposematic individuals show warning coloration -bright colors that signal danger- that may be accompanied by distastefulness or toxicity (Merilaita & Tullberg, 2005). These warning colorations are commonly mimicked (*e.g.* Müllerian or Batesian) by closely related or sympatric species. Mimicry has been studied for more than a hundred years since it was first identified by Henry Walter Bates in 1862. The phenotypes that result from mimicry are highly complex due to all the possibilities in color allocation in a specific wing area or throughout the ventral/dorsal wing regions (Deshmukh et al., 2018). Commonly, natural selection by predators causes unrelated species to evolve into mimics in order to minimize predator attacks. This works by "teaching" predators to associate bright colors with unpalatability, hence increasing survival rates for both species (Deshmukh et al., 2018; Kronforst & Papa, 2015; Pinheiro et al., 2016). Other behaviors such as *visual communication*, *mate choice*, and *general flight behaviors* have significant impacts on wing color. As an example of the role of both visual communication and mate choice on wing pattern evolution, in the Pieridae family, *Eurema hecabe* females prefer males that present UV iridescence in the wings (Kemp, 2008). In *Pararge aegeria*, flight behavior is correlated with wing coloration, with paler males spending more time resting in shaded places relative to darker males -who spend more time flying during the day (Van Dyck et al., 1997).

The Nymphalid Ground Plan

Understanding homology of structures and color patterns serves as a gateway to understand gene homology. A classic example of structure homology is the similarities between limbs of humans, cats, whales, and bats. Like limb homology between vertebrates, homology of wing color patterns in butterflies has been extensively studied. In the 1920's, Süffert and Schwanwitsch, separately described the nymphalid wings as being composed of symmetry systems and pattern elements. Based on these discoveries, (Nijhout, 1978) developed a model that condenses all the patterning elements of the nymphalid butterfly wing into what is known as the Nymphalid 'ground plan' (Figure 2A). This model summarizes the wide variety of wing patterns into symmetry systems, which are mirror image patterns that repeat on the left and right wings of the butterflies. There are three main symmetry systems: (1) the basal symmetry system (B) –patterns at the root of the wings, (2) the central symmetry system (CSS) –delimitated by the elements M^1 and M^2, and (3) the border symmetry system (BoSS). A revised nymphalid ground plan now includes the Discalis I and II elements, the border ocelli, and the proximal and distal parafocal elements. Even though this model was developed based on nymphalid butterflies, it has been applied to other butterfly families including Pieridae and Papilionidae (Martin & Reed, 2014). In 1956, Schwanwitsch described in his work "Color-pattern in Lepidoptera" very specific details about what he understood to be the symmetry systems present in Hesperiidae butterflies. He suggested that members of the Hesperiidae family showed a displacement of the M^1 element in the forewings, some type of "whitening" or lighter coloration developing in the central symmetry system (M^1-M^2) of the hindwings and, lastly, identified additional ground plan elements in *Pyrgus sidae* (Figure 2C) that belong to the border symmetry system (Schwanwitsch, 1956).

Genes underlying butterfly wing patterning

The genetic architecture underlying butterfly wing patterns has been studied in the Nymphalidae family for decades. Table 1 summarizes target genes suggested to control most wing pattern elements in butterflies (Deshmukh et al., 2018; Hanly et al., 2019). *wingless* (*wg*) -a ligand in the Wnt signaling pathway- expresses where the basal, discal, and border wing elements are located (Martin & Reed, 2010; Macdonald et al., 2010), and *aristaless1* and *aristaless2*, have been suggested to serve as pattern precursors for *wg* in the discal elements, DI and DII (Martin & Reed, 2010). In *Heliconius* butterflies, three genes that control most of the wing patterns in many species have been identified: the transcription factor *optix*, the cell cycle regulator *cortex* and the Wnt ligand, *WntA*. The exact genomic regions of these three genes have been identified in *Heliconius erato* races (Van Belleghem et al., 2017). Patterning roles and expression of these three genes vary throughout butterfly species and some examples are presented in Table 2. Since *WntA* and other Wnt pathway components have proven to be such important genes in Nymphalidae butterfly wing pattern determination, characterizing the presence and function of these and other genes in the Hesperiidae family is the main focus of this study.

Study system: the silver-spotted skipper, *Epargyreus clarus*

The silver-spotted skipper, *Epargyreus clarus*, is a butterfly belonging to the Hesperiidae family, which until recently were thought not to be in the butterfly clade and considered moths. This family is 79 million years old according to recent estimates (Espeland et al., 2018) implicating that the family is twelve million years younger than the Nymphalidae family -the most studied butterfly family. Studies on the molecular basis of wing patterns in the Hesperiidae family are not as extensive as in the Nymphalidae family, nonetheless, work focused on the evolutionary origin of this family are available. In 2016, an article described a 55 million years old fossil as being a

member of the Hesperiidae family based on the forewing venation fragments found (De Jong, 2016). In addition, phylogenetic studies have shed light into understanding the skipper's relationship to other butterfly families (Espeland et al., 2018; Li et al., 2019). These skippers are recognized for having a single white spot on the ventral side of their hindwings, hence the name "silver-spotted" (Figure 3A-B). Moreover, they are well known for their shelter-making behavior (Figure 3C), and many studies have focused on their host plant preferences (Rosenwald et al., 2017; Weiss et al., 2003). They are widely distributed in North America (from southern Canada to the southern United States) and are one of the most common skippers in the United States, with the bulk of the population located along the East coast (Figure 3D). While most of the studies on *E. clarus* are focused on phylogenetics and understanding the shelter-building behavior, no recent work has tried to understand the molecular basis of wing pattern development, which was accomplished in this study.

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TABLES

Table 1 | Summary of important genes suggested to control wing pattern formation in Nymphalidae butterflies, and the functional annotation of each gene.

Genes	Function identified in butterfly wing pattern development
al1	Yellow/white switch in *Heliconius* butterflies
al2	Determines placement of discal stripe pattern
cortex	Coordinates yellow and white wing patterns in *Heliconius* butterflies
dll	Involved in specification of border ocelli and marginal patterns
hth	Candidate regulator of *optix* in *Heliconius* butterflies
Notch	Delimits dorsal/ventral (DV) boundary of wing disc
optix	Coordinates red wing patterns in *Heliconius* butterflies
Ubx	Determines hindwing fate
wg	Delimits dorsal/ventral (DV) boundary of wing disc, forms a cluster with *Wnt6* and *Wnt10*
Wnt6	Delimits dorsal/ventral (DV) boundary of wing disc, forms a cluster with wg and *Wnt10*
Wnt10	Delimits dorsal/ventral (DV) boundary of wing disc, forms a cluster with wg and *Wnt6*
WntA	Coordinates melanic boundaries and patterns

Table 2 | *Optix*, *cortex* and *WntA* patterning roles and expression in a variety of Nymphalidae butterflies.

Gene	Wing patterning role and/or expression pattern	Butterfly species	Reference
optix	Coordinates red pigmentation	*Heliconius erato* races	Van Belleghem et al., 2017
cortex	Coordinates yellow and white pigmentation	*Heliconius erato* races	Van Belleghem et al., 2017
WntA	Coordinates melanic boundaries and patterns	*Heliconius hecale melicerta*	Huber et al., 2015
		Heliconius hecale zuleika	Huber et al., 2015
		Heliconius morphs	Martin et al., 2012
	Expressed in basal, central and marginal wing patterns	*Euphydryas chalcedona*	Martin et al., 2014
	Delimits central symmetry system and marginal patterns	*Junonia coenia*	Martin et al., 2014
	Expressed in DII, CSS, external patterns, and forewing eyespots	*Vanessa cardui*	Martin et al., 2014
	Expressed in CSS	*Pararge aegeria*	Mazo-Vargas et al., 2017
	Expressed in CSS patterns and silver-spot related expression	*Agraulis vanillae*	Mazo-Vargas et al., 2017
	Intervenous expression	*Danaus plexippus*	Mazo-Vargas et al., 2017

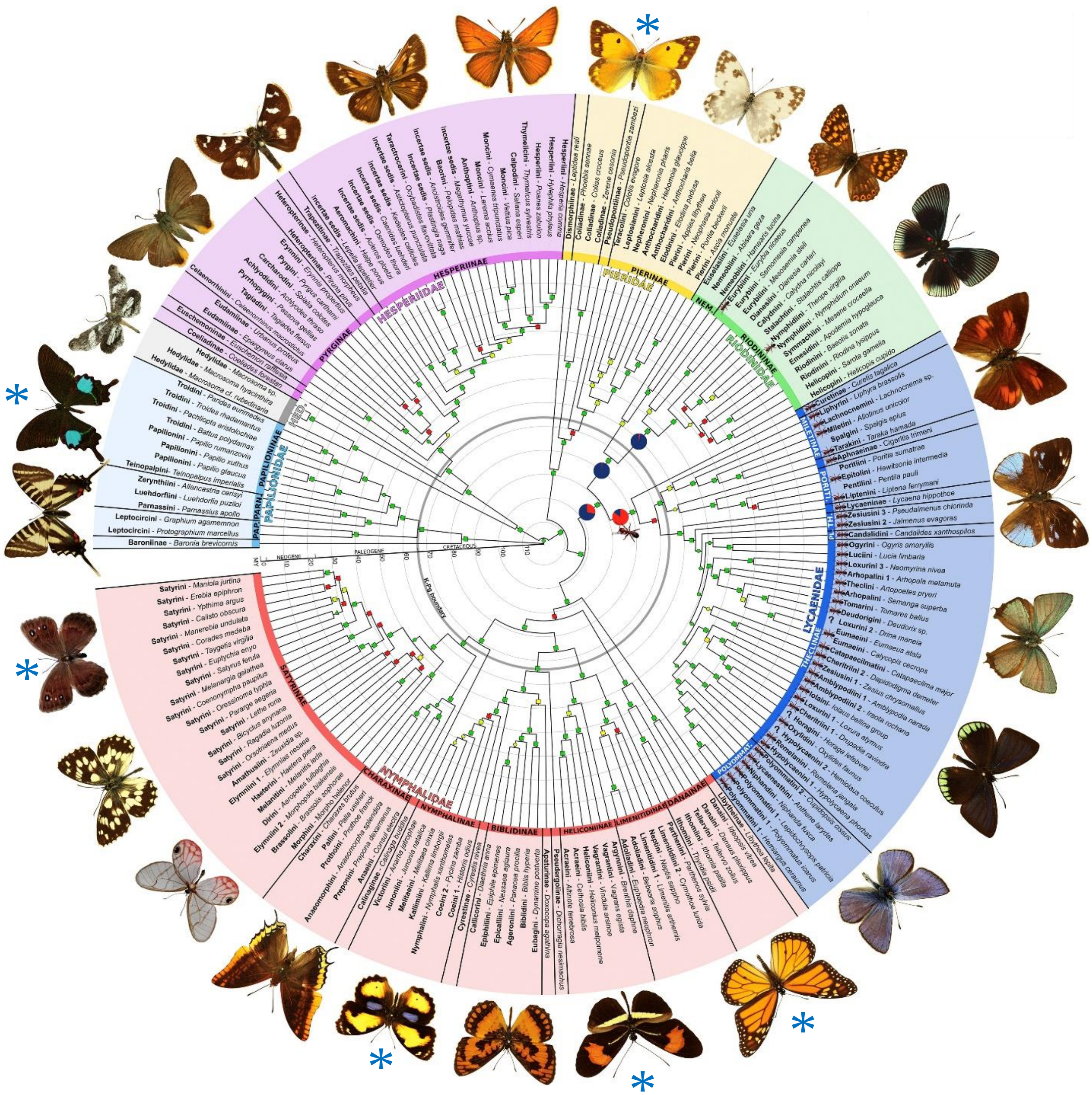
FIGURE LEGENDS

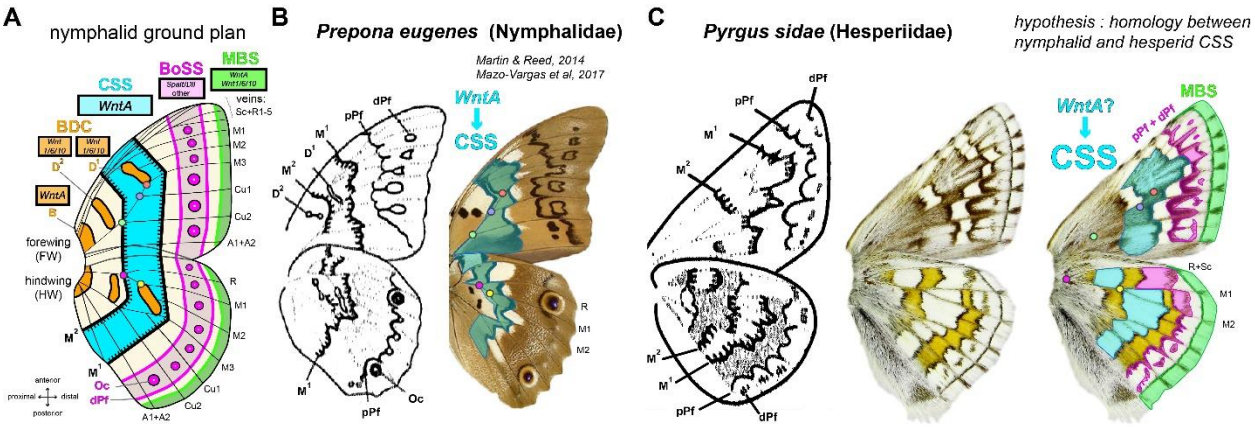
Figure 1. Dated phylogeny tracing the divergence of butterfly families, including Nymphalidae and Hesperiidae. This phylogeny was generated using maximum likelihood of a dataset containing 207 taxa and more than 150,000 base pairs. The approximate time of divergence between Nymphalidae (red) and Hesperiidae (purple) is indicated. Molecular studies on wing pattern development have been performed in some of the species/subfamilies presented here (blue asterisk). Figure reproduced from Espeland et al., 2018 with the permission of the publisher.

Figure 2. Extrapolation of the nymphalid ground plan to skipper butterflies (Hesperiidae). A-B. Summary of the revised nymphalid ground plan and its consecutive symmetry systems, or "stripes", juxtaposed along the proximo-distal axis. orange: Baso-Discal Complex (BDC) patterns; blue: Central Symmetry System (CSS); magenta: Border Ocelli Symmetry System (BoSS), including dPf; green: Marginal Band System (MBS). Dots show wing topological landmarks corresponding to vein crossings. Key patterning genes are shown for each symmetry system, with *WntA* known to induce the CSS in all nymphalids tested to date. Line drawing adapted from the predictions of Boris Schwanwitsch (Schwanwitsch, 1956) **C.** Positional homology of the nymphalid ground plan system applied to the hesperid *Pyrgus sidae,* as proposed by Schwanwitsch (1956). If his model is true, skippers have a CSS predicted to express *WntA* and developmentally homologous to the ones of nymphalids.

Figure 3 | Phenotypic characteristics, distribution, and shelter making of the silver-spotted skipper. The silver-spotted skipper is characterized for having dark brown wings that span approximately from 1.5 to 2.5 inches. In the middle of both the ventral **(A**) and dorsal (**B**) forewings, an orange stripe runs antero-posteriorly. The ventral hindwings possess a white or silver diamond-shaped spot. This silver spot is not present in the dorsal hindwing. **C.** An example of a shelter constructed by a 2nd instar caterpillar (yellow arrow). These shelters are made throughout the five caterpillar stages and vary in sizes depending on the stage. **D.** The silver-spotted skippers are distributed throughout North America, from southern Canada to southern United States (distribution map obtained from https://www.butterfliesandmoths.org/species/Epargyreus-clarus).

FIGURES




A
nymphalid ground plan
BoSS
MBS
CSS
WntA
veins:
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BDC
M1
M2
M3
Cu1
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A1+A2
forewing
(FW)
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(HW)
R
M1
M2
M3
Cu1
Cu2
A1+A2
anterior
proximal
distal
posterior
Oc
dPf
B
Prepona eugenes (Nymphalidae)
Martin & Reed, 2014
Mazo-Vargas et al, 2017
WntA
CSS
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C
Pyrgus sidae (Hesperiidae)
hypothesis : homology between
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CSS
MBS
pPf + dPf
R+Sc
M1
M2

A

B

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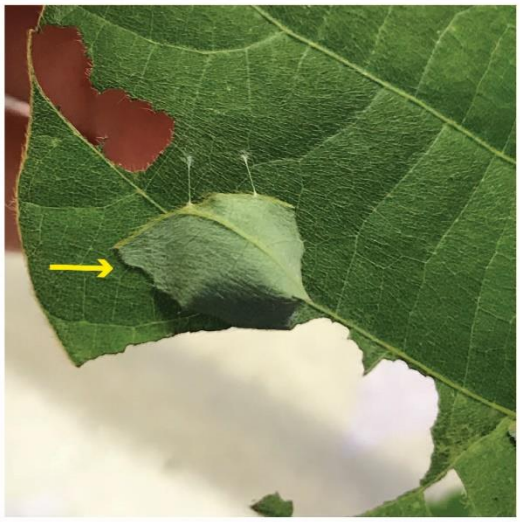
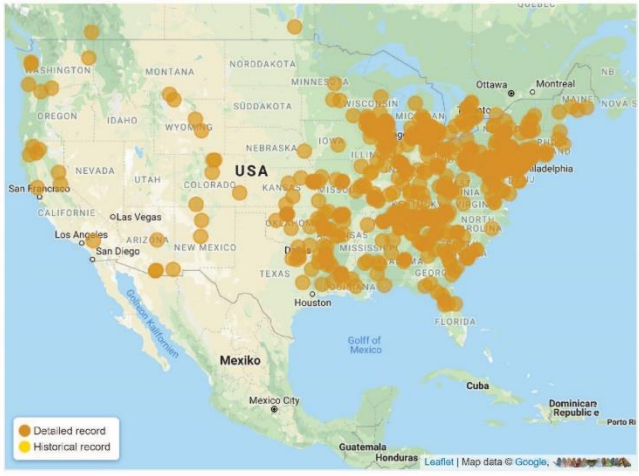


D


MONTANA
NORDDAKOTA
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Las Vegas
Los Angeles
San Diego
NEW MEXICO
TEXAS
Houston
Golf of Mexico
Mexiko
Mexico City
Cuba
Guatemala
Honduras
Ottawa
Montreal
Detailed record
Historical record
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QUESTIONS AND HYPOTHESES

Comparing wing patterning genes between two Lepidopteran families

There is extensive evidence for gene homology within species of the Nymphalidae family. Many species have been shown to share a simple yet modular set of genes that controls a wide variety of wing patterns. In his work, Schwanwitsch suggested that wings of Hesperiidae butterflies present elements and symmetry systems similar to those found in the nymphalid ground plan. We know that these two families share a physical wing map that coordinates and delimits patterns along the wings but ***are these phenotypic wing pattern boundaries genetically homologous between Nymphalidae and Hesperiidae?*** Based on the evidence of wing pattern convergence within nymphalid butterflies and Schwanwitsch's work, I predicted that spatial expression analyses and sequencing results would show convergence of wing patterning genes between these two families that diverged more than 80 Mya.

Testing the presence of *WntA* and other Wnt components in *E. clarus*

As discussed in the introduction, *WntA* and other Wnt signaling pathway components such as *wg*, *Wnt6* and *Wnt10*, have been implicated in controlling a wide variety of patterns in several Nymphalidae species (see introduction). *WntA*, is involved in boundary delimitation and melanic pigment control in many nymphalids. In this study, I aimed to determine whether *WntA* is also involved in boundary delimitation and melanic pigment control in the Hesperiidae family by examining its expression in the imaginal wing discs of fifth instar silver-spotted skippers. More specifically, I aimed to examine whether ***WntA or a Wnt pathway component were responsible for pattern boundary determination in the silver-spotted skipper.*** I predicted that genes controlling color boundaries and specific patterns in the wings of Nymphalidae butterflies, such as *WntA* and other Wnt genes, would also be present in the silver-spotted skipper. Particularly, I

predicted that such genes would be present in the silver-spotted skipper's hindwings where a silver spot is located in the middle of a brown wing, based on previous studies suggesting that some of the silver spots located on *Agraulis vanillae* butterflies are being controlled by *WntA*.

Identifying differentially expressed genes throughout development, between forewings and hindwings, and between wing compartments

Previous work done on differentially expressed genes shows a relationship between time in development and expression pattern in nymphalids. For example, in *Vanessa cardui*, the patterns suggested to be the presumptive eyespots in the adult butterfly begin to express later in the fifth instar stage. In *Heliconius* butterflies, genes such as *cortex* and *WntA* start expressing early in development while *optix* expression begins later in the pupal stage. I aimed to determine ***if patterning genes were differentially expressed during three developmental stages of the silver-spotted skipper.*** Since previous studies suggest that certain genes express earlier than others in development, I hypothesized that RNA sequencing results would indicate developmental stage-specific differential expression between fifth instar (D0) imaginal wing disks, 36 h (D1) pupae, and 48 h (D2) pupae. Moreover, I expected to observe some parallels between my work and previous gene expression work that could help identify the fundamental genes controlling a butterfly's wing development. In addition to developmental stage differential expression, I aimed to determine ***whether there was differential gene expression between forewings and hindwings of the three developmental stages as well as between wing compartments of pupal stages***. Previous studies have shown that specific genes tend to overexpress either in forewings or hindwings. For example, *Ultrabithorax* (*Ubx*) is overexpressed in hindwings of many butterfly species and the overexpression correlates to its function of determining hindwing fate. Additionally, it has been shown that genes may be differentially expressed between wing

compartments (*e.g.* proximal, medial, and distal sections of the wing). In this part of my work I predicted to observe similar expression patterns, including the overexpression of *Ubx* in the hindwings, and the differential expression of genes between the medial compartment relative to the proximal and distal compartments based on the presence of differently pigmented patterns in the medial compartment of both forewings and hindwings.

CHAPTER 1

CHARACTERIZING THE EXPRESSION OF WNT GENES IN THE SILVER-SPOTTED SKIPPER

INTRODUCTION

As previously mentioned, *WntA* and other Wnt pathway genes have been the focus of many studies aiming to determine the origin and molecular basis of butterfly wing patterns. These studies have attempted to pinpoint the specific time in development where these patterning genes begin to express. A commonly used technique to achieve this is *in-situ* hybridization (ISH). ISH can determine the spatial expression of mRNA in specific cells and tissue samples. In this method, a probe containing the complementary sequence of the mRNA target is designed. This technique has been applied to determine the spatial expression of many butterfly wing pattern genes during different developmental stages including but not limited to *WntA*, and other Wnt components, *cortex*, and *optix*. By using a nonradioactive digoxigenin (DIG) probe containing the complementary sequence for mRNA of previously identified butterfly wing pattern related genes, I identified the spatial expression of different gene targets in the fifth instar imaginal wing discs of the silver-spotted skipper.

Pharmacological drug injections of heparin and dextran sulfate have been used in model organisms such as mice (Fuerer et al., 2010) and *D. melanogaster* (Hufnagel et al., 2006) to test the functionality of specific molecules. Heparan sulfate proteoglycans (HSPGs) are glycoproteins present in the extracellular matrix (ECM) of cells. Heparin binds to molecules such as Wnt ligands in the ECM and improves these molecules' transportation and secretion between cells. Dextran sulfate, on the contrary, delays and/or inhibits their transport. A previous study tested the effects of these two drugs on multiple nymphalid butterflies (*e. g. E. chalcedona* and *J. coenia*). The results suggested that heparin injections caused Wnt gain-of-function effects and dextran sulfate injections caused Wnt^{+} patterns to contract in a dose dependent manner (Martin & Reed, 2014). Another study compared the effects of heparin injections with the results of *WntA* knockouts in

Agraulis vanillae (Mazo-Vargas et al., 2017). Heparin injections caused *WntA*$^{+}$ patterns to expand and *WntA*$^{-}$ patterns to contract. When CRISPR was performed and *WntA* was knocked out, the opposite was observed and *WntA*$^{+}$ patterns disappeared. The effects of heparin have also been tested on families other than Nymphalidae [*e.g.* Papilionidae, Erebidae and Crambidae (Soukarov, 2018)], indicating that even though phenotypic effects caused by heparin injections are mostly similar, there are some differences between families that serve as a gateway to understand the evolution of wing patterning mechanisms. Since these experiments have been tested and proven to work not only on Nymphalidae but in other butterfly and moth families, I performed heparin and dextran sulfate injections on the silver-spotted skipper to determine whether there were Wnt pathway related molecules controlling wing patterning in Hesperiidae, and if such molecules were present, identify to what extent their function was being conserved.

MATERIALS AND METHODS

Tissue collection

Epargyreus clarus caterpillars were collected from stocks maintained at the Weiss Lab in Georgetown University, Washington DC from April to June 2018 for *in-situ* hybridization assays and from June to July 2019 for pharmacological drug injections. First instar caterpillars were fed with young kudzu *Pueraria montana/lobata* leaves and second instar to fifth instar caterpillars were fed with older kudzu leaves. The age of the kudzu leaves correlates with the caterpillar stages, providing not only a nutrition source but also the space needed to create their leaf shelters. All individuals were reared inside plastic containers located in a growth chamber with the following settings:

Temperature – 32° C

Alternating photoperiods – 16 hours of "daylight"/ 8 hours of darkness

Caterpillars used for *in-situ* hybridization assays were reared until they reached the pre-pupal stage and were then dissected. In the pre-pupal stage, the body of the caterpillars changes from a light green color to pink. All the individuals obtained from June to July 2019 were reared until they reached the pupal stage at which point, they were injected with pharmacological drugs. After emerging, they were left in a cage for 2 to 4 hours before moving to a -20 °C freezer for storage and, later photography.

Tissue dissection and *in-situ* hybridizations

Following the 4-day protocol by Martin & Reed (2014), *Epargyreus clarus* fifth instar larvae were cold anaesthetized for 10 to 15 minutes and forewings and hindwings were dissected as a whole in PBS using fine forceps and dissecting scissors. Imaginal wing disks were transferred

to 1.5 mL tubes containing fixative (250 µL of formaldehyde 37% in 750 µL of PBS containing 50 mM EGTA) for 40 minutes on ice and then rinsed and washed four times in PBT (50 mL of PBS and 200 µL of Tween20 25%) for 15 minutes each. For long-term storage, samples were preserved by progressive dehydration by increasing concentration of MeOH in PBS (33%, 66% and 100% MeOH in PBS) and then storing at -20 °C. To use this tissue, progressive rehydration in 66%, 33% MeOH in PBS was performed with a final wash in PBS (each wash 2 to 5 minutes). For *in-situ* hybridization, wing disks were incubated 2 minutes at room temperature with 25 µg/mL proteinase K in PBT followed by a rinse and wash for 5 minutes in Stop Solution (10 mL glycine 10 g/L, 5 mL PBS 10x, 200 µL Tween20 25% and 38.4 mL of sterile dH_2O) and two 5 minute washes in PBT. The next step was peripodial membrane removal from the surface of the wing discs followed by a post-fixation in 150 µL of formaldehyde 37% and 850 µL of PBT for 20 minutes on ice. After post-fixation, four washes of 15 minutes in PBT and two additional washes of 5 minutes in 50:50 PBT:PreHyb (For 50 mL of PreHyb: 10 mL of sterile dH_2O, 25 mL of formamide, 12.5 mL of 20X SSC and 200 µL of Tween20 25%) were performed. Pre-incubation in PreHyb at room temperature for 10 minutes was followed by an hour incubation in PreHyb at 63 °C. The last step for day 1 was incubation in 100 µL of hybridization buffer (For 50 mL of Hybridization buffer: 5 mL sterile dH_2O, 5 mL glycine 10 g/L, 25 mL formamide, 12.5 mL 20x SSC, 200 µL of Tween20 25% and 500 µL of Salmon sperm DNA) and 1.0 µL of 151 ng/mL *WntA* probe, 0.3 µL of 90 ng/mL *Wnt7* or 0.5 µL of 65 ng/mL *Wnt10* for 18 to 30 hours at 63 °C.

After the hybridization step was completed, on day 2 of the protocol the samples were washed six times in prehybridization buffer for 5 to 30 minutes at 63 °C with the last wash left overnight. On day 3, the tissues were washed one more time in prehybridization buffer at 63 °C for 5 minutes and then transferred to room temperature. At room temperature, a 5-minute wash in

PreHyb:TBT 50:50 was followed by four washes of 5 minutes in TBT-BSA (For 50 mL: TBS 50 mL, 200 µL of Tween20 25% and 0.05g bovine serum albumin). For secondary detection of the probes, a 30-minute wash in TBT-BSA was followed by a 2 hours incubation at room temperature with a 1:3000 dilution of anti-digoxigenin alkaline phosphatase (Roche Applied Science). After the 2-hour incubation, three washes of 5 minutes and seven washes of 15 minutes in TBT-BSA on ice were completed, leaving the last wash overnight at 4 °C. On the last day, three 5-minute washes in freshly made Alkaline phosphatase buffer were followed by a 2 to 4 hours stain with BM Purple (Roche Applied Science) at room temperature and in the dark. To wash off the staining solution, the samples were washed two times with PBT 2 mM ethylene diamine tetraacetic acid (EDTA), mounted on slides containing 60% glycerol/PBT 2 mM EDTA and photographed with a digital camera on a stereomicroscope.

Determining time after pupation before performing pharmacological drug injections

When fifth instar larvae reached pre-pupal stage, they were moved from the plastic containers to individual plastic cups and assigned a number. The cups were placed in a dark box to avoid disrupting pupation and a clock was set behind them. Time-lapse photography using a Nikon digital camera was perform on 49 silver-spotted skippers to determine specific time after pupation (Figure 4). Determining time after pupation allowed to identify early pupae and this was important because the earlier the injections were performed, the higher the possibilities of observing phenotypic variation. Time after pupation was calculated by subtracting the time at the moment of the injections from the time when pupation occurred (obtained from looking at the time-lapse photography). This was achieved for 36 pupae since the remaining 13 were out of camera focus and pupation time could not be recorded. Table 3 summarizes other information recorded for each individual (*e. g.* weight before injection).

Heparin and dextran sulfate injections

Two protocols (Martin & Reed, 2014; Serfas & Carroll, 2005) were followed to perform pharmacological drug injections of heparin and dextran sulfate, with concentrations of 5 µg/µL and 2.5 µg/µL, respectively. Using glass capillary needles on a micro-injector, 36 pupae reared at 32 °C were injected with heparin or dextran sulfate 2 to 15 hours after pupation directly on the lower abdomen. Twenty individuals were injected with heparin and sixteen with dextran sulfate. Injected drug dosage varied from 7.5 µg to 25 µg and the details for each individual can be found in Table 3. Out of the 36 pupae injected, 34 emerged approximately 10 days after injection. Emerged individuals were stored at -20 °C and later photographed using a Nikon digital camera to visually determine wing pattern variation.

RESULTS

WntA and *Wnt10* are expressed in the wings of *Epargyreus clarus*

In this part of the study, I tested the mRNA spatial expression of three Wnt genes, *WntA, Wnt7,* and *Wnt10* using *in-situ* hybridization. Of these three genes only *WntA* and *Wnt10* were expressed in the fifth instar wing discs of the silver-spotted skipper. *WntA* was the most anticipated gene in this study since it has been identified in many nymphalid species and controls a variety of pattern boundaries (Mazo-Vargas et al., 2017). In the silver-spotted skipper, *WntA* was expressed in both forewings and hindwings of fifth instar caterpillars (Figure 5). In both wings, *WntA* was expressed in the central symmetry system. In the forewings, *WntA* expression marked the melanic contours of the adult orange spot [*e. g.* above Cu1, it marked the distal boundary of the orange spot and below Cu1, the proximal boundary of the orange spot (Figure 5B-D)]. *WntA* expression on the hindwings marked the presumptive location of the adult silver spots. The expression pattern in the hindwings was particularly easier to describe since both the fifth instar pattern and the adult silver spots share a diamond-like shape (Figure 5E-G).

Another gene identified in the larval stage of silver-spotted skippers was *Wnt10*. This gene has been previously described in the peripheral tissue and DI/DII forewing elements of *E. chalcedona,* and there is evidence suggesting that it forms some sort of complex with two other Wnt genes, *wingless* (*wg*) and *Wnt6* (Martin & Reed, 2014). In the silver-spotted skipper, *Wnt10* expression was observed in the peripheral tissue of both forewings and hindwings, but no discal expression was observed (Figure 5H-I).

Heparin injections resulted in Wnt-positive pattern expansion

In the ISH experiments, I was able to identify the expression of two Wnt genes, *WntA* and *Wnt10*. These two genes are ligands belonging to the Wnt signaling pathway, and for that, I decided

to manipulate the pathway using pharmacological injections of heparin and dextran sulfate. Of the 36 pupae injected, 34 emerged (individuals #12 and #18 in Table 3 did not emerge). After emerging, individuals injected with heparin (N = 18) resulted in a wide range of phenotypic variation on their wings (Figure 6). As the heparin dosage increased, the phenotypic variations relative to wild-types also increased, suggesting a dose-dependent effect. Individuals injected with 7.5 µg and 8.0 µg looked like wild-types, and the ones injected with 25 µg showed the highest phenotypic variation (Figure 6A). The variation was observed in both forewings and hindwings as well as on both sides of the wings. In the forewings, the orange spots began to disappear in a dose-dependent manner (Figure 6B, top). Heparin effects on the hindwings caused drastic phenotypic variation in the silver spots. As heparin dosage increased, the hindwing silver spots expanded in all directions (Figure 6B, bottom). These results indicate that heparin induces Wnt gain-of-function effects on *E. clarus* wing patterns and suggest that heparin is directly binding to *WntA*.

Dextran sulfate injections (N = 16) did not result in any pattern variation at all, contrary to what I expected (Serfas & Carroll, 2005). These results could be true negatives suggesting that Wnt ligands are not sensitive to dextran sulfate in Hesperiidae as they are in Nymphalidae butterflies. Alternatively, the results could be due to 1) the drug not being freshly made, 2) a drug concentration lower than needed or 3) technical errors while injecting the pupae.

DISCUSSION

In this chapter, my goals were to determine whether any molecules of the Wnt signaling pathway were present in *Epargyreus clarus* and if so, determine their function in wing patterning. I focused on this group of genes because they have been associated with wing pattern formation and boundary determination in many butterfly species of the Nymphalidae family, including but not limited to: *J. coenia*, *P. aegeria*, *V. cardui*, *D. plexippus*, *E. chalcedona*, *A. vanillae* and *Heliconius* butterflies (Martin et al., 2012; Martin & Reed, 2010, 2014; Mazo-Vargas et al., 2017; Van Belleghem et al., 2017).

For the *in-situ* hybridization experiments, I selected three Wnt genes implicated in butterfly wing patterning, *WntA*, *Wnt7* and *Wnt10*. As expected, *WntA* expressed in the fifth instar wing discs of the silver-spotted skipper. In the forewings, *WntA* marked the melanic boundary of the adult orange spot which is located in the CSS, a patterning role that has been described in *Heliconius* butterflies [*e. g. H. erato demophon* and *H. sara sara* (Mazo-Vargas et al., 2017)]. Even though *WntA* is broadly attributed the role of boundary formation and melanic pattern development, in *A. vanillae* it determines the location and shape of some of the silver spots (Martin & Reed, 2014). In the hindwings of the silver-spotted skipper, *WntA* determined the location of the silver spots. The *WntA* ISH results coincide with the predictions made by Schwanwitsch in 1956. There is dislocation or displacement of CSS pattern elements in the forewings and lighter coloration is developing in the CSS of Hesperiidae hindwings. In order to fully understand the patterning roles of *WntA* in the silver-spotted skipper, further experiments such as *WntA* knockouts using CRISPR need to be performed. As part of this project, I tried *WntA* knockouts in the silver-spotted skipper with no success. The eggshells resulted too tough and kept breaking the glass

needles. In the future, CRISPR injections could be performed with needles consisting of tougher materials such as quartz and by developing protocols to soften the eggshell prior to injections.

Of the remaining Wnt genes, I was able to observe *Wnt10* expression in the fifth instar wing discs, no *Wnt7* expression was identified. As with *WntA*, I expected to see *Wnt10* expressing in the wing discs, since its expression was described in the peripheral tissue of *E. chalcedona* wing discs (Martin & Reed, 2014). In line with these expectations *Wnt10* expression was indeed limited to the forewing and hindwing peripheral tissue in *E. clarus*.

Heparin injections performed on eighteen silver-spotted skipper pupae, helped confirm the patterning role of Wnt pathway components in both forewings and hindwings. Heparin is known to bind to Wnt ligands. Based on this, I inferred that heparin was binding to either *WntA* or to another Wnt component not identified here. Injecting heparin in increasing dosage caused the orange spots on the forewings to disappear. Heparin injections and ISH results suggest that the orange spot is disappearing because the melanic boundaries distal to the spot and anterior to vein Cu1 are expanding towards the proximal wing section and the melanic boundaries proximal to the spot and posterior to vein Cu1 are expanding towards the distal wing section of the forewings. In the hindwings, as in the forewings, the effects of heparin injections were dose-dependent. As heparin dosage increased, the hindwing silvers spots expanded in all directions. The effects of heparin observed in the silver-spotted skipper coincide with the Wnt gain-of-function results observed in other butterfly and moth families, confirming the conservation of wing patterning mechanisms between distantly related species. Dextran sulfate injections caused no phenotypic variation in the silver-spotted skipper relative to the wild-type individuals, suggesting that the Wnt signaling pathway is not sensitive to this drug in the Hesperiidae family.

It is important to continue generating data on both *in-situ* hybridization experiments and pharmacological drug injections to obtain a complete picture of the patterning roles of Wnt genes in the silver-spotted skipper. For ISH experiments, it would be best to generate probes for other known Wnt components such as *wg, Wnt6,* and *frizzled* genes to test whether they share functions with nymphalid butterflies. As for pharmacological drug injections, I would suggest repeating the dextran sulfate injections, to determine whether the negative results obtained here are true negatives or if they were caused by experimental error.

With the *in-situ* hybridization and heparin injection experiments, I was able to confirm two of my main hypotheses. First, I determined that there is a conserved set of genes that is controlling wing patterning in both Nymphalidae and Hesperiidae butterflies. As Schwanwitsch suggested in 1956, Hesperiidae butterflies develop wing pattern elements that are present in the nymphalid ground plan, specifically the CSS in both forewings and hindwings of the silver-spotted skipper. In brief, I confirmed that the phenotypic pattern boundaries observed in nymphalids and hesperids possess developmental homology.

Second, I aimed to identify whether *WntA* or other Wnt genes were involved in boundary delimitation of patterns in Hesperiidae as in Nymphalidae. Towards this goal, I identified two Wnt genes, *WntA* and *Wnt10*. Both genes were expressed on the fifth instar wing discs of the silver-spotted skipper and their roles were conserved; *WntA* determined pattern boundaries in both forewings and hindwings and *Wnt10* was expressed in the peripheral tissue of both wings. The area of the wing discs that expresses *Wnt10* is lost once caterpillars reach pupal stage and the adult wings start to develop. With the ISH and heparin injection results I was able to confirm both convergence of patterning genes and conservation of gene function between nymphalid butterflies and a representative of the Hesperiidae family, *E. clarus*.

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Table 3 | Heparin and dextran sulfate injection details including whether the injections caused phenotypic variations.

Individual #	Time AP	Weight (mg)	Drug injected	µg injected	PV	Individual #	Time AP	Weight (mg)	Drug injected	µg injected	PV
1	10.3	534	dextran sulfate	12.5	no	19	6.4	533	dextran sulfate	7.5	no
2	6.0	561	dextran sulfate	12.5	no	20	5.5	547	heparin	7.5	no
3	3.3	547	dextran sulfate	12.5	no	21	10.2	438	heparin	7.5	no
4	2.0	627	dextran sulfate	12.5	no	22	9.3	458	heparin	7.5	no
5	3.3	659	dextran sulfate	12.5	no	23	8.5	514	heparin	12.5	yes
6	5.6	560	dextran sulfate	12.5	no	24	12.4	594	heparin	12.5	yes
7	6.2	560	dextran sulfate	12.5	no	25	14.3	532	heparin	12.5	yes
8	5.5	598	dextran sulfate	12.5	no	26	6.2	424	heparin	10	yes
9	4.5	536	dextran sulfate	12.5	no	27	2.4	537	heparin	20	yes
10	13.2	636	dextran sulfate	8	no	28	7.5	647	heparin	20	yes
11	11.2	575	dextran sulfate	8	no	29	9.3	710	heparin	20	yes
12	10.1	561	heparin	8	no	30	15.3	491	heparin	7.5	no
13	15.1	545	dextran sulfate	20	no	31	5.3	647	heparin	7.5	no
14	9.2	586	dextran sulfate	20	no	32	10.3	504	heparin	12.5	yes
15	10.1	532	heparin	10	yes	33	9.0	529	heparin	12.5	yes
16	10.4	505	heparin	10	yes	34	3.2	408	heparin	10	yes
17	8.1	446	dextran sulfate	7.5	no	35	6.1	627	heparin	7.5	no
18	6.2	509	heparin	7.5	no	36	12.1	553	dextran sulfate	7.5	no

AP: after pupation
PV: phenotypic variation
Injections of 25 µg were conducted in a separate experiment and details are not included in the table.

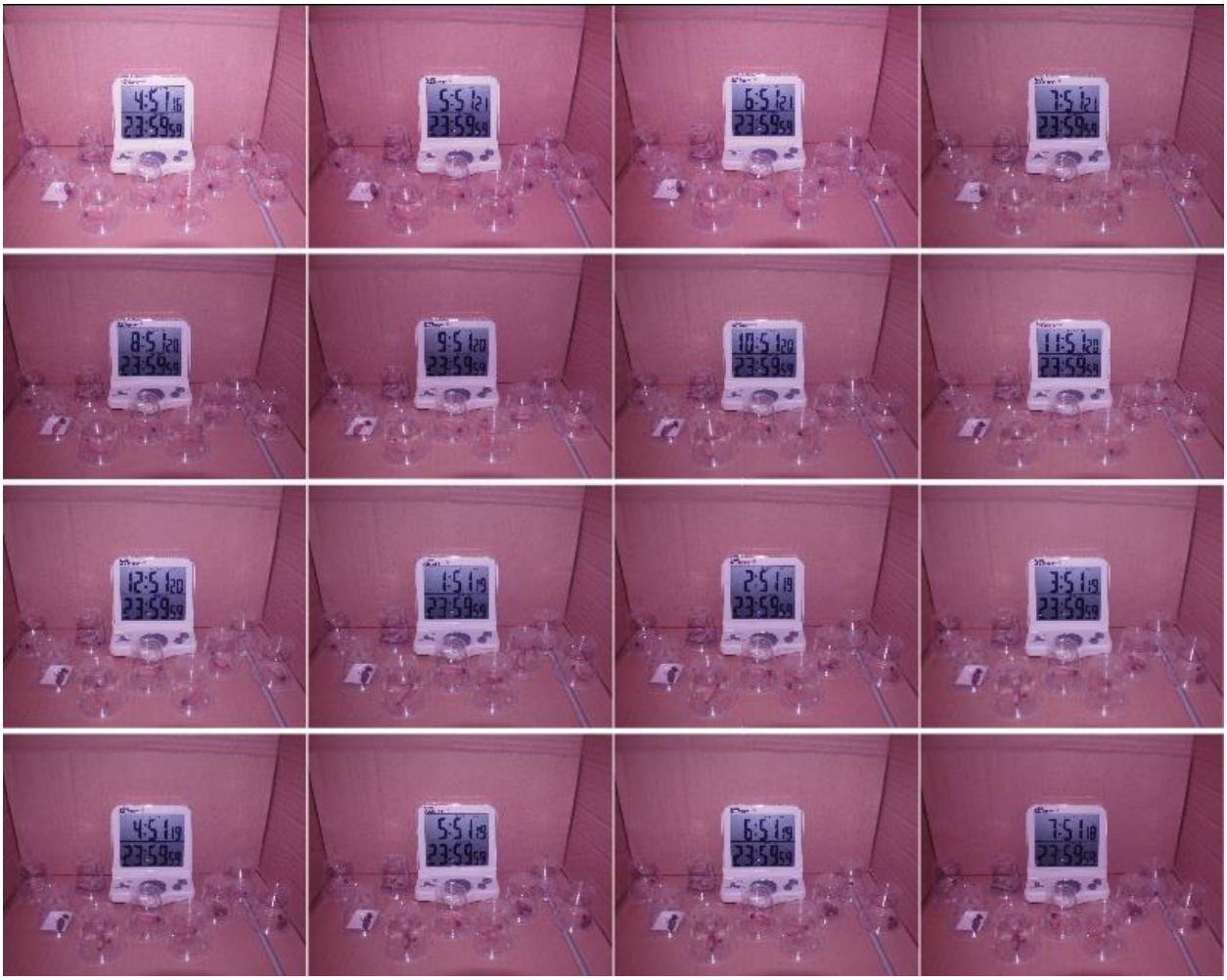
FIGURE LEGENDS

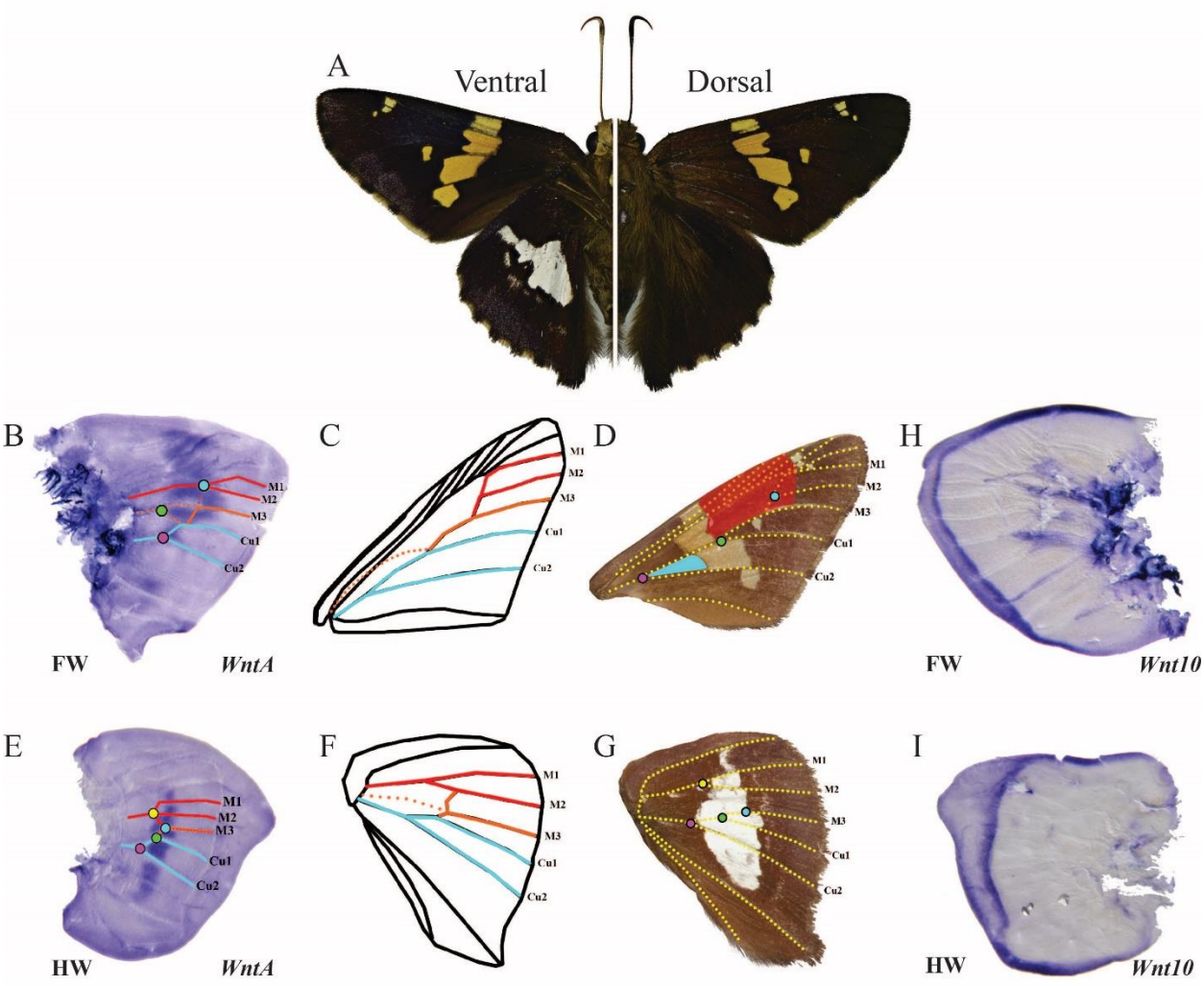
Figure 4 | Example of time-lapse photography setting used to determine time after pupation. Each of the twelve photos shows nine silver-spotted caterpillars that had reached the pre-pupal stage and were undergoing the pupation process. This particular session was started at 4:51 pm and ended at 7:51 am the next morning. The digital camera was set to take automatic pictures every five minutes, images here represent photos taken every hour.

Figure 5 | Expression of *WntA* and *Wnt10* in both forewings and hindwings of the silver-spotted skippers. A. Ventral and dorsal sides of an *Epargyreus clarus* adult. **B-D.** *WntA* expression in the forewings of the silver-spotted skipper. **B.** *WntA* is expressed in the central symmetry system of fifth instar forewing imaginal discs. Colored veins correlate to adult veins in (C), and colored dots correlate to adult vein intersections in (D). **C.** Silver-spotted skipper forewing vein anatomy. **D.** Adult forewing with veins (dashed yellow lines) and vein intersections (colored dots) identified. Red: section of distal melanic pattern that expands proximally in heparin injected individuals, cyan: proximal melanic pattern that expands distally after heparin injections. **E-G.** *WntA* hindwing expression in the silver-spotted skipper. **E.** In the hindwings, *WntA* also expresses in the central symmetry system where the adult silver spot develops. Colored veins correlate to adult veins in (F), and colored dots correlate to adult vein intersections in (G). **F.** Hindwing vein anatomy. **G.** Adult hindwing with veins and vein intersections identified as in (D). **H-I.** Expression of *Wnt10* in the peripheral tissue of both forewing and hindwing imaginal wing discs, respectively.

Figure 6 | Heparin induces Wnt gain-of-function effects. A. Heparin injections caused wing pattern diversity directly dependent on the dosage. Individuals injected with 7.5 µg and 8.0 µg resembled wild-types (not shown), and skippers injected with 10 µg, 12.5 µg, 20 µg, and 25 µg resulted in a gradient of phenotypic variation. **B.** Superimposition of the patterns obtained from heparin injections. (Top image) Forewing orange spot, blue: pattern of wild-type individuals, green: pattern of individuals injected with 10 µg, pink: pattern of individuals injected with 12.5 µg. Injections of 20 µg and 25 µg caused orange spots to disappear. (Bottom image) Hindwing silver spot, blue: pattern of wild-type individuals, purple: pattern of individuals injected with 20 µg, red: pattern of individuals injected with 25 µg. **C.** Magnification of patterns in (B).

FIGURES




A
Ventral
Dorsal
B
M1
M2
M3
Cu1
Cu2
FW
WntA
C
M1
M2
M3
Cu1
Cu2
D
M1
M2
M3
Cu1
Cu2
H
FW
Wnt10
E
M1
M2
M3
Cu1
Cu2
HW
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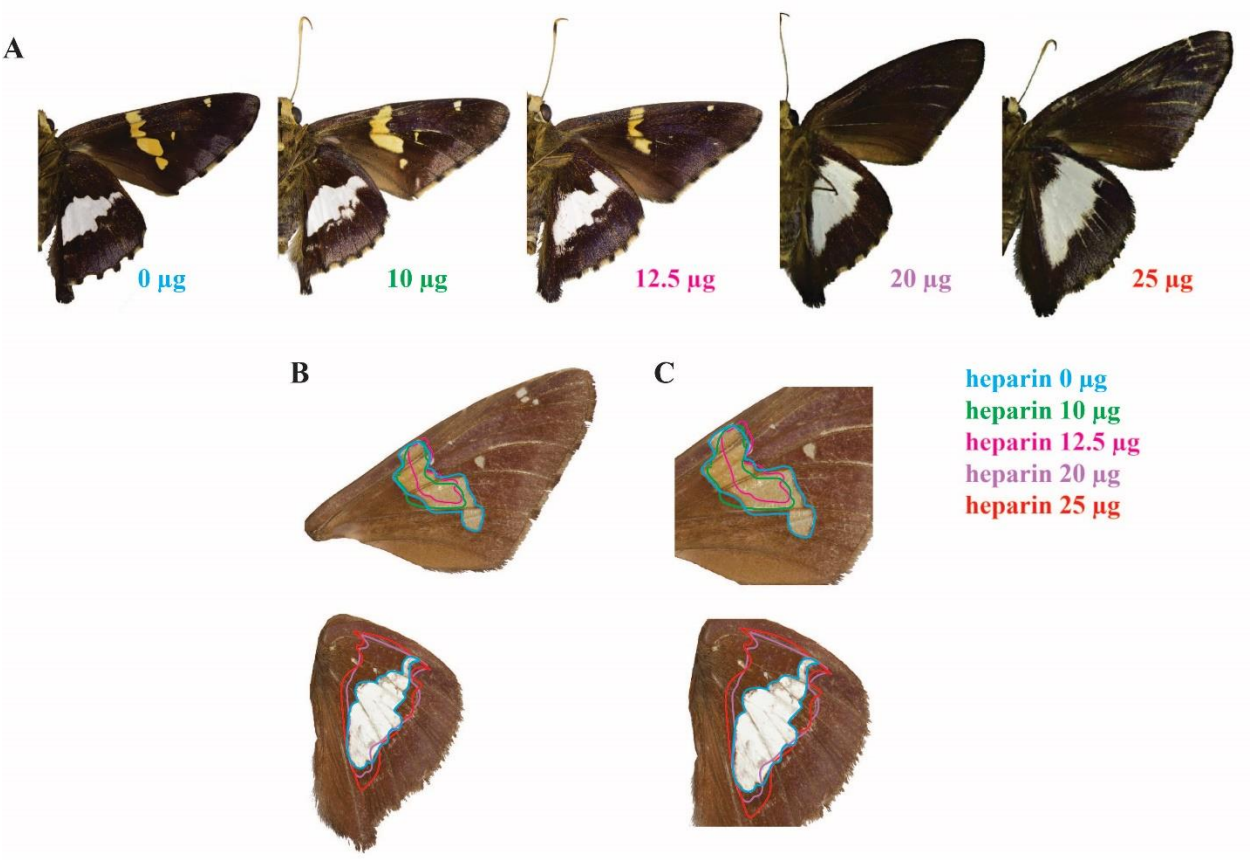
Cu1
Cu2
I
HW
Wnt10


A
0 µg
10 µg
12.5 µg
20 µg
25 µg
B
C
heparin 0 µg
heparin 10 µg
heparin 12.5 µg
heparin 20 µg
heparin 25 µg

CHAPTER 2

CHARACTERIZING DIFFERENTIALLY EXPRESSED GENES BETWEEN WING TYPES, BETWEEN WING COMPARTMENTS AND THROUGHOUT THREE DEVELOPMENTAL STAGES

INTRODUCTION

In the first chapter of my work, I was able to determine the presence, expression, and function of two Wnt genes, *WntA* and *Wnt10*, by performing *in-situ* hybridization experiments on fifth instar caterpillar wing discs and injecting heparin to pupae. According to literature, certain Wnt genes in nymphalids work as complexes to perform either the same function or complement each other (Martin & Reed, 2014). For this reason, I wanted to know what other Wnt genes, if any, were being expressed in the silver-spotted skipper. I also aimed to identify genes outside of the Wnt signaling pathway to better understand the transcriptomic landscape of *E. clarus*. In addition to describing a portion of the genes present in this skipper's genome, I wanted to characterize genes that were being differentially expressed not only throughout development but also between forewings and hindwings, and between wing compartments by performing RNA-sequencing.

RNA sequencing is a technique that uses next generation sequencing to determine whether genes are being expressed in specific tissues and the level of expression. This technique measures the quantity of mRNA and gives short sequences as output that can then be aligned to a reference genome to determine gene identity, location, and expression level. A comparative transcriptomic sequencing of two well-known *Heliconius* species was previously performed to determine the variations in the transcriptomic landscape that cause phenotypic variation in wing patterns (Hanly et al., 2019). With this project, I proposed to use RNA-sequencing to determine the general transcriptomic landscape of the silver-spotted skipper, as well as identify differentially expressed genes in three separate analyses. First, between wing type (*e. g.* forewing versus hindwing), second between wing compartments (*e. g.* proximal versus medial versus distal), and third during three developmental stages: (*e.g.* fifth instar larvae, 36 h pupae, and 48 h pupae). For the compartment

analyses, I compared forewing and hindwing compartments separately and did not include fifth instar wing discs because they were not dissected into compartments.

MATERIALS AND METHODS

Tissue collection and dissection

Epargyreus clarus caterpillars were collected for RNA extraction from stocks maintained at the Weiss Lab in Georgetown University, Washington DC from March to July 2018. All caterpillars were fed and reared inside plastic containers located in a growth chamber with the same settings as in the previous chapter. Once caterpillars reached fifth instar stage, they were divided into two groups: (1) caterpillars that would reach pre-pupal stage and be dissected, and (2) caterpillars that would reach pupal stage and be dissected 36 h or 48 h after pupating. Dissections were performed in cold PBS. Larval wing disks were dissected as a whole and stored in RNAlater (Thermo Fisher, Waltham, MA), separating forewings from hindwings in different 1.5 mL Eppendorf tubes. Pupal wings were carefully removed from the cuticle and the peripodial membrane was dissected to reveal the wing tissue. Then the wings were cut using microdissection scissors into six compartments: FW1 (proximal forewing), FW2 (medial forewing), FW3 (distal forewing), HW1 (proximal hindwing), HW2 (medial hindwing), and HW3 (distal hindwing) using the developing veins as guides (Figure 7), and then stored in RNAlater. All samples were stored at -80 °C for later RNA extractions. A total of three biological replicates were used for the analyses.

RNA extraction, library preparation, and sequencing

RNA extraction was performed using a Trizol/RNeasy protocol. Wing tissue was transferred into 2 mL tubes containing 500 µL of Trizol Reagent (Invitrogen, Carlsbad, CA), and homogenized using one stainless steel bead per tube and a tissue lyser. The homogenizing step was performed for two minutes at frequency 30. Homogenate was then stored at room temperature for five minutes and 200 µL of chloroform were added to each tube shaking vigorously for 15 seconds. After that, samples were left to rest for 3 minutes at room temperature and then

centrifuged at 13,000 rpm for 15 minutes at 4 °C. For chloroform phase extraction, the aqueous top phase was removed from the tubes and transferred to new tubes. Aqueous phase volume was measured and the same volume of 70 % EtOH (ethanol) was added drop by drop to avoid localized precipitation. The purification step was performed using the RNeasy kit (Qiagen, Valencia, CA). Up to 700 µL of each sample was loaded into a RNeasy column seated in a collection tube and then centrifuged 30 seconds at 10,000 rpm and the flow-through was discarded. Next, 700 µL of buffer RW1 were added into each column and centrifuged for 30 seconds at 10,000 rpm. Columns were transferred into new 2 mL collection tubes and 500 µL of buffer RPE were added, samples were then centrifuged once again for 30 seconds at 10,000 rpm. After discarding flow-through, another 500 µL of buffer RPE were added and the samples were centrifuged for 2 minutes at 10,000 rpm. Columns were transferred a third time to a new collection tube and centrifuged for 2 minutes at 12,000 rpm, next the columns were transferred to a new 1.5 mL collection tube and 30 µL of RNase-free water were pipetted directly into the column membrane. Samples stayed at room temperature for 2 minutes and were centrifuged one last time for 1 minute at 10,000 rpm to allow the RNA to dilute. RNA was then treated with DNAse I (Ambion, Naugatuck, CT). For 30 µL of sample the following mixture was made and added to the column: 3.53 µL of 10x buffer and 1.76 µL of DNAse I. Samples were incubated at 37 °C for 10 minutes and 5 µL of inactivation reagent (DRR) was added and mixed by gentle flickering. After adding the DRR, the samples were left at room temperature for 2 minutes, centrifuged 1 minute at 10,000 rpm and the supernatant transferred to a new 1.5 mL tube. Samples were then quantified and stored at -80 °C.

cDNA synthesis, library preparation, and sequencing were performed by the Sequencing and Genomics Facility (University of Puerto Rico Rio Piedras, San Juan, Puerto Rico). Samples were sequenced at either 75 SE or 150 PE with Illumina NextSeq.

Verifying read quality and removing Illumina adapters

To verify the quality of the fastq files containing the RNA-seq raw reads, FastQC was performed on all 40 samples. This tool provides multiple statistics and reports including *per base sequence quality* and *per sequence GC content* which allows to determine if the reads are damaged or contaminated. All 40 sequenced samples resulted in quality scores higher than 30, disregarding the need to perform extreme trimming. Nonetheless, Illumina adapters needed to be removed and for this Trimmomatic (Bolger et al., 2014) version 0.39 was used. For PE samples basic command was performed with slight changes: SLIDINGWINDOW:4:20 and MINLEN:85. As for the SE samples, the default parameters were used.

Generating a *de novo* transcriptome for the silver-spotted skipper

All sequences obtained for *E. clarus* were assembled using Trinity (Haas et al., 2013) transcriptome assembler and a total of 529,323 contigs were generated. Using TransDecoder and the Trinity output, a GFF3 annotation was generated which contained 357,804 genes. The average gene count in butterflies spans from 16,000 to 20,000 genes, the extremely high number of genes obtained here might be explained by (1) the presence of high haplotype variation, (2) the possibility of genes splitting into multiple annotations or (3) environmental contamination (e.g. bacterial contamination).

Aligning sequenced reads to the *Epargyreus clarus* reference genome

Before this study, no genome had been generated for the silver-spotted skipper. For this reason, we generated a reference genome which contains 426,663,605 bases and has a N50 of 3.7 Mb. All 40 reads were aligned to the reference genome using Hisat2 (Kim et al., 2019). Hisat2 indexes were generated from the reference genome with the *hisat2-build* option. These indexes were then used to align reads with the *hisat*2 option. The highest alignment percentages for unique

mapping were achieved using default parameters. Since output files from Hisat2 were in SAM format, the *samtools view* option was used to transform them into BAM format. These files were then sorted with *samtools sort* and indexed using *samtools index*.

Identifying differentially expressed genes

Determining how many counts aligned to each gene was achieved with htseq-count (Anders et al., 2015) *union* mode and parameters *–format = bam*, *--type = gene* and *–idattr = ID*. Counts were analyzed with the R package DESeq2 (Love et al., 2014) using three different design formulas, *~ wing-type* for forewing vs hindwing analysis, *~ compartments* for forewing and hindwing compartment analyses, and *~ day* for developmental stages analysis. Since larval wing discs were dissected as a whole, they were not included in the compartment analyses just in the wing type and developmental stage comparisons.

RESULTS

Genes identified in the silver-spotted skipper

One aim of this study was to identify genes controlling wing patterns in the silver-spotted skipper, specifically genes belonging to the Wnt signaling pathway. For this, I selected Wnt genes that had been described in Nymphalidae and recovered a total of eight including *WntA*. I used NCBI's BLAST option for nucleotides and searched the resulting coordinates on the skipper genome using IGV. After identifying the different Wnt genes, I determined whether they were expressed in my samples. (Table 4). In addition to Wnt genes, other genes were identified in the silver-spotted skipper's genome (Table 5), including *Ultrabithorax* (*Ubx*), *homothorax* (*hth*) and *distal-less* (*dll*). All genes identified, were tested for differential expression (DE) in three separate analyses: forewing versus hindwing DE, between wing compartments DE, and between developmental stages DE. For analysis purposes only, each developmental stage was assigned a day as follows: fifth instar (D0), 36 h (D1) and 48 h (D2).

Ubx is overexpressed in the hindwings of *E. clarus*

The first differential expression analysis performed was between forewing and hindwing samples. Of the samples sequenced, half corresponded to forewings (N = 20) and the other half to hindwings (N = 20). To confirm the analysis' efficiency, the first gene characterized was *Ubx*. In nymphalids, this gene determines hindwing fate (*e. g.* which wing will become a hindwing) and therefore is highly expressed in hindwings relative to forewings. I observed the same expression pattern in the sliver-spotted skipper. *Ubx* was overexpressed in the hindwing samples of fifth instar caterpillars, with an average read count of 1,189. (Figure 8). In addition to *Ubx*, other genes were differentially expressed in either forewings or hindwings. Two Wnt genes were overexpressed in forewings: *WntA*, and *fz1* (Figure 9). *WntA* expression was enriched in fifth instar forewings

relative to 36 h and 48 h forewings (Figure 9A). *fz1* was also differentially expressed in forewings, the overexpressed samples correspond to a fifth instar and a 48 h pupa (Figure 9B). *Homothorax* (*hth*) was another gene differentially expressed in the forewings, overexpress samples correspond to forewings of 36 and 48 h pupae (Figure 10A). The last gene identified to be overexpressed in forewings was *bab2* showing higher expression in 48 h individuals (Figure 10B).

In this analysis, two genes other than *Ubx* were also overexpressed in the silver-spotted skipper's hindwings. *Distal-less* (*dll*), showed higher expression on 48 h hindwings (Figure 11A). On the other end, *wg* showed higher expression on fifth instar hindwings relative to pupal hindwings (Figure 11B). The remaining eight genes were not differentially expressed neither on forewings nor hindwings of the silver-spotted skipper. A summary of all genes identified and their respective read count for all samples is included in Table 6. A total of 73,693 genes with read counts higher than 10 were identified, of those, 1,682 had an adjusted p-value < 0.05 (Figure 12).

Differential expression in the proximal, medial, and distal wing compartments

For forewing and hindwing compartment DE analysis, I only considered samples of 36 h and 48 h pupae since fifth instar wing discs were dissected as a whole and not by wing compartments. In previous studies, specific genes have been described to be differentially expressed in the proximal (FW1), medial (FW2), and distal (FW3) compartments of butterfly wings. Of these genes, *hth* and *Hr38* tend to be overexpressed in the proximal and medial compartments of *Heliconius* butterflies, respectively (Hanly et al., 2019). *Dll* has been identified as differentially expressing in distal wing compartments (Reed & Gilbert, 2004). With the following analyses, I tested differential expression of these and other genes in forewing and hindwing compartments separately.

Forewing compartment differential expression analysis revealed some expected and some interesting results. In the silver-spotted skipper, *hth* expression was enriched in the proximal compartment (FW1) of 36 h pupae as expected, but showed a different expression pattern in 48 h individuals with overexpression on the proximal and distal compartments relative to lower expression in the medial compartment (Figure 13A). *WntA* was also overexpressed but in the proximal compartment of day 2 pupae (Figure 13B). Contrary to the medial compartment overexpression expected, *Hr38* was differentially expressed in the proximal compartment of a 48 h pupae, this could be due to the way wings were dissected [*e.g.* small sections of the medial forewings were distributed between FW1 and FW3 compartments in order to just include the medial orange spot (Figure 14A)]. Another gene differentially expressed in the proximal compartment was *optix*, which was also overexpressed in 48 h samples (Figure 14B). Other genes showed higher expression levels in the distal or FW3 compartment. In addition to being overexpressed in the distal compartment of butterfly wings, in some cases *dll* expresses in a gradient throughout the wing. This gradient expression pattern was observed in the silver-spotted skipper, specifically in 48 h pupae (Figure 15A). The gradient expression was also observed for *bab2* and it was also seen in 48 h pupae (Figure 15B).

Besides differential expression in either FW1 or FW3 compartments, several genes resulted in lower expression in the medial or FW2 compartment relative to the expression levels of FW1 and FW3. Figure 16 shows two examples of genes with this expression pattern (*e. g. wg* and Wnt6) in addition, *Wnt11, fz1*, and *ci* also showed this expression pattern (not presented here). Of the five genes, four are members of the Wnt signaling pathway: *wg, Wnt6, Wnt11,* and *fz1*. This is particularly important because as previously stated, some Wnt genes are involved in medial forewing spot development and in the silver-spotted skipper there is an under expression of such

genes. In this analysis, a total of 56,360 genes with read counts higher than 10 were identified and 14 had an adjusted p-value < 0.05 (Figure 17).

For hindwing compartment differential expression analysis, only 36 h and 48 h tissue were used. Similar to what occurred in forewing compartment DE analysis, *hth* and *Hr38* were overexpressed in the proximal hindwing compartments of day 1 and day 2 pupae, respectively (Figure 18). In addition to these two genes, *optix* and *inv* were also differentially expressed in HW1 compartments. *Dll* showed the gradient expression seen in forewing compartment analyses but the differences between hindwing compartment expression levels were less drastic (Figure 19). The rest of the genes analyzed in this part did not show significant enough differential expression to be considered. In this analysis, a total of 37,999 genes with read counts higher than 10 were identified (Figure 20).

Genes tend to overexpress in fifth instar wing discs

The last differential expression analysis performed was between the three developmental stages: fifth instar, 36 h pupa and 48 h pupa. First, I tested the differential expression of all Wnt genes identified in this study. Of the eight Wnt genes described above, four were overexpressed in the wings of fifth instar caterpillars. *WntA* and *Wnt10*, the two genes I identified in the *in-situ* hybridization experiments, were over expressed in all the wing samples corresponding to fifth instar larvae (Figure 21). *wg*, and *Wnt11* showed the same expression pattern, with overexpression in both forewings and hindwings of fifth instar caterpillars (Figure 22). In addition to Wnt genes, I identified other genes overexpressed in the fifth instar wings discs. *Ubx* was overexpressed in fifth instar caterpillars, specifically in the hindwings, as expected (Figure 23A), and *ci* was overexpressed in both forewings and hindwings of fifth instar caterpillars (Figure 23B).

In addition to genes that were only overexpressed in fifth instar caterpillars, there were genes that had high expression levels in both fifth instar and 48 h stages but had low expression in the 36 h stage. These genes were *fz1*, a member of the Wnt signaling pathway, and *Hr38* (Figure 24). In the developmental stage differential expression analysis, a total of 73,653 genes were identified that contained ten or more read counts (Figure 25).

DISCUSSION

In this chapter, I was able to identify and describe the differential expression of a total of sixteen genes in the silver-spotted skipper transcriptome. In addition to identifying Wnt genes and Wnt constituents which was the focus of this chapter, I also was able to describe other genes such as *Ubx*, *hth*, and *dll*, which have been identified before in Nymphalidae butterflies.

In the forewing versus hindwing differential expression analysis, my goal was to determine whether genes such as *Ubx* were expressing in the same manner as in nymphalid butterflies. As expected, *Ubx* was drastically enriched in the hindwings of fifth instar caterpillars. This result is extremely relevant when considering the possible conservation of wing patterning genes between butterfly families since *Ubx* is involved in hindwing fate of Nymphalidae butterflies. *wg* and *dll* were also overexpressed in the hindwings of *E. clarus*. Genes like *WntA*, *fz1*, *hth*, and *bab2* were overexpressed in the forewings. Since the presence of these genes have been confirmed, performing *in-situ* hybridization experiments to identify their spatial expression in the silver-spotted skippers may provide a better understanding of their function in wing color pattern and shape development.

The second differential expression analysis was performed to determine whether genes known to differentially expressed in the proximal (FW1), medial (FW2), and distal (FW3) wing compartments of Nymphalidae butterflies, were present in the silver-spotted skipper and conserved their expression patterns. In the forewing compartment analysis three expression patterns were observed: 1) overexpression in the proximal forewing compartment (*e. g. hth*, *WntA*, *Hr38*, and *optix*), 2) gradient-like expression with lower expression in the proximal forewing compartments and higher expression in the distal compartments (*e. g. dll* and *bab2*), and 3) overexpression in both proximal and distal forewing compartments relative to the medial compartments (*e. g. wg* and

Wnt6). Some of this expression patterns are interesting like the under expression of *hth* (in D2), *wg*, and *Wnt6* in the medial forewing compartments because that is where the adult orange spot develops in the silver-spotted skipper. *Hr38* showed a different expression pattern when it was overexpressed in FW1 compartments instead of FW2 like anticipated.

In the hindwing compartment differential expression analysis, I was able to detect differential expression in only a few of the selected genes. As in the forewing DE analysis, *hth* and *Hr38* were overexpressed in the proximal hindwing compartments, while *dll* showed a slight gradient pattern. In previous studies, genes like *ci* and *inv*, have been described to overexpress in the anterior and posterior compartments of hindwings, respectively (Hanly et al., 2019). In the silver-spotted skipper this was difficult to identify because of the pupal cutting scheme I used – instead of cutting the hindwings into two half and obtaining anterior and posterior compartments, I cut the pupal wings to obtain a middle compartment where the silver spot develops. In future studies, cutting hindwings in half will provide a better understanding on whether *ci* and *inv* share expression homology with nymphalids. Even though this was a hindwing differential expression analysis, *Ubx* was not identified because all 36 h and 48 h samples had read counts lower than 10 for *Ubx*, and therefore not considered.

The last analysis was the developmental stage differential expression. In general, genes tended to overexpress in forewings and hindwings of fifth instar larvae. This is expected because it is known that genes can start to express and develop in patterns as early as the fifth instar stage, specifically some Wnt genes that develop early like *WntA*. Other Wnt genes were overexpressed in the fifth instar stage as well (*e. g. wg*, *Wnt10*, and *Wnt11*). In addition to Wnt genes, *Ubx* and *ci* were overexpressed in fifth instar caterpillars relative to 36 h and 48 h pupae. Like in *Heliconius* butterflies, *optix* expression skyrocketed later in development in the 48 h pupal samples. All results

obtained from the differential expression analyses of Wnt genes and others suggest, that not only there is gene homology between Nymphalidae and Hesperiidae but also expression patterns of key genes such as *Ubx, hth* and *dll* are being shared between these two distantly related families.

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TABLES

Table 4 | Wnt signaling pathway genes and components identified in the silver-spotted skipper regardless differential expression.

	Developmental stages and wing compartments													
	Fifht Instar		36 hour pupa						48 hour pupa					
Wnt gene	**FW**	**HW**	**FW1**	**FW2**	**FW3**	**HW1**	**HW2**	**HW3**	**FW1**	**FW2**	**FW3**	**HW1**	**HW2**	**HW3**
WntA	x	x							x	x	x			
Wnt4	x	x							x					
Wnt6			x		x			x	x		x	x		x
Wnt10	x	x												
Wnt11	x	x	x	x	x	x	x	x	x	x	x	x	x	x
fz1	x								x		x			
fz2	x	x	x	x	x	x	x	x	x	x	x	x	x	x
wg	x	x							x	x	x			

An x was assigned to tissue samples with read counts than 10.

Table 5 | Summary of genes identified in the silver-spotted skipper's genome, the coordinates, and organisms matched on NCBI.

Gene	Location in *E. clarus* genome	Gene ID *E. clarus* Genome	Organism matched	Percent Identity (%)
WntA	Sc0000003:2,868,509-2,876,661	Gene.264598::TRINITY_DN61072_c0_g2::g.264598::m.264598.path1	*Heliconius erato*	83
wg	Sc0000038:314,630-316,776	Gene.32547::TRINITY_DN57439_c0_g1::g.32547::m.32547.path1	*Epargyreus clarus*	99
Wnt4	Sc0000003:2,880,992-2,902,056	Gene.264595::TRINITY_DN61072_c0_g2::g.264595::m.264595.path1	*Danaus plexippus plexippus*	79
Wnt6	Sc0000038:261,768-261,821	Gene.231410::TRINITY_DN48494_c1_g2::g.231410::m.231410.path1	*Bicyclus anynana*	81
Wnt10	Sc0000038:215110-214353	Gene.306070::TRINITY_DN57944_c1_g1::g.306070::m.306070.path5	*Bicyclus anynana*	80
Wnt11	Sc0000036:1635569-1642909	Gene.30365::TRINITY_DN60947_c2_g1::g.30365::m.30365.path1	*Vanessa tameamea*	81
fz1	Sc0000042:3154328-3154850	Gene.242589::TRINITY_DN52426_c1_g1::g.242589::m.242589.path5	*Danaus plexippus plexippus*	81
fz2	Sc0000044:620550-621694	Gene.149963::TRINITY_DN43493_c1_g2::g.149963::m.149963.path1	*Vanessa tameamea*	90
hth	Sc0000083:1445144-1444850	Gene.248917::TRINITY_DN58465_c2_g1::g.248917::m.248917.path1	*Vanessa tameamea*	94
dll	Sc0000051:2757652-2757795	Gene.271115::TRINITY_DN52689_c1_g1::g.271115::m.271115.path5	*Heliconius melpomene rosina*	88
ci	Sc0000075:681991-682166	Gene.58960::TRINITY_DN48687_c3_g2::g.58960::m.58960.path5	*Heliconius melpomene rosina*	84
inv	Sc0000051:707945-707841	Gene.169799::TRINITY_DN60618_c2_g1::g.169799::m.169799.path2	*Precis coenia*	84
bab2	Sc0000008:359382-360590	Gene.306076::TRINITY_DN32816_c0_g1::g.306076::m.306076.path1	*Papilio xuthus*	77
optix	Sc0000080:498857-499315	Gene.111935::TRINITY_DN48770_c2_g3::g.111935::m.111935.path1	*Heliconius melpomene rosina*	83
Hr38	Sc0000042:535218-534190	Gene.89723::TRINITY_DN48593_c2_g6::g.89723::m.89723.path3	*Agrotis ipsilon*	82
Ubx	Sc0000022:830,846-831,149	Gene.306176::TRINITY_DN41481_c0_g1::g.306176::m.306176.path1	*Junonia coenia*	90

hth=homothorax, dll=distal-less, ci=cubitus interruptus, inv=invected, Ubx=Ultrabithorax

Table 6 | Genes identified in the silver-spotted skipper genome and the read counts corresponding to each sample tested in all differential expression analyses.

Sample ID	*WntA*	*wg*	*Wnt4*	*Wnt6*	*Wnt10*	*Wnt11*	*fz1*	*fz2*	*hth*	*dll*	*ci*	*inv*	*bab2*	*optix*	*Hr38*	*Ubx*
FW0_Ec5TH_O0	279.62	449.66	42.21	0.50	16.54	8303.46	0.50	247.54	6.92	481.74	3356.33	744.81	112.79	1216.43	6728.21	6.92
FW0_Ec5TH_Q0	232.43	793.15	74.41	3.05	18.34	8576.94	23.44	612.19	3.05	428.68	2149.07	245.18	140.68	619.84	6400.33	3.05
FW0_Ec5TH_S0	182.89	969.68	114.94	0.50	57.72	8530.01	0.50	683.58	7.65	376.01	3215.61	819.48	132.82	937.49	5179.00	7.65
HW0_Ec5TH_P0	181.20	860.29	41.30	0.50	50.05	8446.89	3.41	271.55	3.41	589.24	2562.40	656.28	233.66	373.56	6182.28	1204.21
HW0_Ec5TH_R0	180.94	1323.73	53.96	0.50	57.31	8711.78	7.18	1012.97	3.84	605.31	2900.92	234.40	200.99	267.82	6827.18	1256.90
HW0_Ec5TH_T0	154.39	1235.87	34.70	0.50	56.07	7361.43	0.50	436.51	9.05	530.56	3274.88	731.46	274.08	804.13	4634.21	1107.63
FW1_Ec36H_F2	0.50	0.50	0.50	8.48	0.50	919.41	0.50	434.02	88.27	1031.11	0.50	1.16	0.50	131.49	912.10	0.50
FW1_Ec36H_I2	0.50	0.50	0.50	14.15	0.50	1026.63	0.50	649.17	83.59	840.28	0.50	1.09	0.50	49.17	646.80	0.50
FW1_Ec36H_L2	0.50	1.51	0.50	10.60	0.50	960.74	1.51	690.14	116.62	749.71	0.50	2.52	0.50	74.21	723.46	0.50
FW2_Ec36H_G2	0.50	0.50	0.50	4.58	0.50	771.07	0.50	248.81	12.74	984.41	2.83	0.50	0.50	109.50	1264.19	0.50
FW2_Ec36H_J2	0.83	0.83	0.50	5.72	0.50	1052.23	0.50	322.35	3.44	909.26	0.50	1.81	0.50	48.16	613.19	0.50
FW2_Ec36H_M2	0.50	0.50	0.50	9.37	0.50	793.93	0.50	346.52	4.30	811.67	0.50	0.50	0.50	57.54	676.06	0.50
FW3_Ec36H_H2	0.50	1.34	0.50	17.39	0.50	925.82	0.50	262.22	4.72	1106.49	0.50	2.19	0.50	122.92	854.90	0.50
FW3_Ec36H_K2	0.50	0.50	0.50	18.58	0.50	1089.85	0.50	213.13	0.50	822.35	1.12	2.99	0.50	44.77	591.63	0.50
FW3_Ec36H_N2	0.50	0.50	0.50	20.14	2.14	885.74	0.50	464.39	12.77	906.19	0.50	1.32	0.50	48.77	758.11	0.50
HW1_Ec36H_H1	0.50	0.50	0.50	1.18	0.50	937.62	0.50	453.51	1.85	921.39	0.50	1.18	0.50	38.36	719.23	0.50
HW1_Ec36H_K1	0.50	0.50	0.50	5.38	0.50	885.35	1.96	976.13	30.27	919.52	0.50	1.96	0.99	41.99	723.32	1.48
HW2_Ec36H_F1	1.03	0.50	0.50	0.50	1.03	857.11	1.03	284.44	3.17	948.37	0.50	0.50	0.50	128.06	821.35	0.50
HW2_Ec36H_I1	0.50	0.50	0.50	2.67	0.50	942.71	0.50	330.17	4.40	912.39	0.93	3.53	0.50	40.35	649.87	0.50
HW2_Ec36H_L1	0.50	0.50	0.50	1.76	1.13	902.77	0.50	524.78	5.52	864.47	1.13	1.76	0.50	62.66	688.66	1.13
HW3_Ec36H_G1	0.50	0.50	0.50	12.94	0.50	881.48	0.50	316.27	5.34	1066.66	1.88	1.19	0.50	124.87	826.90	0.50
HW3_Ec36H_J1	0.50	0.50	0.50	19.17	1.88	1069.62	0.50	240.47	0.50	860.78	1.88	4.65	0.50	41.99	739.76	1.19
HW3_Ec36H_M1	0.50	0.50	0.50	2.22	2.22	856.44	0.50	424.59	4.81	866.78	1.36	0.50	0.50	45.32	676.28	0.50
FW1_Ec48H_D0	182.40	71.96	19.99	0.50	0.50	3151.32	26.49	149.92	7.00	585.19	364.31	481.24	1130.90	2787.52	9108.65	0.50
FW1_Ec48H_R2	0.50	0.50	0.50	16.69	1.14	941.31	0.71	639.92	57.58	859.94	0.71	1.56	0.50	80.80	557.27	0.50
FW2_Ec48H_E0	15.66	51.05	0.50	0.50	5.55	2381.29	0.50	30.83	10.61	713.22	162.25	505.98	2204.37	1365.28	1577.58	0.50
FW2_Ec48H_P2	1.05	0.50	0.50	6.56	1.60	899.61	0.50	559.90	12.06	937.60	0.50	3.25	0.50	86.94	1750.27	0.50
FW2_Ec48H_S2	0.50	0.50	0.50	7.58	0.50	989.79	0.94	579.39	15.09	894.71	0.94	2.27	0.50	72.59	533.84	0.50
FW3_Ec48H_F0	23.53	69.60	0.50	0.50	0.50	2795.40	15.86	15.86	8.18	822.08	292.28	453.52	3002.71	875.83	3271.45	31.21
FW3_Ec48H_Q2	0.50	0.96	0.50	15.69	0.50	988.00	0.50	1337.42	88.89	990.76	0.50	2.80	0.96	95.80	1607.20	0.50
FW3_Ec48H_T2	0.50	1.25	0.50	18.50	0.88	743.48	0.88	605.84	11.75	1410.33	0.50	1.63	0.88	47.38	496.70	0.50
HW1_Ec48H_I0	0.50	0.50	0.50	4.13	0.50	666.10	2.31	943.59	13.20	2073.48	0.50	7.75	0.50	33.15	1241.02	0.50
HW1_Ec48H_N1	0.50	0.50	0.50	12.80	1.06	902.23	1.06	490.78	13.92	964.85	0.50	0.50	0.50	71.50	516.49	0.50
HW1_Ec48H_Q1	0.50	0.50	0.50	3.19	0.50	976.20	0.50	480.63	3.86	1103.11	1.84	3.19	0.50	59.59	585.38	1.17
HW2_Ec48H_J0	0.50	0.50	0.50	0.50	0.50	568.73	4.41	686.03	8.32	2206.97	0.50	3.11	0.50	33.08	368.03	0.50
HW2_Ec48H_O1	0.50	0.50	0.50	2.32	0.50	941.27	0.50	447.82	3.53	1009.25	2.32	0.50	0.50	82.44	524.91	1.11
HW2_Ec48H_R1	0.50	0.50	0.50	2.23	1.36	989.27	0.50	507.85	10.01	1061.01	0.50	3.09	0.50	61.87	550.20	0.50
HW3_Ec48H_K0	0.50	0.50	0.50	1.83	0.50	543.79	1.83	1051.30	0.50	3093.26	0.50	1.83	1.83	28.33	306.60	0.50
HW3_Ec48H_P1	0.50	0.50	0.50	17.77	0.50	1088.67	0.92	406.46	2.61	1020.45	0.92	1.76	0.50	78.41	579.12	0.50
HW3_Ec48H_S1	0.50	0.50	0.50	4.53	0.50	928.43	0.50	449.13	2.11	1001.86	0.50	3.73	0.50	73.12	633.91	1.31

FIGURE LEGENDS

Figure 7 | Vein anatomy of the silver-spotted skipper and dissection scheme used for tissue collection. A. Drawn vein anatomy of the silver-spotted skipper. **B.** Same vein anatomy as in (A) superimposed on an adult skipper (dashed blue lines). **C.** Scheme used for RNA-sequencing tissue collection. After removing the peripodial membrane, wings were cut into compartments (dashed blue lines) following developing vein location (pink dots) as guides. Compartments are identified by colored triangles [*e. g.* blue=proximal forewing (FW1), yellow=medial forewing (FW2), red=distal forewing (FW3), green=proximal hindwing (HW1), purple= medial hindwing (HW2), and orange=distal hindwing (HW3)].

Figure 8 | Differential expression of *Ubx* in the silver-spotted skipper wings. In the forewing versus hindwing differential expression analysis, *Ubx* was overexpressed in the hindwings of fifth instar caterpillars relative to the forewings of all developmental stages.

Figure 9 | Wnt genes overexpressed in the forewings of the silver-spotted skipper. A. *WntA* was overexpressed in the forewings of fifth instar larvae and slightly enriched in the forewings of 48 h pupa relative to the hindwings of all stages. **B.** *fz1* had a differential expression pattern similar to that of *WntA* with overexpression in the forewings of fifth instars and a slight overexpression in 48 h pupa.

Figure 10 | Other genes overexpressed in the forewings of the silver-spotted skipper. A. In the forewing versus hindwing differential expression analysis, *hth* expression was higher in the forewings of 36 and 48 h pupae. **B.** *bab2* was overexpressed in the forewings of 48 h pupae.

Figure 11 | Genes enriched in the hindwings of the silver-spotted skipper. A. In the forewing versus hindwing differential expression analysis, *dll* was overexpressed in the hindwings of day 2 pupae. **B.** On the other hand, *wg* overexpression was observed in fifth instar hindwings.

Figure 12 | Genes identified in the forewing versus hindwing analysis with read counts higher than 10. Dots represent the 73,693 genes identified with read counts higher than 10. A total of 1,682 genes had an adjusted p-value < 0.05 (red dots). Dots above the red line represent genes overexpressed in hindwings relative to forewings and dots below the red line represent genes under expressed in hindwings relative to forewings of the silver-spotted skipper.

Figure 13 | *hth* and *WntA* are overexpressed in the proximal forewing compartments of the silver-spotted skipper. A. In the forewing compartment differential expression analysis, *hth* was overexpressed in the proximal forewing compartment of day 1 pupae and showed a different expression pattern in day 2 individuals. **B.** *WntA* showed low levels of expression in all forewing compartments of 36 h pupae but was overexpressed in the proximal compartment of 48 h pupae.

Figure 14 | Two other genes were overexpressed in the proximal forewing compartments. **A.** *Hr38* was overexpressed in the proximal compartment of day 2 pupae, which was unexpected and probably due to the cutting scheme used. **B.** *optix* was also overexpressed in 48 h FW1 compartments.

Figure 15 | *Dll* and *bab2* expressed in a gradient throughout the forewing compartments of the silver-spotted skipper. Both *dll* (**A**) and *bab2* (**B**) were expressed in a gradient with low

expression in the proximal compartments, medium expression in the medial compartments, and high expression in the distal compartments of day 2 pupae.

Figure 16 | Five genes were under expressed in FW2 compartments relative to FW1 and FW3. A. *wg* was under expressed in the medial forewing compartment of day 2 individuals. **B.** *Wnt6,* showed the same expression pattern in both day 1 and day 2 individuals. *Wnt11*, *fz1*, and *ci* were also under expressed in FW2 compartments (not showed).

Figure 17 | Genes identified in the forewing compartment analysis with read counts higher than 10. Dots represent the 56,360 genes identified with read counts higher than 10. A total of 14 genes had an adjusted p-value < 0.05 (red dots). Dots above the red line represent genes overexpressed in FW3 relative to FW1 and dots below the red line represent genes under expressed in FW3 relative to FW1 of the silver-spotted skipper.

Figure 18 | *hth, Hr38* showed overexpression in the proximal hindwing compartments of the silver-spotted skipper. A. *hth* was overexpressed in HW1 compartments of 36 h pupae. **B.** On the contrary, *Hr38* was overexpressed in HW1 compartment of 48 h pupae.

Figure 19 | *Dll* showed a slight gradient expression pattern in 48 h pupae . In the hindwing compartment differential expression analysis, *dll* showed low expression on the proximal compartments, medium expression in the medial compartments, and slightly higher expression in the distal compartments.

Figure 20 | Genes identified in the hindwing compartment analysis with read counts higher than 10. Dots represent the 37,999 genes identified with read counts higher than 10. Dots above the red line represent genes overexpressed in HW3 relative to HW1 and dots below the red line represent genes under expressed in HW3 relative to HW1 of the silver-spotted skipper.

Figure 21 | Genes identified in ISH experiments are overexpressed in fifth instar caterpillars. **A.** In the developmental stage differential expression analysis, *WntA* was overexpressed in fifth instar caterpillars relative to 36 h and 48 h pupae. **B.** *Wnt10* was also overexpressed in fifth instar caterpillars.

Figure 22 | *wg* and *Wnt11* are also overexpressed in the fifth instar stage. Both *wg* (**A**),and *Wnt11* (**B**) showed higher expression levels in forewings and hindwings of fifth instar caterpillars relative to the other two stages.

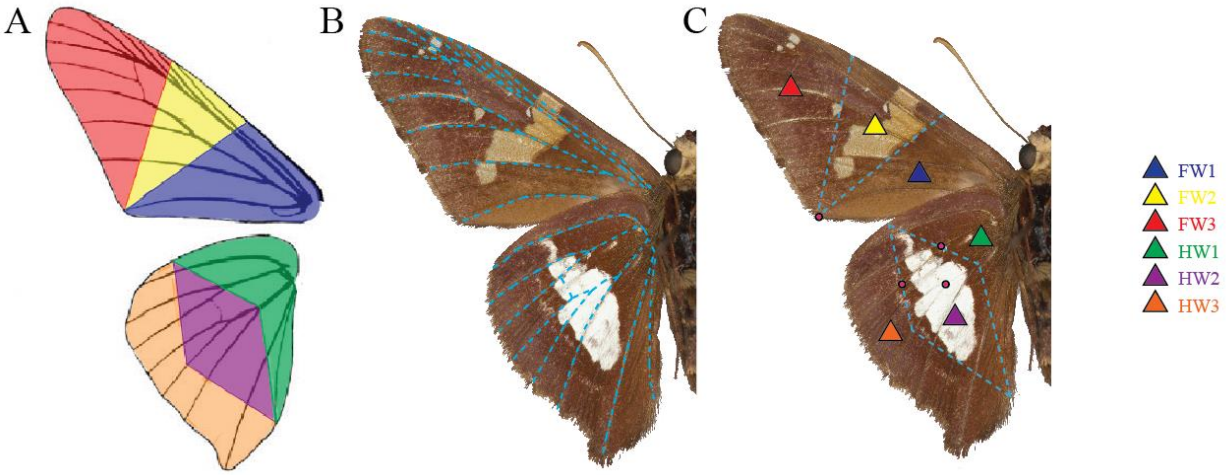
Figure 23 | Other genes overexpressed in the fifth instar stage. *Ubx* (**A**) overexpressed in the fifth instar hindwings, while *ci* (**B**) expression was higher in both forewings and hindwings of fifth instar caterpillars.

Figure 24 | Two genes were overexpressed in fifth instar and 48 h stages. Both *fz1* (**A**) and *Hr38* (**B**) were over expressed in fifth instar caterpillars and 48 h pupae relative to 36 h pupae.

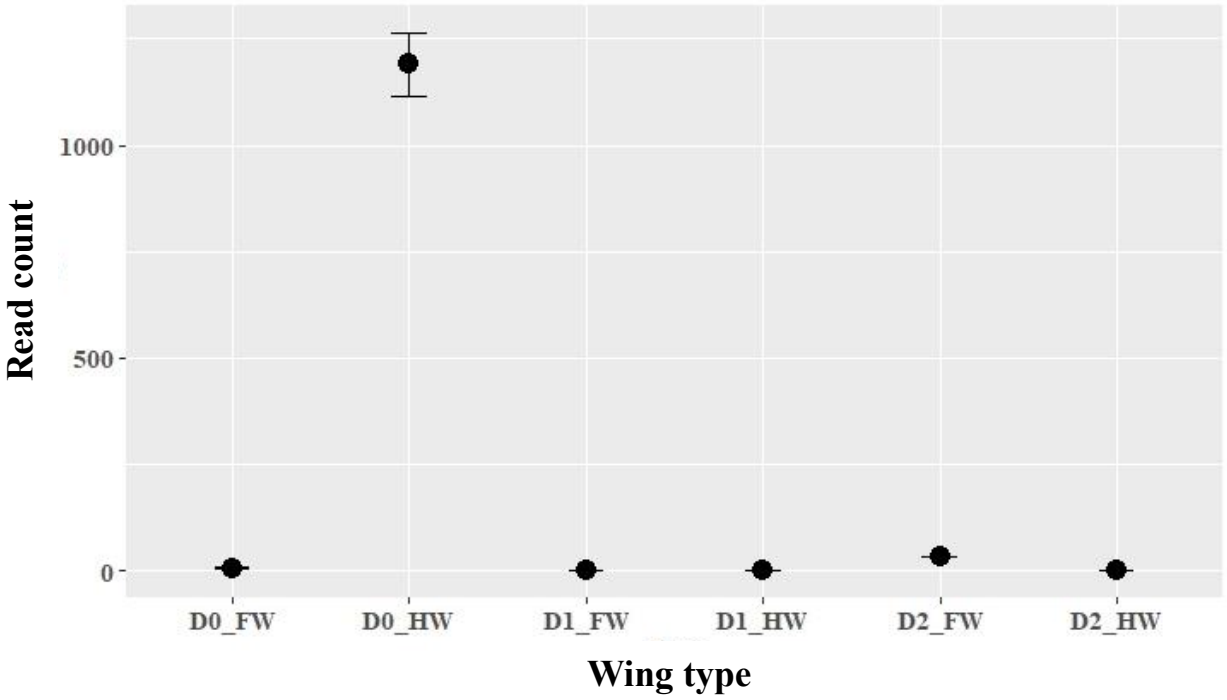
Figure 25 | Genes identified in the developmental stage analysis with read counts higher than 10. Dots represent the 73,653 genes identified with read counts higher than 10. Dots above the red

line represent genes overexpressed in 48 h pupae relative to fifth instar caterpillars and dots below the red line represent genes under expressed in 48 h pupae relative to fifth instar caterpillars of the silver-spotted skipper.

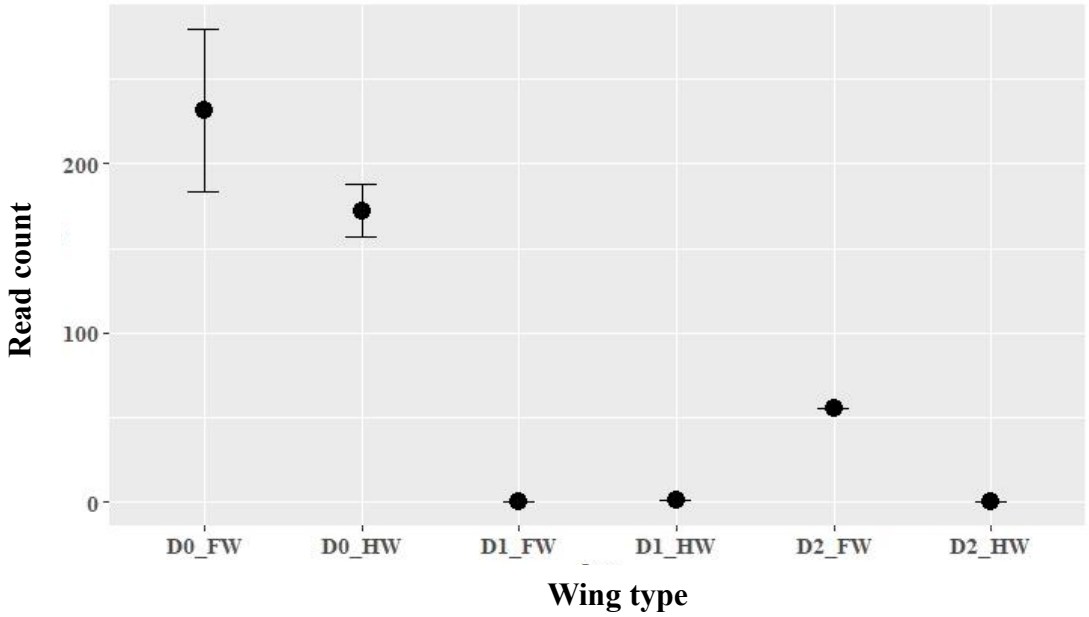
FIGURES



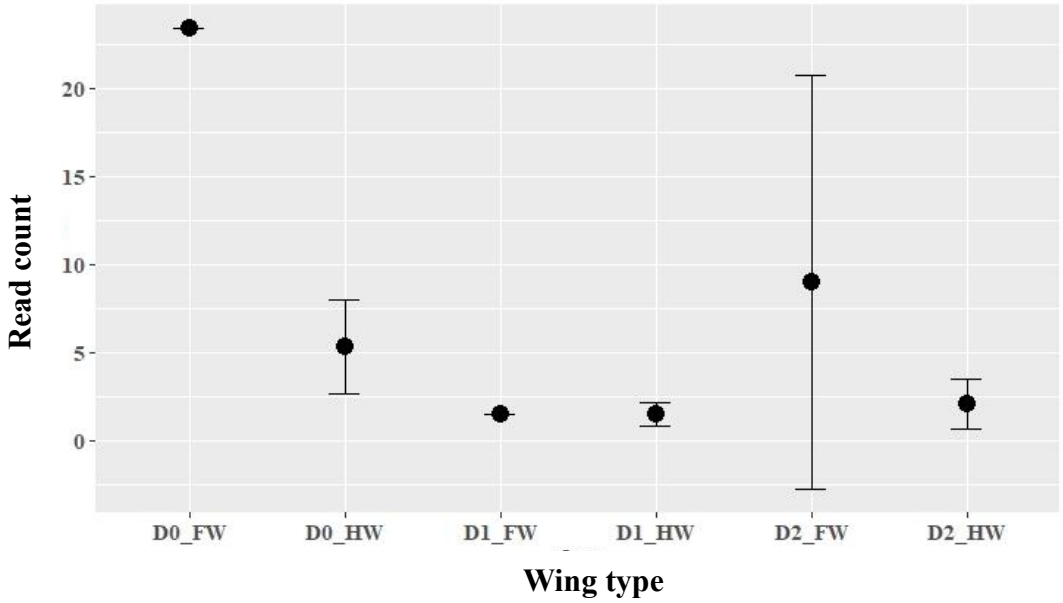
Ultrabithorax differential expression in forewing versus hindwing analysis



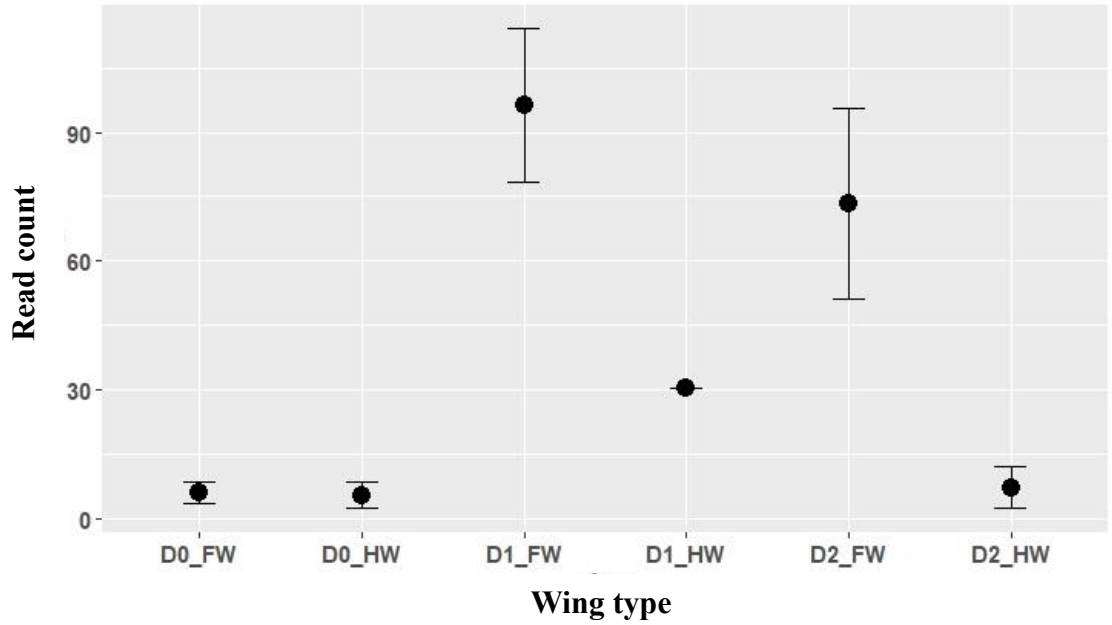
A ***WntA*** **differential expression in forewing versus hindwing analysis**



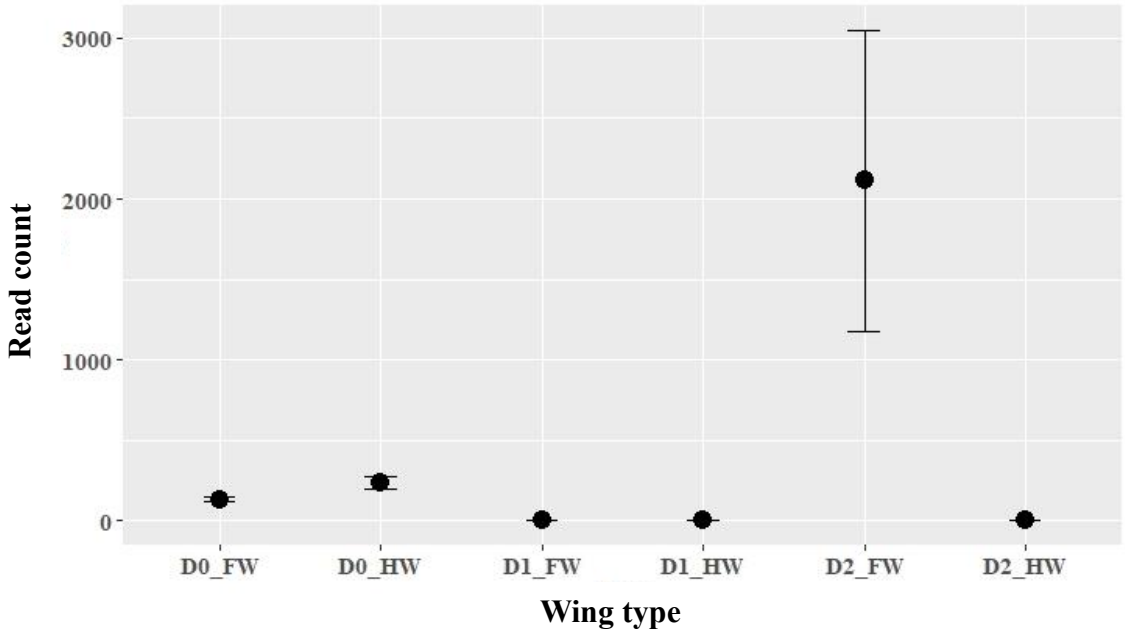
B ***fz1*** **differential expression in forewing versus hindwing analysis**



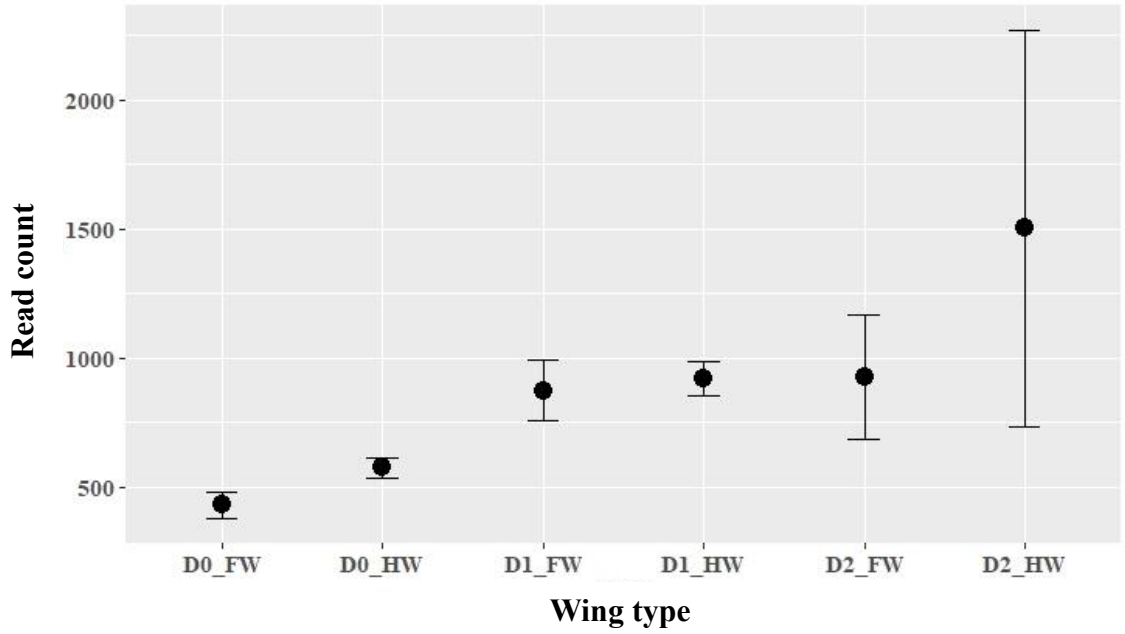
A ***hth*** **differential expression in forewing versus hindwing analysis**



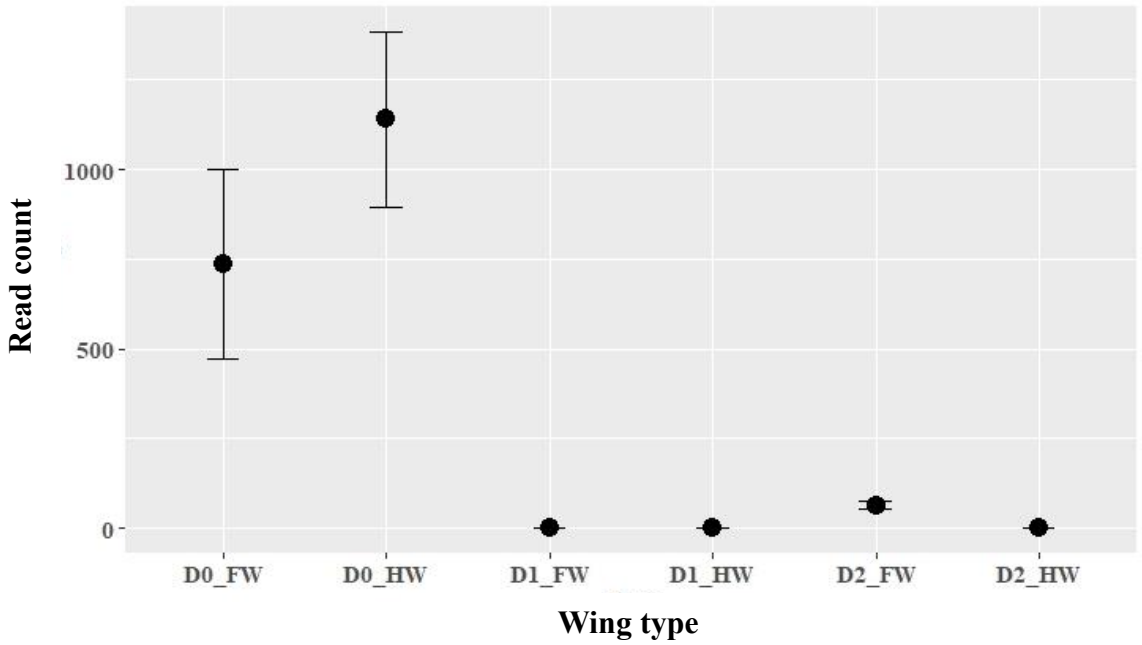
B ***bab2*** **differential expression in forewing versus hindwing analysis**



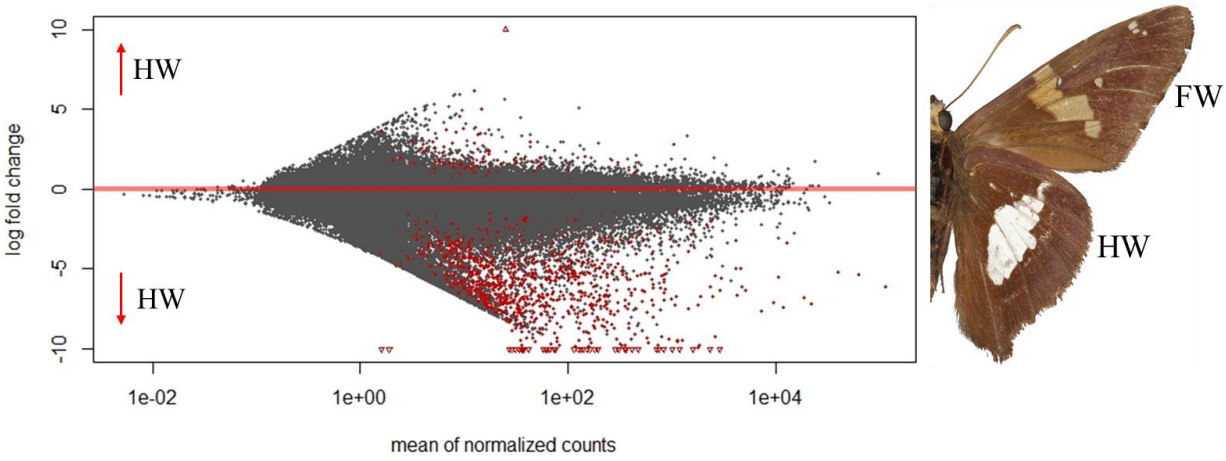
A ***dll*** **differential expression in forewing versus hindwing analysis**



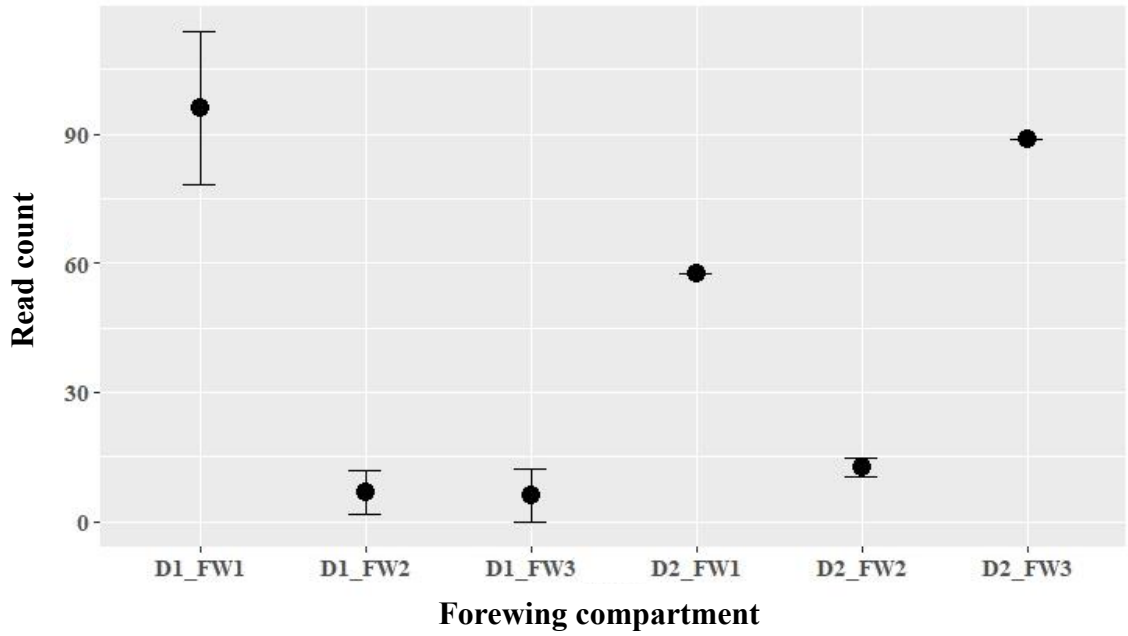
B ***wg*** **differential expression in forewing versus hindwing analysis**



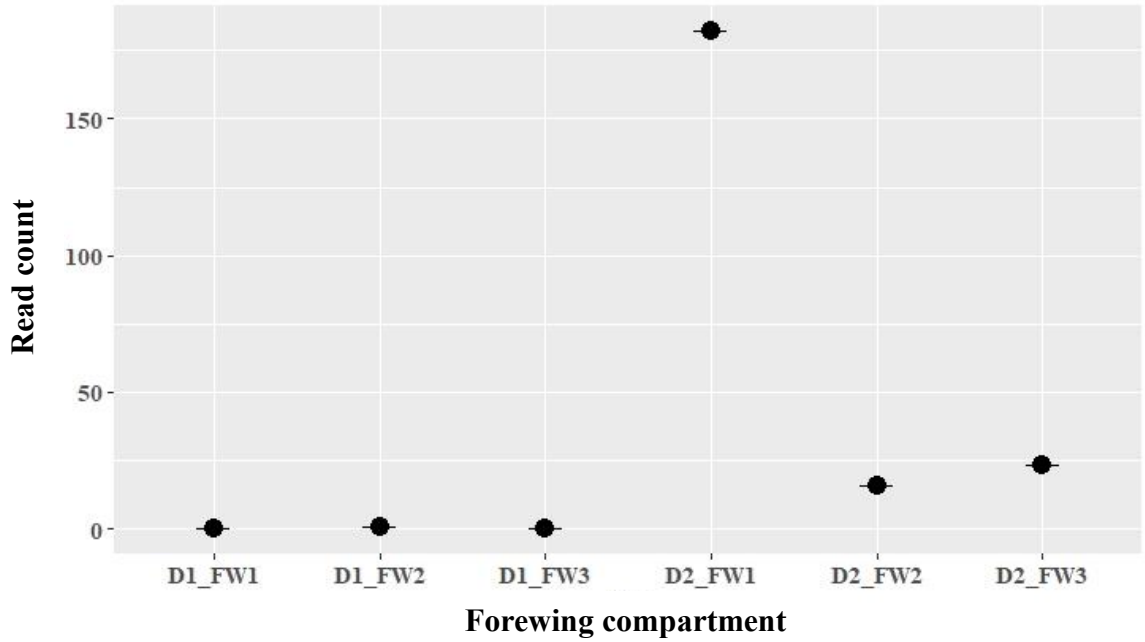
Genes identified in forewing versus hindwing analysis with read counts > 10



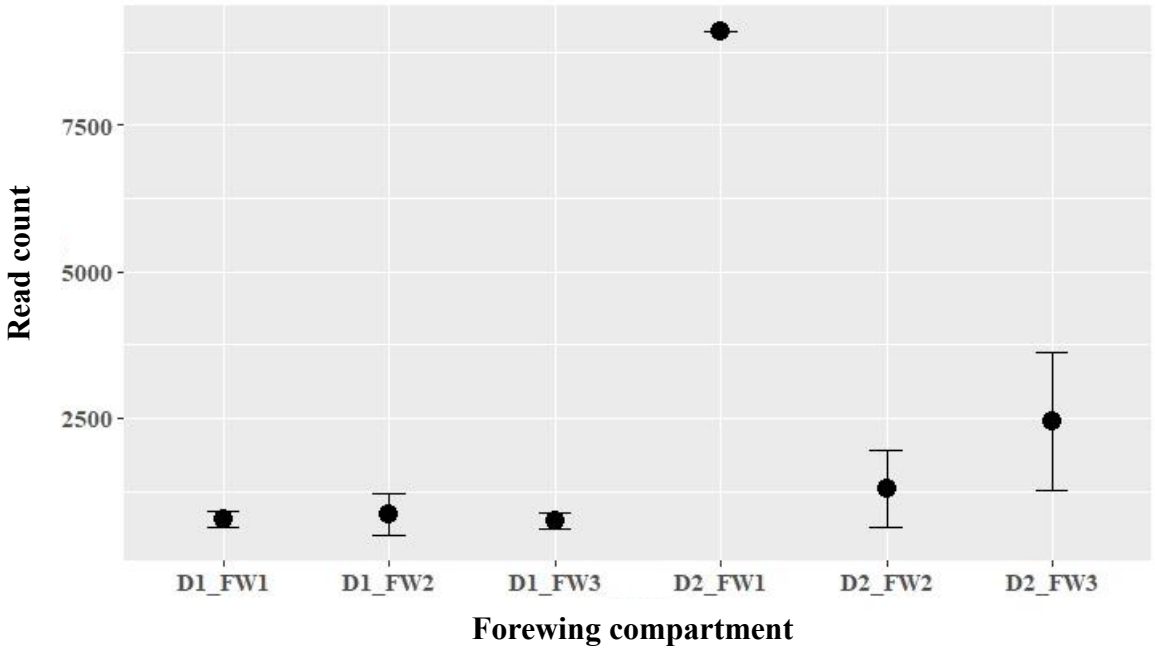
A ***hth*** **differential expression in forewing compartment analysis**



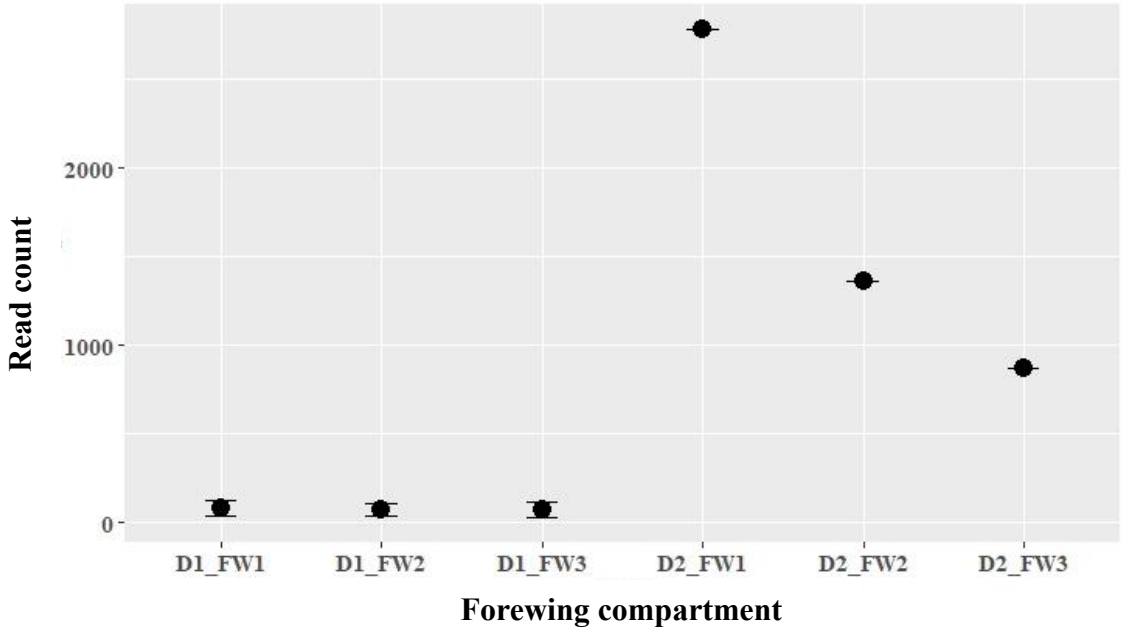
B ***WntA*** **differential expression in forewing compartment analysis**



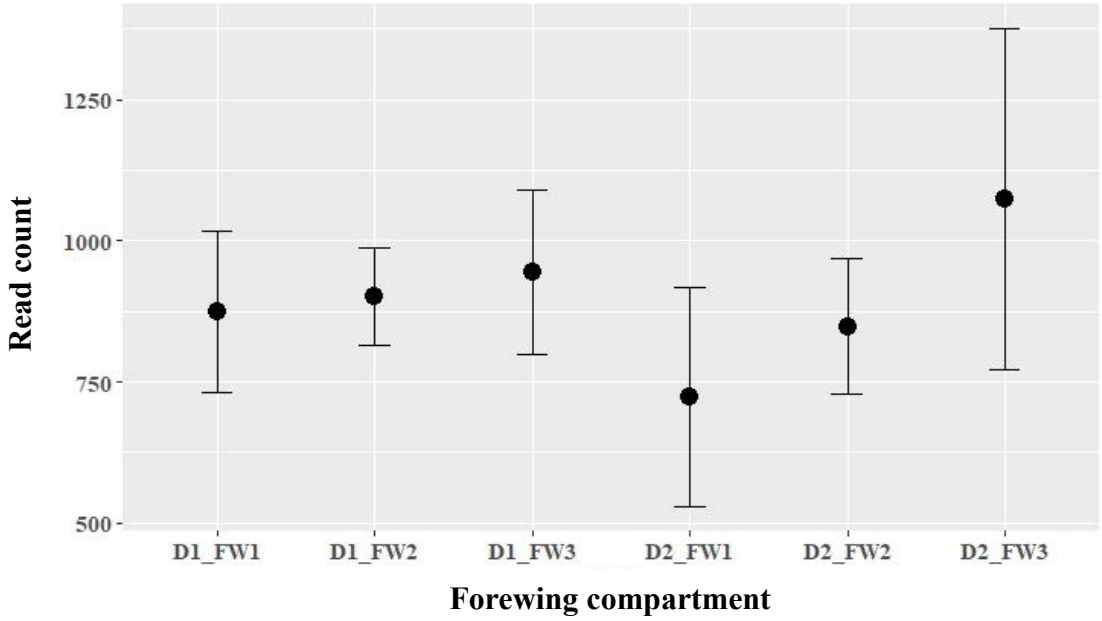
A ***Hr38*** **differential expression in forewing compartment analysis**



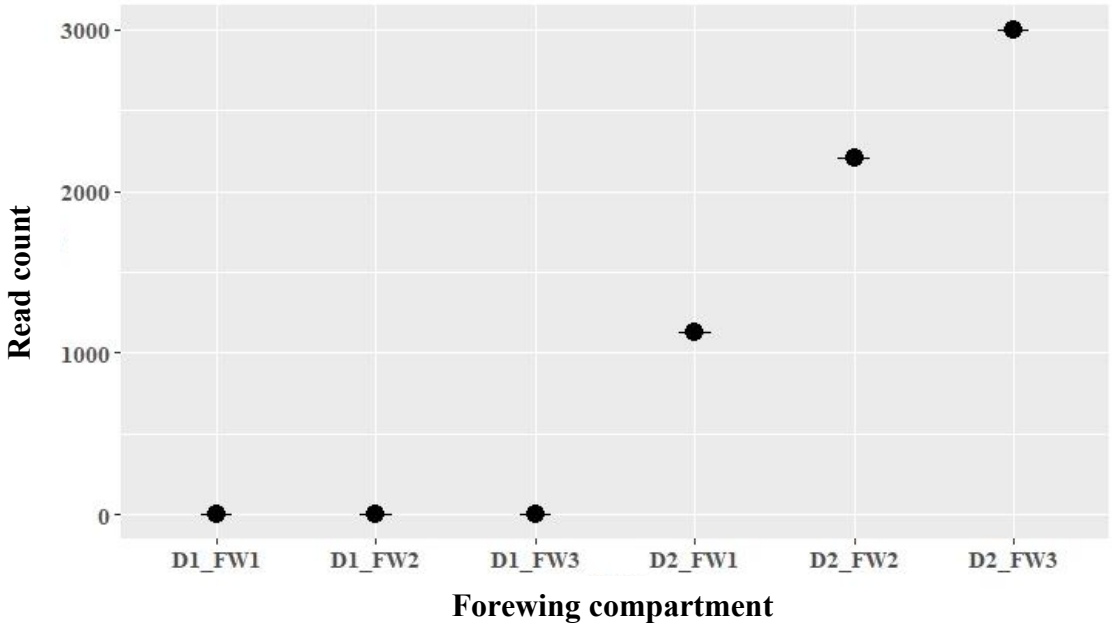
B ***optix*** **differential expression in forewing compartment analysis**



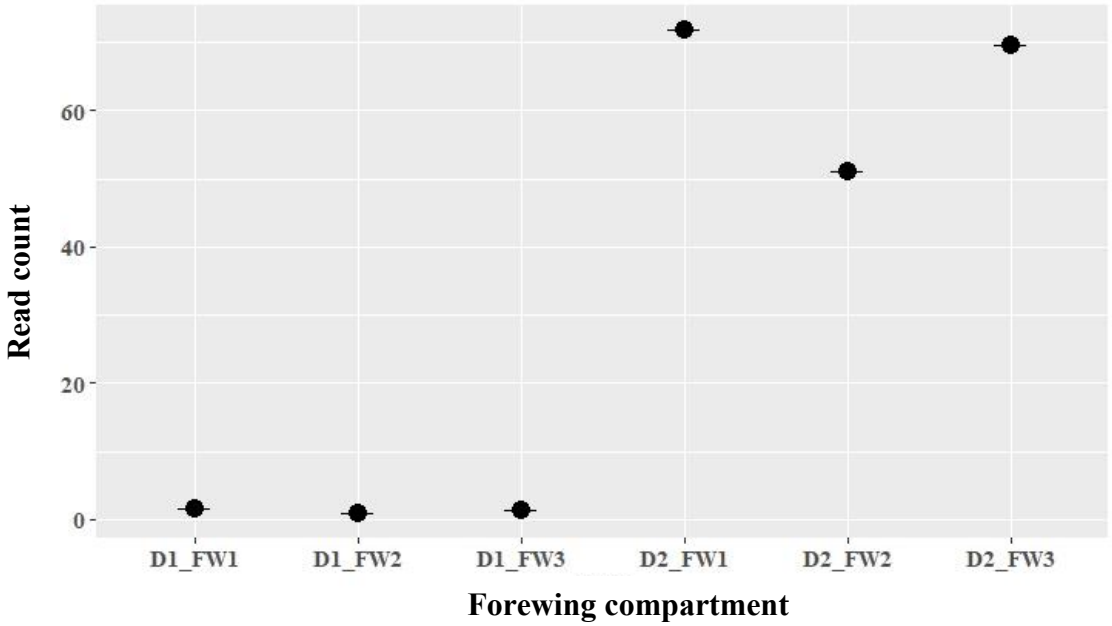
A ***dll*** **differential expression in forewing compartment analysis**



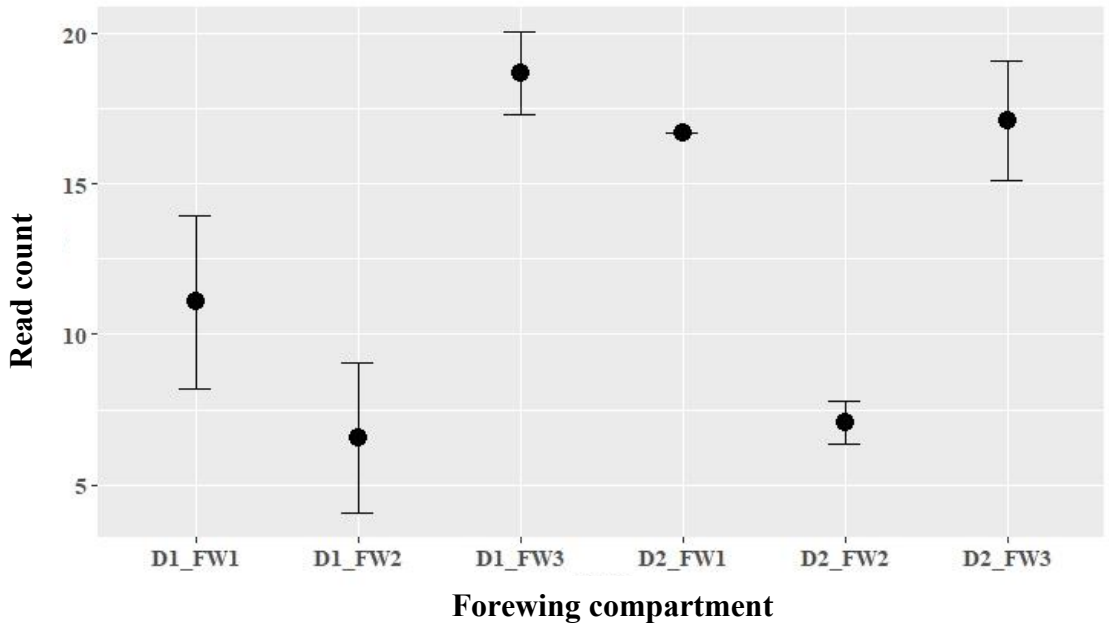
B ***bab2*** **differential expression in forewing compartment analysis**



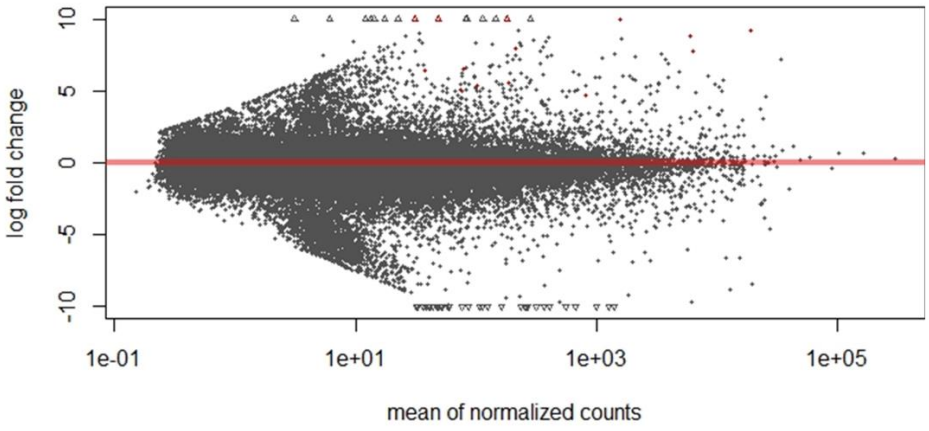
A ***wg*** **differential expression in forewing compartment analysis**

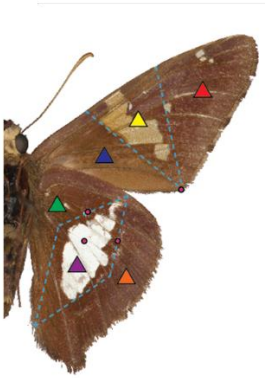


B ***Wnt6*** **differential expression in forewing compartment analysis**

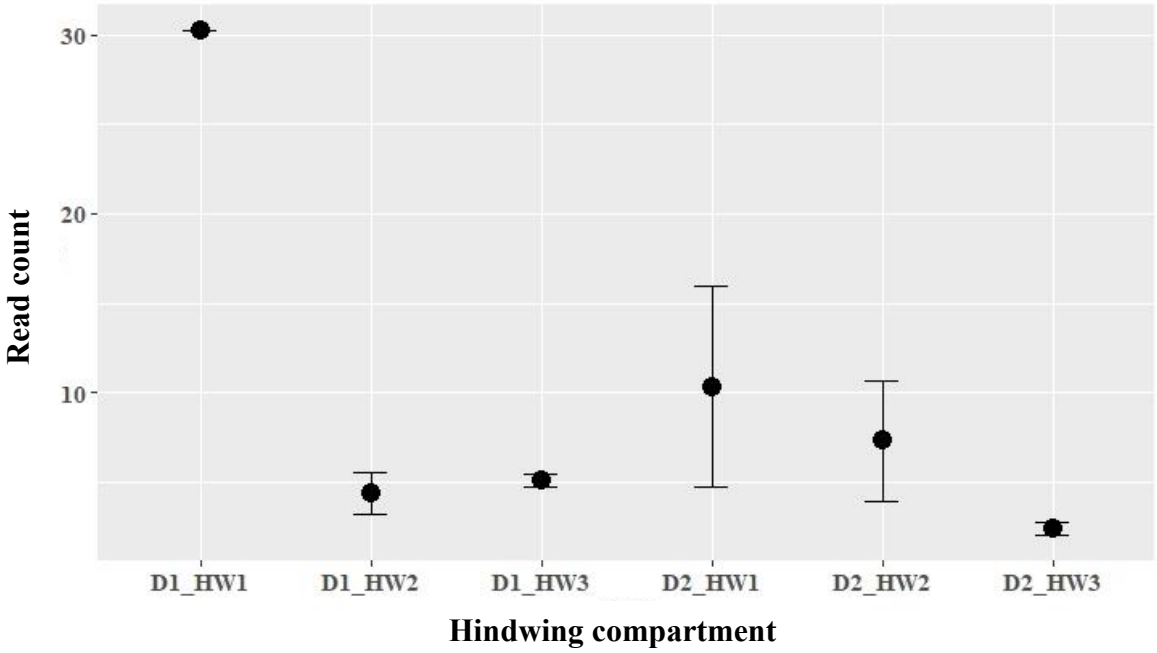


Genes identified in forewing compartment analysis with read counts > 10

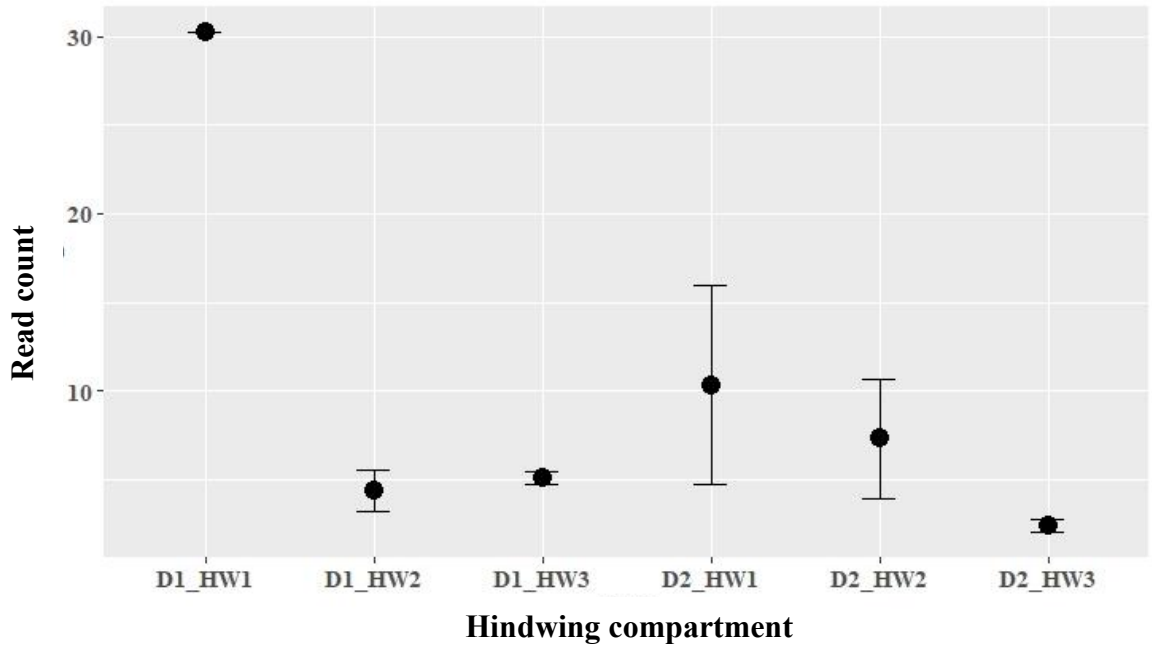




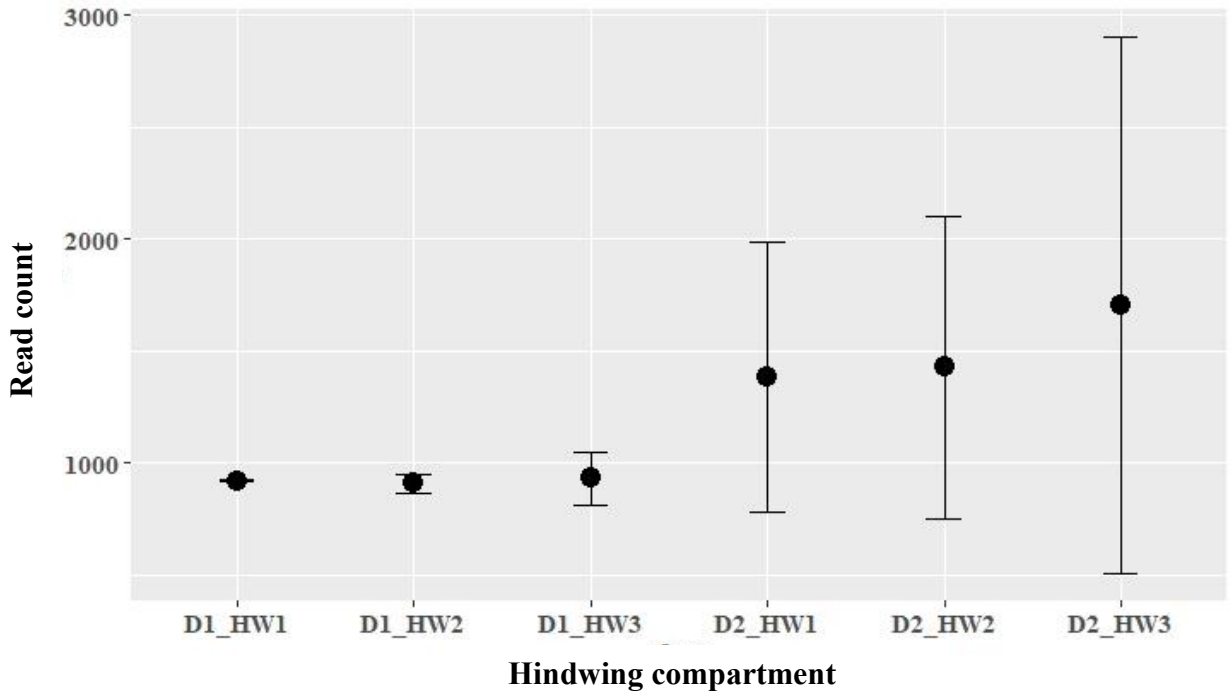
A ***hth*** **differential expression in hindwing compartment analysis**



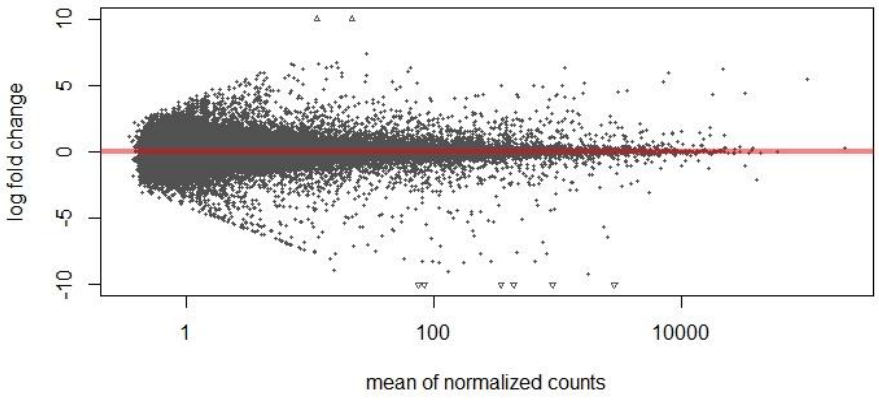
B ***Hr38*** **differential expression in hindwing compartment analysis**

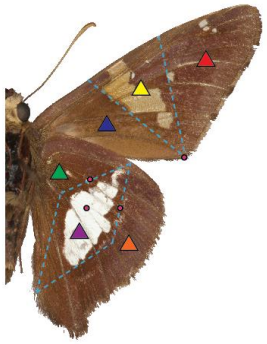


dll differential expression in hindwing compartment analysis

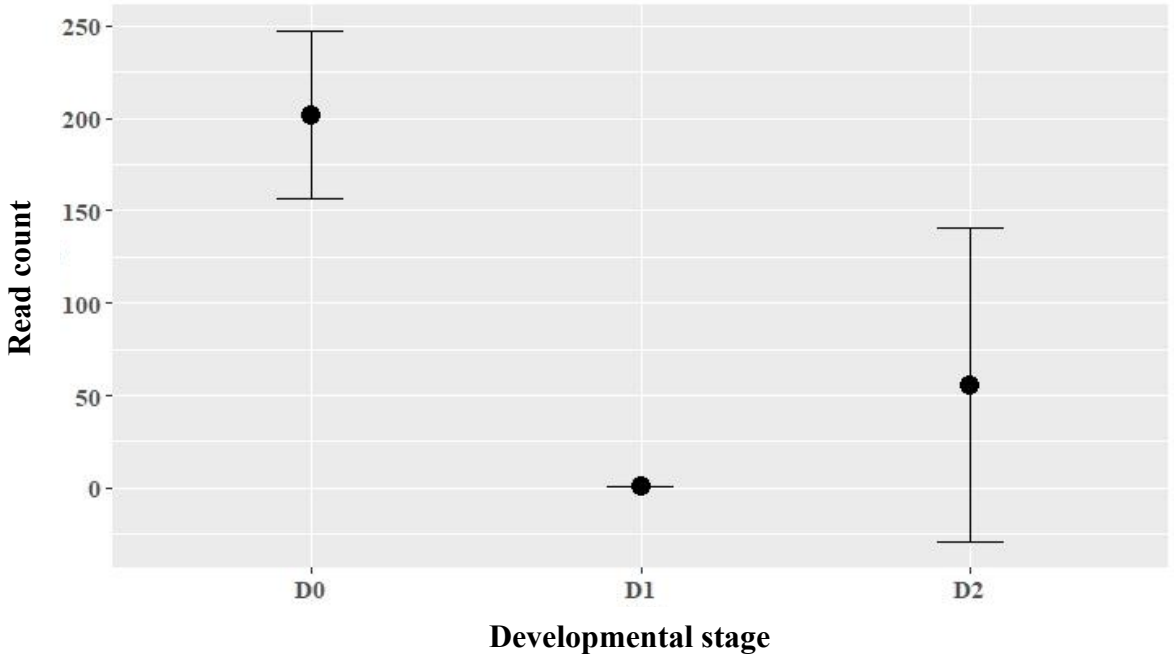


Genes identified in hindwing compartment analysis with read counts > 10

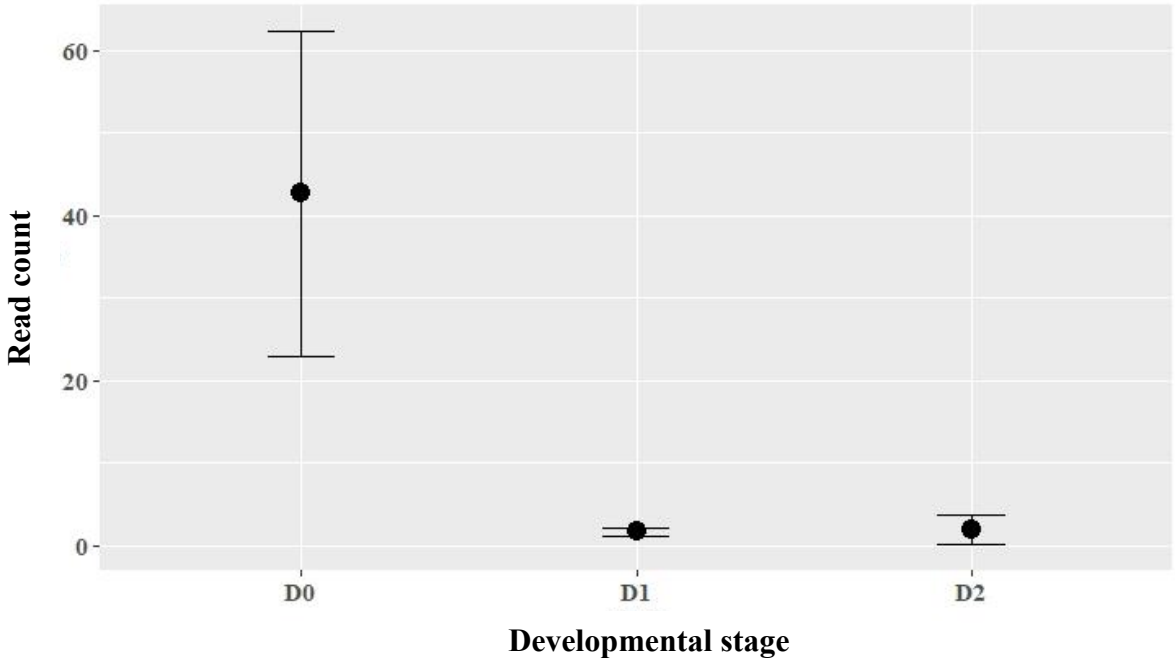




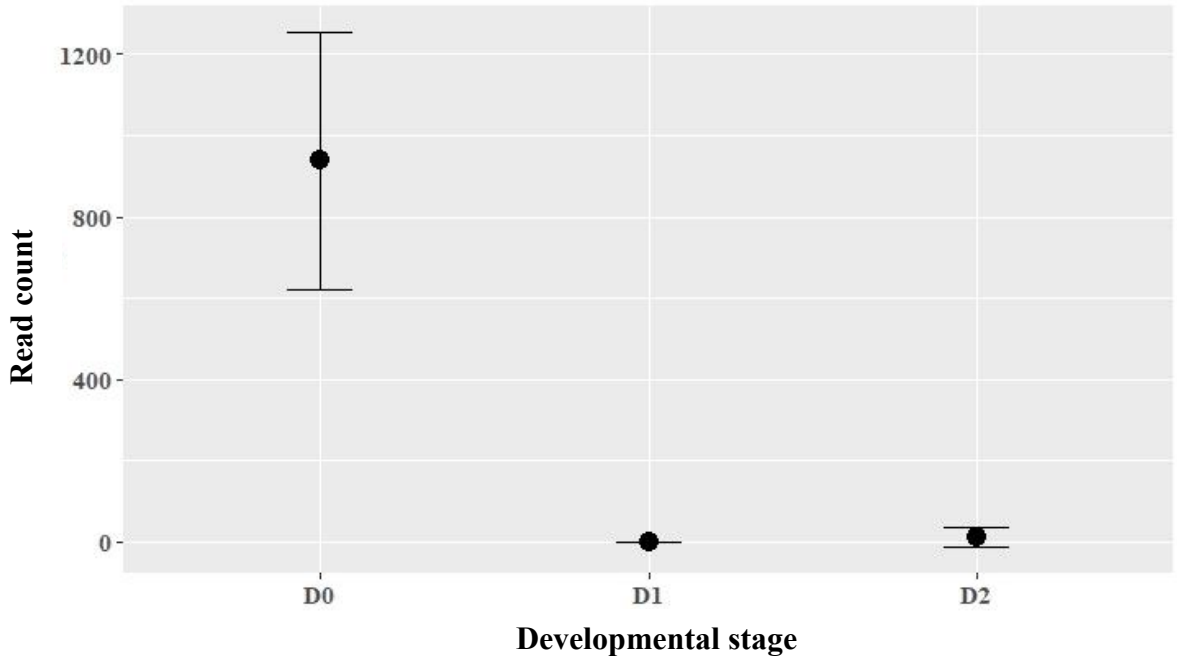
A ***WntA*** **differential expression in developmental stage analysis**



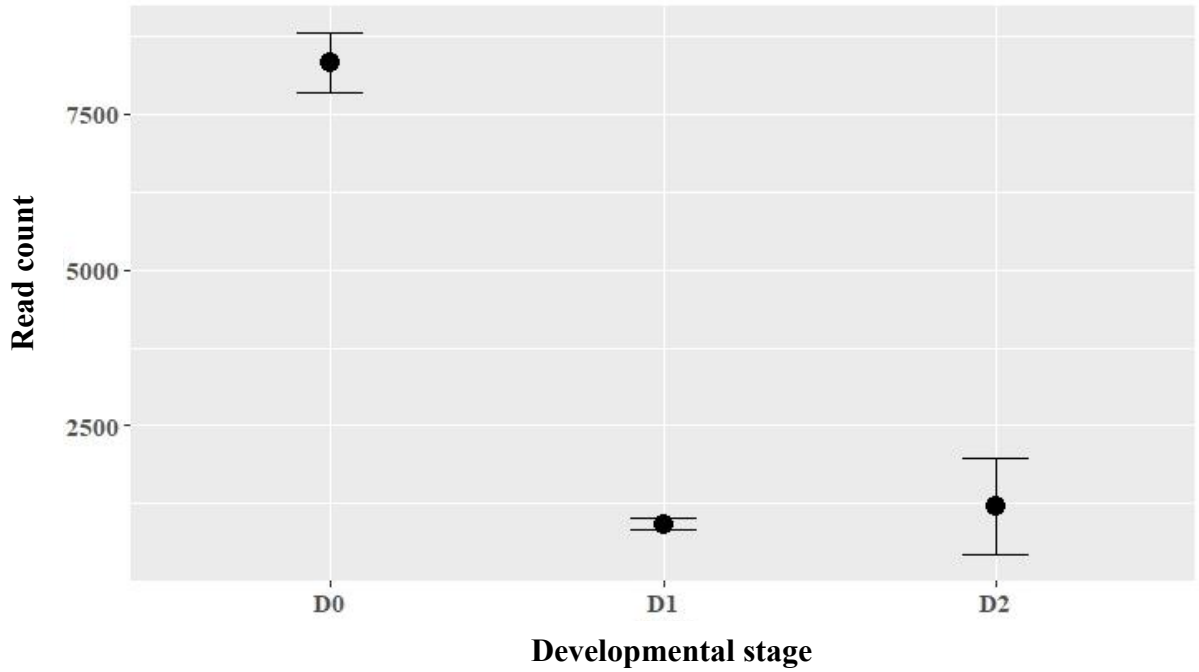
B ***Wnt10*** **differential expression in developmental stage analysis**



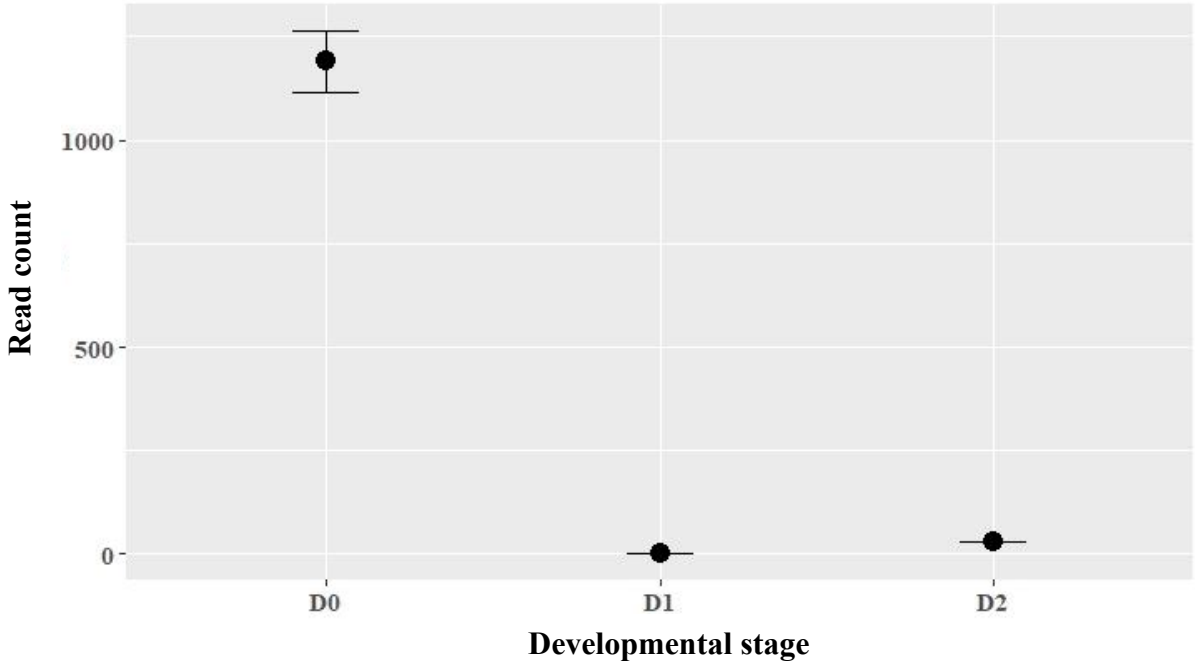
A ***wg*** **differential expression in developmental stage analysis**



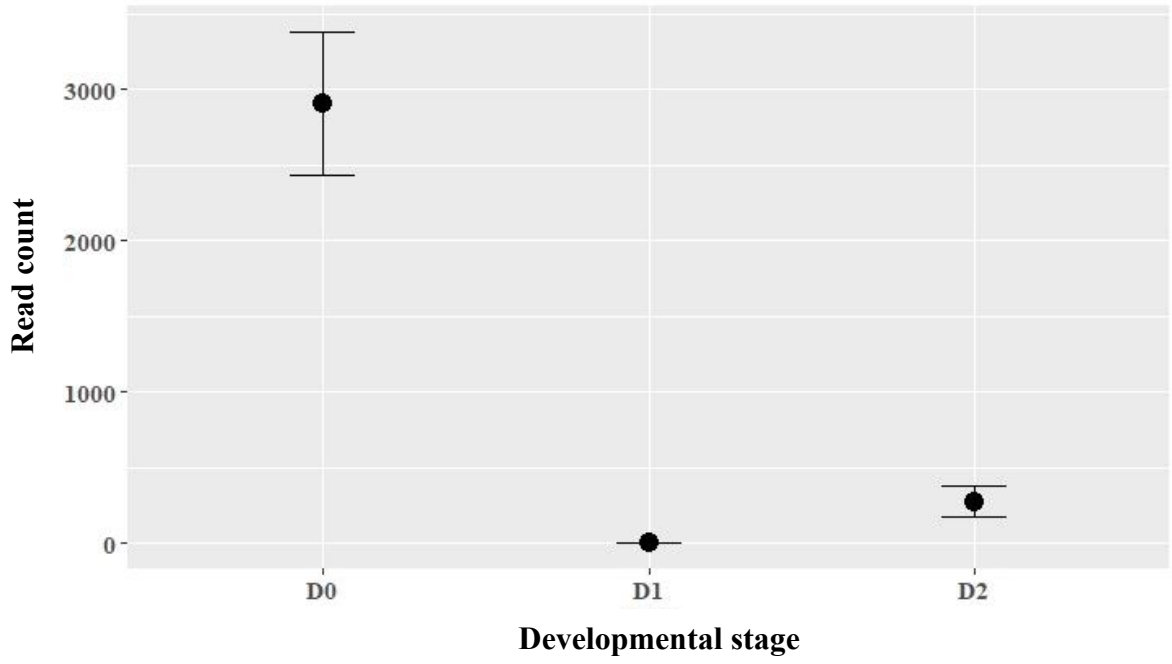
B ***Wnt11*** **differential expression in developmental stage analysis**



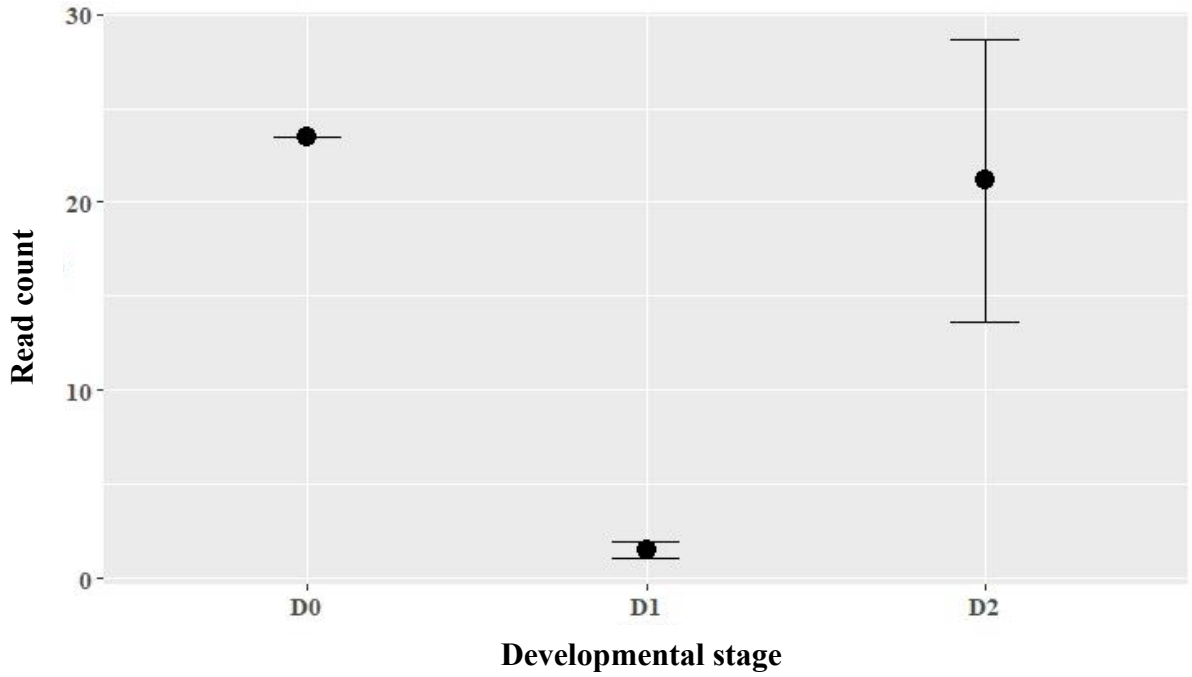
A ***Ubx*** **differential expression in developmental stage analysis**



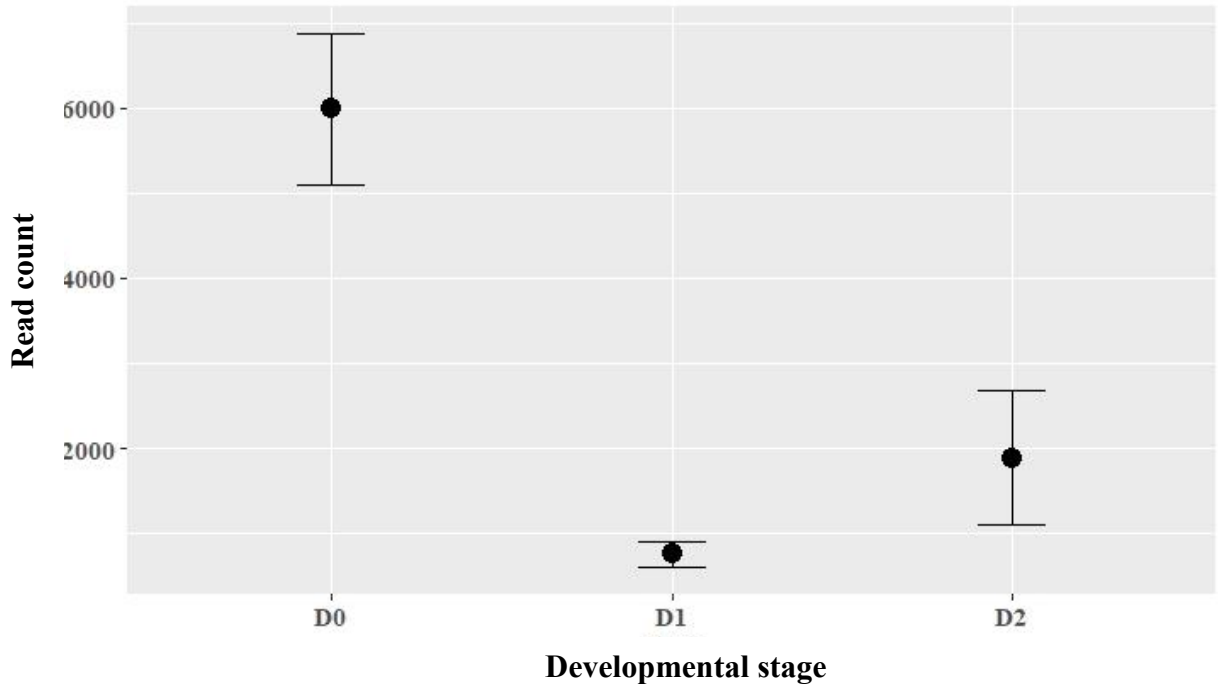
B ***ci*** **differential expression in developmental stage analysis**



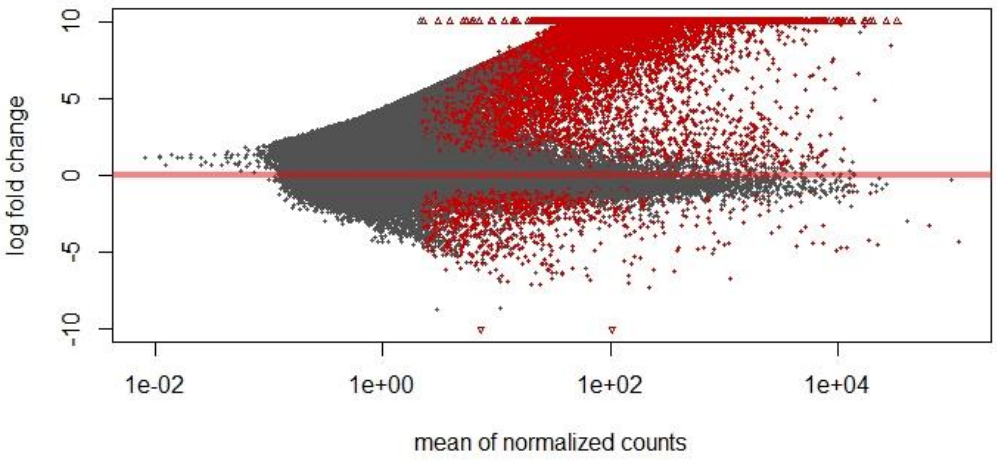
A ***fz1*** **differential expression in developmental stage analysis**



B ***Hr38*** **differential expression in developmental stage analysis**



Genes identified in hindwing compartment analysis with read counts > 10



CONCLUSION

With this project, I was able to characterize fifth instar mRNA expression of two Wnt genes, *WntA* and *Wnt10* through *in-situ* hybridization experiments. I also determined the effects of two drugs, heparin, and dextran sulfate, on wing patterning in a member of the Hesperiidae family and obtained insight into some of the patterning roles of Wnt genes in the silver-spotted skipper. I selected known patterning genes in Nymphalidae and was able to identify some of them in the genome of *E. clarus*. And lastly, I was able to describe the differential expression patterns of the sixteen genes identified.

The results obtained here suggest that indeed there is genetic homology of wing patterning genes between two distantly related butterfly families, Hesperiidae and Nymphalidae. Additionally, the ISH experiments and pharmacological drug injections confirmed the presence of Wnt genes in the silver-spotted skipper and showed that both *WntA* and *Wnt10*, share their patterning roles with member of the Nymphalidae family. The results obtained in the differential expression analyses confirmed that in the silver-spotted skipper many genes tend to differentially express depending on the developmental stage and tissue that we analyze.

The results obtained here are not only novel since the molecular basis of wing patterning has never been studied on the silver-spotted skipper but also help us understand better the mechanisms of wing patterning in general. By describing the phenomenon of wing pattern diversity of a poorly studied butterfly family and proving that there is convergence and conservation of patterning genes with one of the most studied butterfly families, Nymphalidae, I provide further evidence of how nature tends to repeat itself.