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Vein patterning during juvenile wing development in *Oncopeltus fasciatus* and *Jadera haematoloma*

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Vein patterning during juvenile wing development
in *Oncopeltus fasciatus* and *Jadera haematoloma*

An Honors Thesis

Presented to

The Faculty of The Department of Biology

Colby College

in partial fulfillment of the requirements for the

Degree of Bachelor of Arts with Honors

by

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ABSTRACT

Wings are a defining characteristic of all pterygote insects and are agreed to originate from a single common winged ancestor. However, essentially nothing is known about the molecular mechanisms that regulate wing development and patterning outside of Endopterygota, and the vast majority of this knowledge comes solely from *Drosophila melanogaster*. There is reason to suspect that the drastic developmental differences that exist between Endopterygota and Exopterygota have resulted in changes in the mechanisms, timings, or even genes that govern wing development. This study examined the roles of several genes known to be involved in *D. melanogaster* wing development and patterning in two exopterygote species, *Oncopeltus fasciatus* and *Jadera haematoloma*, and is the first to address molecular wing patterning outside of Endopterygota. Our results suggest that provein identity is established in early nymphal instars, and that the *spalt* transcription factor plays a role in maintaining vein development in both species.

INTRODUCTION

A majority of the estimated 5.5 million insect species inhabiting the earth today are winged (Pterygota), a trait at least partially responsible for their enormous success (Grimaldi and Engel, 2005; Stork et al., 2015). While there have been several alterations to wing function and structure in various orders (*e.g.* dipteran halteres and coleopteran elytra), the basic structure and placement of adult pterygote wings is highly conserved across species. The ground plan of the adult insect wing consists of a bilayer of dead cells enclosing a pattern of ectodermal veins, which act both as structural support and as conduits for hemolymph, tracheae, nerves, and other factors like melanin precursors (Liu et al., 2014). Pterygote wings have been subject to intensive study for hundreds of years and remain a common research topic today. Examining vein development and placement within the wing is a common method for understanding how various signaling processes work together to develop the wing itself. Therefore, with advances in developmental and molecular biology, the processes in both wing and vein development have been thoroughly investigated and described in orders that fully metamorphose (the Holometabola or Endopterygota). Most of what is known comes from the common fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae) (Blair, 2007). Meanwhile, these processes in winged, partially metamorphosing orders (Exopterygota) have received scant attention (Brisson et al., 2010). As a result of this neglect, essentially nothing is known about exopterygote wing development or how what is known from endopterygote species applies to these orders. There is reason to suspect that variations exist in the molecular mechanisms of wing and vein development between various insect orders of insects due to differences in wing growth, form, and function. These variations are likely to be most

pronounced between endopterygote and exopterygote orders due to drastic differences of how wing development proceeds in each. A better understanding of this topic may contribute to ongoing debates such as that over the evolutionary origin of wings and will provide a basis for additional study to broaden the understanding of pterygote wing development beyond Endopterygota alone.

The study presented here is the first to examine how orthologs of several well-understood *D. melanogaster* genes involved in wing and vein development act in similar processes in two exopterygote species through knockdown of gene expression by RNA interference (RNAi). The exopterygote species used in this project were the large milkweed bug, *Oncopeltus fasciatus* (Hemiptera: Lygaeidae), and the red-shouldered soapberry bug, *Jadera haematoloma* (Hemiptera: Rhopalidae). Our goal was to establish how gene functions necessary for wing and vein development compare between Endopterygota and Exopterygota. I present data showing vein identity in the primordial wing tissue is likely established prior to the fourth instar in both species, *spalt* expression is required for veins to fully develop, and that *serum response factor* is necessary for adhesion of the dorsal and ventral wing epithelia.

BACKGROUND

THE ENDOPTERYGOTE WING: OBSERVATIONS OF FORM, DEVELOPMENT, AND PATTERNING FROM *DROSOPHILA MELANOGASTER*

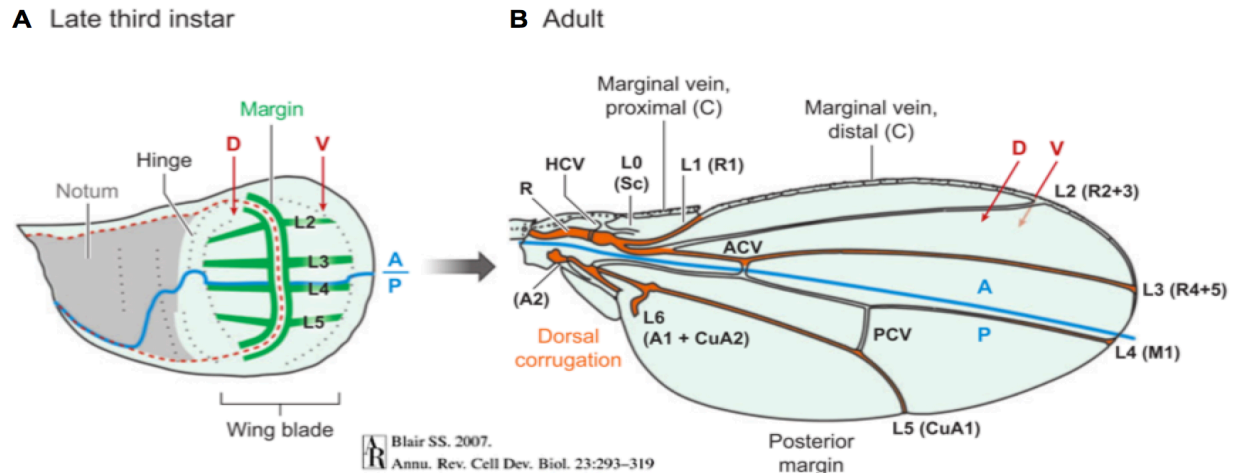


Figure 1. The wing imaginal disc during the late third instar (A) and the adult wing (B) of *Drosophila melanogaster*. (A) Areas expressing high levels of EGF signaling are shown in green. The anterior-posterior compartment boundary is shown in blue, and the dorsal-ventral compartment boundary is denoted by a dotted orange line. The proveins are labeled using the standard “L” nomenclature. (B) Veins are labeled using both the “L” nomenclature and a more general nomenclature developed by Stark et al. (1999). This figure was originally presented in Blair (2007).

Endopterygotes, such as the Diptera, Coleoptera, and Hymenoptera, undergo complete metamorphosis between juvenile and adult life stages. During this process, some species completely restructure their bodies, developing wings and other appendages from internal groups of cells called imaginal discs (Švácha, 1992). While not all species develop their appendages in this way, wings are known to arise from imaginal discs in Diptera, Coleoptera, and Hymenoptera (Angelini and Kaufman, 2005) Švácha, 1992). Wing imaginal discs and their development are best understood in *D. melanogaster*. This species’ wing disc consists of an epithelial sack of cells that grows through three larval instars. Early in development, the wing disc is divided along two spatial axes into anterior/posterior and dorsal/ventral compartments, which prevent cell movement between compartments as shown in Figure 1A (reviewed by Blair, 1995). At the onset of metamorphosis, the forewing disc everts, telescopes outward, inflates, and flattens before assuming its final shape and

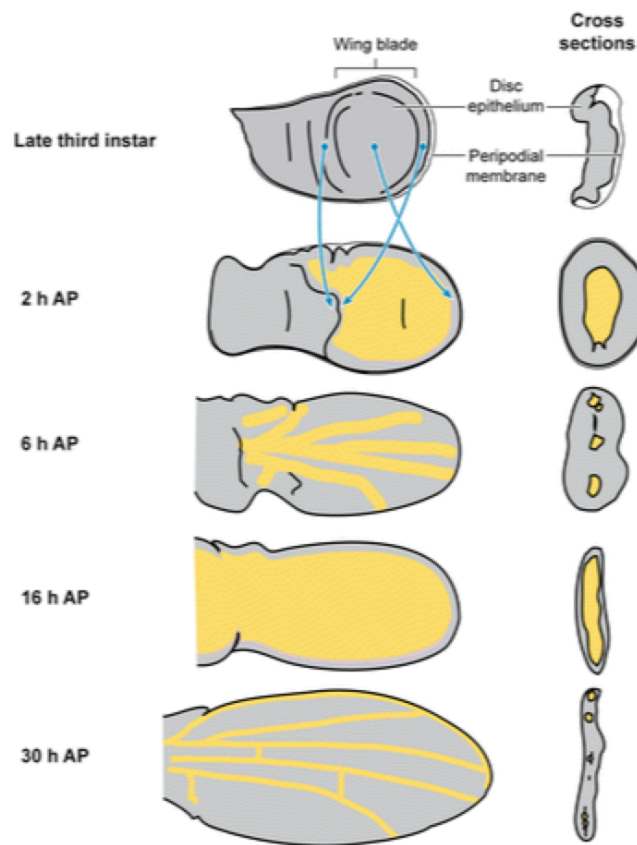


Figure 2. Development of the *Drosophila melanogaster* wing imaginal disc into the adult wing. Regions in which the dorsal and ventral epithelia are not in direct contact are shown in yellow. Timings are denoted on the left. The various shapes of the developing wing disc are shown in the middle and cross sectional views are shown on the right. This figure was originally presented in Blair (2007).

pattern over the course of 30 hours post-pupariation (Figure 2) (Blair and Palka, 1985).

Vein placement is determined through the interaction of a number of signaling gradients that occur in the wing

Blair SS. 2007.
Annu. Rev. Cell Dev. Biol. 23:293–319

disc prior to its eversion. Cells designated to participate in vein development are referred to as proveins. *Drosophila* has five provein regions including the prospective wing margin (Figure 1A). The wings of *D. melanogaster* have five longitudinal veins (denoted L1-L5 from anterior to posterior), three cross veins, the anterior cross vein (ACV) between L3 and L4, the posterior crossvein (PCV) between L4 and L5, and the humeral crossvein located in the proximal-anterior portion of the wing hinge, and finally two truncated veins (L0 and L6) (Figure 1B). The proveins are refined during wing development after pupariation, in which time they take on the traditional pattern of a *D. melanogaster* including the crossveins. Additionally, the proveins are generally called by the shorthand of the vein they will develop into. For example, the L2 provein is the provein area that will develop into L2.

Multiple signaling pathways and expression gradients are responsible for patterning different parts of the *D. melanogaster* wing in the mid to late third instar wing disc. Epidermal growth factor (EGF) signaling is required for both initiating and maintaining provein and longitudinal vein development, processes that are also accomplished to some degree by both decapentaplegic (Dpp) signaling and wingless (wg) signaling (Blair, 2007). *Serum response factor (srf)*, meanwhile, is necessary for determining non-vein, or intervein, cell fates and is down regulated in regions of high EGFR signaling (Montagne et al., 1996). Provein placement is regulated by various gradients depending on the provein in question. Marginal vein development (L1) is determined by wingless expression secreted from cells along to the dorsal-ventral compartment boundary. L2 and L5 are both placed based on a gradient of Dpp, a secreted morphogen, which diffuses from cells anterior of the anterior-posterior compartment boundary. However, the mechanisms by which each provein is placed differ. L2 is placed according to expression levels of two *spalt* transcription factors (*sal*) regulated by Dpp concentration. High Dpp leads to high *sal* expression, which inhibits L2 provein identity. Lower levels of *sal* expression specify L2 identity by promoting expression of two *knirps* transcription factors (*kni*), which are expressed in the L2 provein (de Celis and Barrio, 2000). Meanwhile, L5 provein identity is inhibited by high *sal* expression, but it is not known if *sal* plays other roles in L5 placement. Another transcription factor, *optomotor blind (omb)*, promotes necessary L5 provein identity at the posterior limit of *omb* expression, determined by the Dpp gradient, by initiating transcription of genes in the iroquios complex (*iro-C*) and *abrupt (ab)*. Another secreted signaling protein, Hedgehog, determines the placement of the L3 and L4 proveins at the anterior and posterior limits of *hedgehog* expression. L3 develops a bit anterior to the

anterior-posterior compartment boundary while L4 develops directly posterior to this boundary cell identity.

Work in *D. melanogaster* has primarily utilized a genetic tool called mosaic analysis. In this method, flies have been genetically manipulated so that random populations of cells in the wing express different copies of genes than those around them. This can result in islands of cells that express a nonfunctional copy of a gene that may be necessary for some process, allowing the researcher to examine what occurs in a small region of cells without that gene, as opposed to dealing with pleiotropic effects that a traditional null allele may present. The processes and pathways in wing development are thoroughly reviewed by Blair (2007), and a reader is encouraged to visit his work and citations for further information.

Studies of *Tribolium castaneum* (Tomoyasu et al., 2005; Tomoyasu et al., 2009) and winged castes of some ant species (Abouheif and Wray, 2002) have shown that similar genes, interactions, and timings to those found in *D. melanogaster* pattern the wings of these insects, leading to the conclusion that wing development, and therefore wing vein development, is likely well conserved across the holometabolous insects. However, the wings of flies, beetles, and ants are all highly derived in that their development differs significantly from other orders', especially those outside of Endopterygota (Grimaldi and Engel, 2005). As no studies have been carried out to identify any discrepancies between the mechanisms of exopterygote and endopterygote wing development, our work provides a necessary starting point for beginning to understand any differences that may exist.

THE EXOPTERYGOTE WING: OBSERVATIONS OF FORM AND DEVELOPMENT FROM HEMIPTERA

External wing development, such as that observed throughout Exopterygota, is accepted as the plesiomorphic state for all Pterygota (Grimaldi and Engel, 2005). If this shared ancestral origin of wings is true, similarities in developmental mechanisms and gene functions in the wing development of all pterygotes should be taken as a null hypothesis. However, in the 406 million years since the emergence of Pterygota and the accompanying adaptive radiations pterygotes have undergone (Misof et al., 2014), there is cause to assume that unique methods of wing development and its components have emerged.

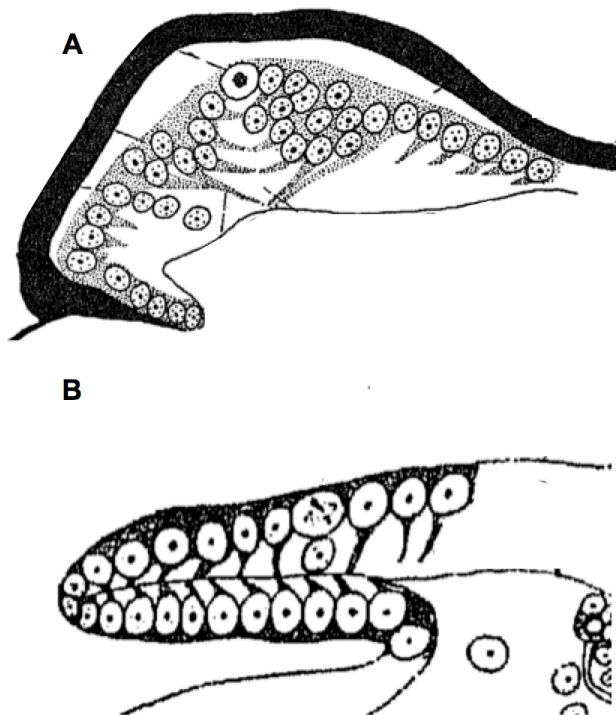


Figure 3. Depictions of cross sections of the first (A) and second (B) instar forewing pads of *Oncopeltus fasciatus*. (A) The first instar wing pad consists of a small evagination of the dorsal cuticle on the second thoracic segment. Some cells occupy this space, but have not yet formed a bilayer. (B) By the second instar, the primordial wing cells have formed a bilayer around a middle membrane. This early establishment of a bilayer in the wing primordia differs greatly from *Drosophila melanogaster* where the wing disc only becomes a bilayer very late in development. Modified from Nivedita (1982).

Exopterygotes progress from hatchling to adult by molting through a series of nymphal instars. These insects develop most appendages, like legs and antennae, from embryonic buds rather than from imaginal discs. Hatchlings of these orders bear strong

resemblances to their adult forms, but lack the few structures seen only in adults, such as ocelli, wings, and genitalia. Exopterygote wings grow over the course of nymphal development in a process that has been well studied at a histological level in some species, including one of our study organisms, *O. fasciatus* (Comstock and Needham, 1899; Nivedita, 1982). The *O. fasciatus* wing begins as a wing pad, a population of cells within small dorsal evaginations from the thoracic ectoderm, during the first of five nymphal instars (Figure 3A). These cell populations bear little resemblance to the imaginal discs of Endopterygota. By the second instar, the primordial wing cells within the wing pad form a bilayer (Figure 3B), in a drastic contrast to the late pupal bilayer formation observed in *D. melanogaster*.

Adult exopterygote wings display as much differentiation and specialization as those of the endopterygotes. In the Hemiptera, the forewing has taken on a semi-protective role similar to that of coleopteran elytra, resulting in their being called hemelytra. The hemelytra is classically described as consisting of three regions, the corium (anterior proximal), the clavus (posterior proximal), and the membrane (distal) (Comstock, 1918). The hindwing is fully membranous and used for flight, consisting of a main membrane portion and a smaller proximal posterior lobe called the juga (Comstock, 1918). Both hemipteran species used in our work, *O. fasciatus* and *J. haematoloma*, have long been kept as study systems. Each species develops through five nymphal instars and is susceptible to systemic RNAi (Hughes & Kaufman 2000; Fawcett et al., In Review). *J. haematoloma* is of particular interest to evolutionary developmental biologists due to a nutrition-related wing polyphenism. This polyphenism involves growth of *J. haematoloma*'s forewing membrane and hindwings, as they develop to a varying extent depending on nutritional availability (Carroll et al., 2003). The mechanisms by which the membrane and hindwings are

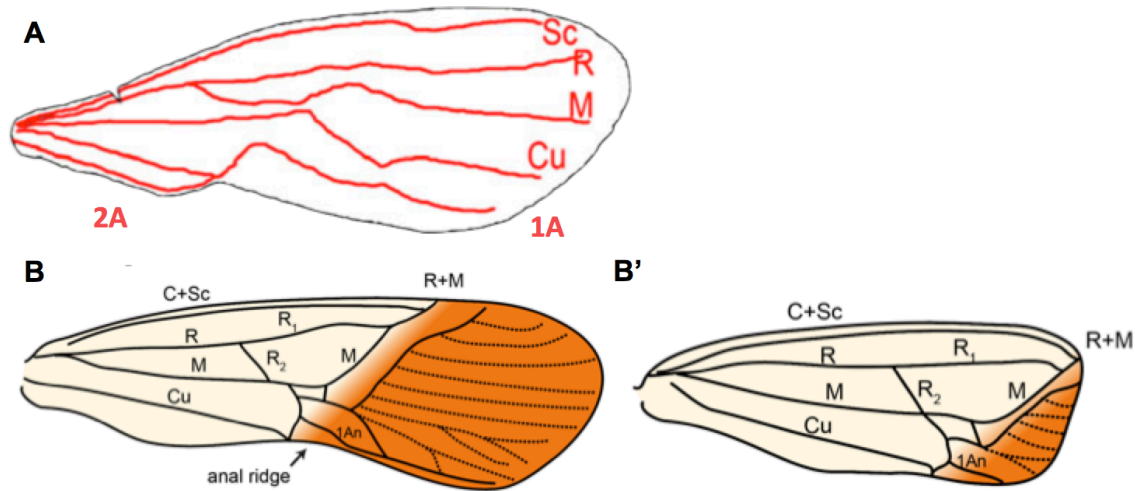


Figure 4. Longitudinal veins of the *Oncopeltus fasciatus* (A) and *Jadera haematoloma* (B and B') forewings. Longitudinal veins are named according to the nomenclature outlined by Nivedita (1982) for *O. fasciatus*. Sc=Sub-costal, R=Radial, M=Medial, Cu=Cubitus, 1A=First-anal, 2A=Second-anal. (A) is adapted from Liu et al. 2014. (B) Vein patterns of the macropterous (B) and brachypterous (B') morphs of *J. haematoloma*. The distal membrane region is highlighted in orange and the vein patterning within it is semi-random between individuals. C = Costal, subscripts denote branches of a vein.

truncated are unknown, but it is believed that the mechanisms themselves are regulated by insulin signaling (Fawcett et al., In Review).

The veins of *O. fasciatus* develop in a set pattern throughout the wing, and are observed to roughly approximate tracheal development in juvenile *O. fasciatus* wing pads (Nivedita, 1982). Figure 4 shows the longitudinal veins of the fore and hindwings of *O. fasciatus* labeled with general nomenclature for hemipteran longitudinal veins. In the forewing from anterior to posterior these veins are referred to as costal (not shown, but it makes up the margin), subcostal, radial, medial, cubital, first anal, and second anal. The hindwing veins follow the same nomenclature but the subcostal vein is largely truncated. These observations suggest that, despite differences in developmental timing between *D. melanogaster* and hemipteran species, vein pre patterning still occurs in the primordial

wing tissue. However, it is entirely unknown if the mechanisms by which the prepatterning occurs are conserved between the species. Additionally, *J. haematoloma* is observed to develop a semi-random pattern of veins in the membrane of its wings while the veins of the corium and clavus develop in invariant patterns (Figure 5). Random patterning of the membranes likely has to do with *J. haematoloma*'s wing polyphenism, but suggests that novel mechanisms have developed to pattern the distal region of this species' wings.

Only one study has examined any aspect of molecular genetics in exopterygote wing development. A study of the pea aphid, *Acyrtosiphon pisum* (Hemiptera: Auchenorrhyncha), identified the presence of genes homologous to those known for their involvement in *Drosophila* wing development, and quantified expression in homogenates of multiple whole aphids (Brisson et al., 2010). However, this study did not examine gene functions or spatial expression patterns within developing wings.

METHODS

(1) Organisms and rearing conditions

The *O. fasciatus* strain used was obtained in 2012 from Carolina Biological Supply Company and has been kept at room temperature in 3.38L terrariums (Carolina Biological #674339A) and fed organic shelled sunflower seeds. Several *J. haematoloma* strains were used. The PK13 and PK15 strains were collected wild from Plantation Key, Florida (24.9777°N, 80.5512°W) in 2013 or 2015 and have been raised in lab in 3.38L terrariums at 26°C on *Cardiospermum halicacabum* seeds (Outsidepride.com Inc., Independence, OR). The AC15, FR15, GR14, and strains were initially collected from sites in Aurora, CO (39.7295°N, 104.8273°W), Frederick, MD (39.3878°N, 77.4093°W), and Greenbrier, VA (38.8802°N,

77.3965°W), respectively. Each has been raised in 3.38L terrariums at 26°C on *Koelreuteria paniculata* seeds (F.W. Schumacher Co. Inc., Sandwich, MA).

After microinjection with dsRNA, bugs of both species were kept in 71cm² petri dishes, provided with their appropriate food source and wet paper towels. Several treatments of *J. haematoloma* were kept individually in 28cm² petri dishes at 26°C with three seeds from the appropriate host species, a wetted section paper towel, and ~2 cm² of cardboard egg carton to aid in molting. Individuals were isolated due to numerous instances of opportunistic cannibalism by treatment-mates, especially during molting. This approach may have caused an overrepresentation of short-winged individuals, however, as every insect reared in isolation that reached adulthood was short-winged.

(2) Double-stranded RNA (dsRNA) synthesis and preparation of injection solution

First, genes of interest were amplified via PCR using Jumpstart Taq ReadyMix (Sigma-Aldrich #P2893, see Table 1 for primers) from cDNA previously cloned into plasmids or Gibson assembly synthetic linear DNAs (Integrated DNA Technologies). Primers included a 5' 20-nucleotide T7 RNA polymerase promoter sequence. Primer information is available in Table 1. The T7-PCR products were then used as template for in vitro transcription with the MegaScript T7 Transcription kit (Thermo Fisher #AM1334) to produce dsRNA that was purified through ammonium acetate (7.5M) and ethanol (100%) precipitation at -20°C for between 20 minutes and overnight followed by 20 minutes of 17,000xg centrifugation at 4°C. Pellets were then washed in 70% ethanol and centrifuged for five minutes at 14,000xg and 4°C. Pellets were then dried via vacuum centrifugation, removing the pellet prior to its becoming a white-opaque color. Finally RNA pellets were

resuspended in nuclease-free water, heated to 98°C and annealed by slow cooling. The resulting double-stranded RNA (dsRNA) was stored at -20°C.

Injection solutions were prepared by diluting dsRNA to 1500, 2000, 2500, or 3000 ng/μL using insect physiological saline and a 1:20 dilution of green McCormick food coloring. Concentrations used for each gene are available in Table 2. Injection solutions were stored at -20°C.

Table 1: Primer information used in dsRNA synthesis

species	gene	oligo sequence	F/R	position	amplicon
<i>J. haematoloma</i>	<i>Dll</i>	taatacgactcactatagggATAGCTATACTGGATATCAC	F	26	161
		taatacgactcactatagggTTCCTCATCTTCTTACCCCT	R	146	
<i>J. haematoloma</i>	<i>omb</i>	taatacgactcactatagggTCTGAACCTCAATGCACAAG	F	341	200
		taatacgactcactatagggATTTTCAACTGCGTTATCTT	R	500	
<i>J. haematoloma</i>	<i>spalt</i>	taatacgactcactatagggGCAAACAACGCCGATAACCC	F	38	220
		taatacgactcactatagggTCGACTCCTTCTTGAGGAG	R	217	
<i>J. haematoloma</i>	<i>srf</i>	taatacgactcactatagggGCGTATGAGCTGTCAACACT	F	31	165
		taatacgactcactatagggGCCTTGCCCGCCTCTGACGT	R	155	
<i>O. fasciatus</i>	<i>spalt</i>	taatacgactcactatagggGATAGGTGCTACCAAAGTC	F	4	241
		taatacgactcactatagggGTTCTGACGACTTACTCAA	R	204	
<i>O. fasciatus</i>	<i>omb</i>	taatacgactcactatagggCTTTCAAAGTCCGAGTTATG	F	36	180
		taatacgactcactatagggTGGCATCTCCGGGTCCGCCT	R	175	
<i>O. fasciatus</i>	<i>EGFR</i>	taatacgactcactatagggAAAGAGTCGTAATGGAATAG	F	3588	242
		taatacgactcactatagggCAATCCAGTTTAAGATTCC	R	3789	
<i>O. fasciatus</i>	<i>bcl2</i>	taatacgactcactatagggGAAACTATCTCACACCCTTG	F	84	278
		taatacgactcactatagggGTCTTATTCTCTCGAGTTC	R	322	

(3) Injections

Bugs were kept anesthetized for the duration of the procedure using carbon dioxide. Injections consisted of between 0.2μL and 1.0μL injection solution delivered via a nitrogen-backed MPPI-3 pressure injector (Applied Scientific Instrumentation) using pulled glass capillary needles. Injections were made ventrolaterally at the 3rd abdominal segment. All individuals injected were fourth instar juveniles, but staging within fourth instar and sex were not considered. This stage was chosen because mortality at earlier stages is high and

because RNAi at this stage has been found to affect the development of adult structures in *O. fasciatus* (Aspiras et al., 2011).

(4) Imaging

Images were captured at 6.5X magnification using a Moticam 5 camera (Motic) mounted on a VistaVision trinocular microscope (VWR).

(5) Statistical Analyses

Fisher's exact test was used to determine if the proportion of a treatment that successfully completed the fifth (adult) molt significantly differed from the same proportion from the *GFP* (*green fluorescent protein*) dsRNA control treatment. Completing adult molt was defined as the bug successfully extracting its whole body from the fifth instar cuticle. Fisher's exact test was also used to determine if frequencies of observed atypical phenotypes in successfully molting individuals in treatments significantly differed from zero. These data are available in Table 2.

RESULTS AND DISCUSSION

Table 2: Tabulated results of RNAi knockdown treatments. ^a Fisher's exact test. * $p < 0.05$.

Target gene	Species	[dsRNA] (ng/μL)	Injected	Completing Adult Molt	p-value ^a	Abnormal Phenotypes	p-value ^a
<i>GFP</i>	Jh	1500/2000	35	30	---	0	1
<i>Dll</i>	Jh	3000	16	7	0.005015 *	7	5.828x10 ⁻⁴ *
<i>omb</i>	Jh	1500	20	14	0.709	0	1
<i>sal</i>	Jh	1500	20	7	0.000221 *	7	5.828x10 ⁻⁴ *
<i>srf</i>	Jh	1500	12	0	1.184e-07*	12	7.396x10 ⁻⁷ *
<i>Egfr</i>	Of	1500	18	8	0.003079 *	1	1
<i>omb</i>	Of	2000	20	17	1	4	1
<i>sal</i>	Of	2000	21	11	0.0115 *	10	3.402x10 ⁻⁵ *

(1) OPTOMOTOR BLIND AND SPALT

In *Drosophila* wing development, the Decapentaplegic (Dpp) signaling pathway plays a role in determining the positioning of longitudinal veins in the developing wing disc via a diffusion-gradient of Dpp produced by cells anterior to the anterior-posterior compartment boundary (Matsuda et al., 2016). Dpp acts as a morphogen and activates several genes across the anterior-to-posterior axis of the wing. Of the genes controlled by Dpp signaling, I examined *spalt (sal)* and *optomotor blind (omb)*, transcription factors shown to play roles in placing L2 and L5 in *D. melanogaster* wings.

(1.a) *Optomotor blind*

Optomotor blind is a transcription factor whose expression is activated by Dpp signaling in the space between the L2 and L5 proveins of the *D. melanogaster* wing disc. While *omb* is expressed across a wide range in the disc, it seems to be only relevant to vein development in the L5 provein where its expression is required to initiate expression of *abrupt* and genes of the *iroquios*-complex (*iro-C*). These genes are necessary for L5 provein development. In *D. melanogaster* mosaic analysis, *omb* null clones overlap the L5 region, no vein formation is observed (Cook et al., 2004). Therefore, I hypothesized *omb* knockdown in *J. haematoloma* and *O. fasciatus* would result in elimination of one or more posterior veins.

RNAi targeting *omb* during the fourth instar in both species did not result in any apparent vein phenotypes and had no significant impact on ability to complete adult molt (*J. haematoloma*: n=20, completed adult molt = 14, Fisher's exact test $p = 0.709$; *O. fasciatus*: n=20, completed adult molt = 17, $p = 1$). These results suggest that if *omb* is involved in

wing or vein development in either species, its role exists prior to the fourth instar. It is possible that activation of *ab* through *brinker (brk)*, another transcription factor expressed posterior to *omb*, is sufficient to promote vein development (Cook et al., 2004). Additional experimentation is required to determine when or if *omb* participates in vein placement in *J. haematoloma* or *O. fasciatus*.

(1.b) *Spalt*

Drosophila has two *spalt (sal)* genes involved in wing development, *spalt major* and *spalt-related*. These genes are transcribed throughout the Dpp signaling gradient at levels depending on Dpp concentration. Together, *salm* and *salr* influence the placement of the L2 vein and inhibit cells from taking on L5 vein identity. Removal of both genes results in a loss of the L2 vein. The most recent model explaining *sal* involvement in L2 development (de Celis and Barrio, 2000) states cells expressing high levels of both genes are unable to transcribe transcription factors of the *knirps*-complex (*kni-C*) which are necessary for L2 identity. As Dpp concentration decreases further from the anterior-posterior compartment boundary less *salm* and *salr* are transcribed. This decrease allows transcription of *kni-C*, which then goes on to activate EGFR signaling necessary for vein development. Therefore, knocking down either *sal* gene would promote expression of *kni-C* more posterior than normal, leading to ectopic L2 development closer to the AP compartment boundary. Posterior of the anterior-posterior compartment boundary, *salm* and *salr* inhibit expression of transcription factors of the *iroquois*-complex (*iro-C*). *iro-C* transcription factors are responsible for L5 identity. Clones lacking *salm*, *salr*, or both genes thus may lead to formation of ectopic L5 anterior to its normal site (Blair, 2007).

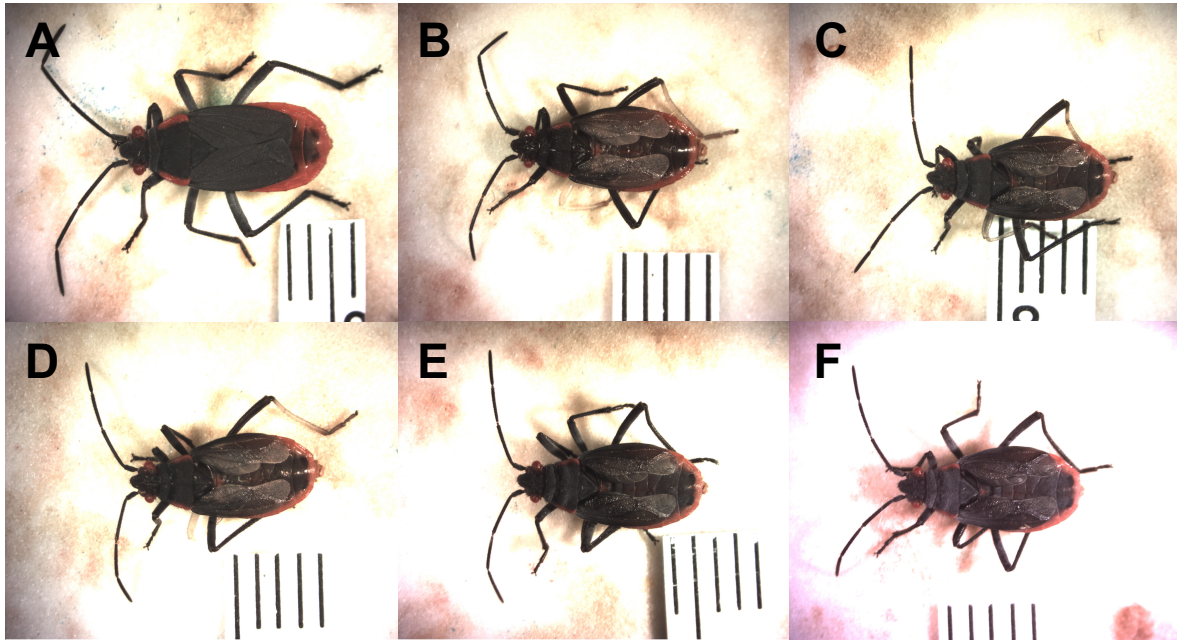


Figure 6. Knockdown of *spalt* in *Jadera haematoloma* significantly delays normal forewing melanization. (A) *gfp* RNAi control within 24 hours of adult molt (B) *sal* RNAi hours within 12 hours of adult molt (C) same individual 7 hours post initial imaging (D) 22 hours post (E) 47 hours post (F) 150 hours post ($n = 7$, $p = 5.828 \times 10^{-4}$)

Only one *spalt* ortholog exists in the genomes of *J. haematoloma* and *O. fasciatus*. Therefore, I hypothesized that ectopic vein phenotypes similar to those observed in *D. melanogaster* would result from *sal* knockdown in these species. No ectopic vein phenotypes or obvious loss-of-vein phenotypes were observed, but distinct atypical forewing pigmentation was observed in both species. Wild type *J. haematoloma* rapidly melanize their wings within a few hours after completing the adult molt. Meanwhile, *sal* knockdown in this species resulted in reduced melanization in the majority of the wing (Figure 6; Fisher's exact test $p = 5.828 \times 10^{-4}$). Some individuals displayed melanization delays in their legs or antennae, but these appeared to resolve within a few days of molting. Similarly, wildtype *O. fasciatus* melanize their wings within hours of molting while *O.*

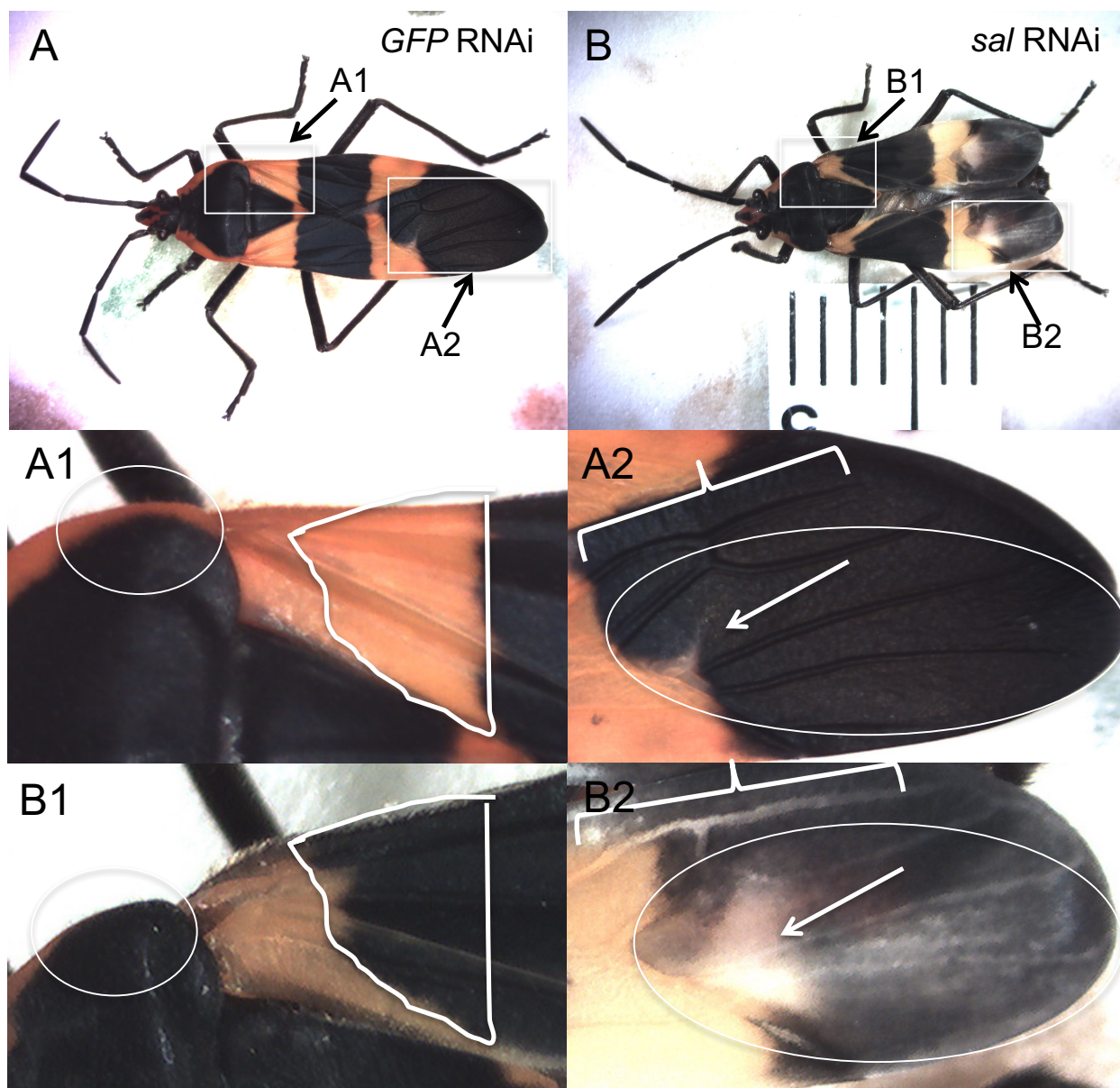


Figure 7. *Sal* knockdown via RNAi results in an increase in pigmentation of the proximal forewing and lateral pronotum (B1) while pigmentation is lost in the distal forewing and first anal vein (B2). (A) *GFP RNAi* (B) a representative *sal RNAi* phenotype. Boxes in both A and B denote zoomed regions. (A1 and B1) Proximal region of the wing and lateral pronotum. RNAi causes an increase in pigmentation of the proximal forewing (outline) and lateral margin of the pronotum (circle). (A2 and B2) Distal region of the wing. Pigmentation decreases in the distal black region (circle) specifically around the point indicated by the arrow. Additionally, the first anal vein (bracket) loses pigmentation suggesting some developmental interference. All atypical phenotypes were observed to some degree in 91% of individuals ($n=11$, $p = 3.402 \times 10^{-5}$)

fasciatus sal knockdown resulted in abnormal melanization characterized by decreased distal forewing pigmentation and increased proximal forewing and lateral pronotal

pigmentation. Additionally, *sal* knockdown appears to eliminate development of the juga, the proximal-posterior lobe, in the *O. fasciatus* hindwing (Figure 7, $n = 11$, $p \rightarrow 0$).

Previous work by Liu and colleagues (2014) demonstrated that intact venation is required in *O. fasciatus* for transport of melanin precursors throughout the wing after adult molt. Similar loss-of-pigment phenotypes have also been made in some *Drosophila* when vein development is interfered with (True et al., 1999). In *O. fasciatus*, increased proximal pigmentation in our trials may arise from two causes. The first explanation is that melanin precursors are being converted to melanin in an atypical pattern due to interference with the pattern of *sal* expression pattern. In this model, *sal* might be required for transcription of inhibitors melanin-producing proteins in the proximal forewing and lateral pronotum, but when it is knocked down the removal of these inhibitors allow for melanin production. It is also possible that normal melanin production patterning is present in the forewing, but precursors cannot be distributed throughout the wing due to incomplete vein development. This model might also lead to the observed buildup of pigment in proximal wing.

Under the second model, I would conclude *sal* expression is required between the fourth instar for maintaining proper vein development, but vein identity is already established. This is a departure from the role of *sal* in *D. melanogaster* wing development, however, most *D. melanogaster* studies focus on late (mid third instar and later) wing development (Blair, 2007) and utilize cell lines rather than systemic knockdowns. It is possible that *sal* plays a similar role in *D. melanogaster* wing development earlier in development, but additional study is needed in that area. Similar results for *sal* knockdown

in *O. fasciatus* and *J. haematoloma* do support functional homology of *sal* in vein development within Hemiptera. The loss of the *O. fasciatus* juga is a novel phenotype that lacks a clear explanation, but provides an interesting objective for future inquiry.

(2) *Distal-less*

Distal-less (Dll) is a gene encoding a homeobox transcription factor necessary for the development of distal regions of appendages in insects including *D. melanogaster* and *O. fasciatus* (Angelini and Kaufman, 2004).

The intersection of *dpp* and *wingless* expression areas mark where *Dll* is expressed in

developing appendages. Previous *Dll* knockdowns in fifth instar *J. haematoloma* (D.R. Angelini, unpublished data) suggest that this gene plays a role in membrane development of long and short-winged morphs. I repeated this knockdown in fourth instar *J. haematoloma* to confirm the effects of *Dll* on membrane development. Individuals subjected to *Dll* RNAi knockdown did display reduced membrane development, but few completed adult molt (Figure 8, n=7). some individuals displayed reduced forewing pigmentation. However, wildtype bugs that experience difficulties getting their wings out



Figure 8. Lateral view of a *Dll* knockdown individual. It is impossible to determine if the stunted growth of the wing membrane region is due to gene knockdown in the wing or pleiotropic molting difficulties resulting from the knockdown.

of the fifth instar cuticle tend to melanize only the proximal portions of the forewing that are exposed to the environment. Therefore, the observed differences in coloration likely had more to do with the *Dll*-knockdown bugs' inability to escape their exuvia than the knockdown effect. The observed reduction of the distal wing support prior findings that *Dll* is required for proper membrane development, and suggest that the gene may be involved in the mechanism for reducing membrane size in short-winged individuals. The observation that some individuals would lose legs is less easily explained, but it may have tie-ins to work that showed *Dll* plays a role in maintaining leg integrity in *Tribolium* (Suzuki et al., 2009).

(3) SERUM RESPONSE FACTOR

Drosophila Serum Response factor (srf) is a transcription factor expressed in intervein regions throughout the wing disc while cells included in proveins down-regulate expression of the gene at an early stage. *D. melanogaster* clones lacking *srf* develop so that in some areas the dorsal and ventral cell layers of the wing are not in contact while extensive ectopic venation occurs elsewhere in the intervein space (Montagne et al., 1996). Similar phenotypes are observed when the a *srf* ortholog is knocked down via RNAi in *T. castaneum* (Tomoyasu et al., 2009) *Srf* knockdown during the fourth instar in *J. haematoloma* resulted in bugs displaying inflated wing pads during the fifth instar and a general inability to complete adult molt ($n=12$, $p = 7.396 \times 10^{-7}$). Furthermore, the wings of bugs attempting adult molt were severely reduced in size and consistently inflated containing a fluid that was presumably hemolymph. No venation was observed in these

bugs' wings, ectopic or otherwise. However, some individuals' wings displayed bristle patterns roughly approximating the normally occurring venation pattern.

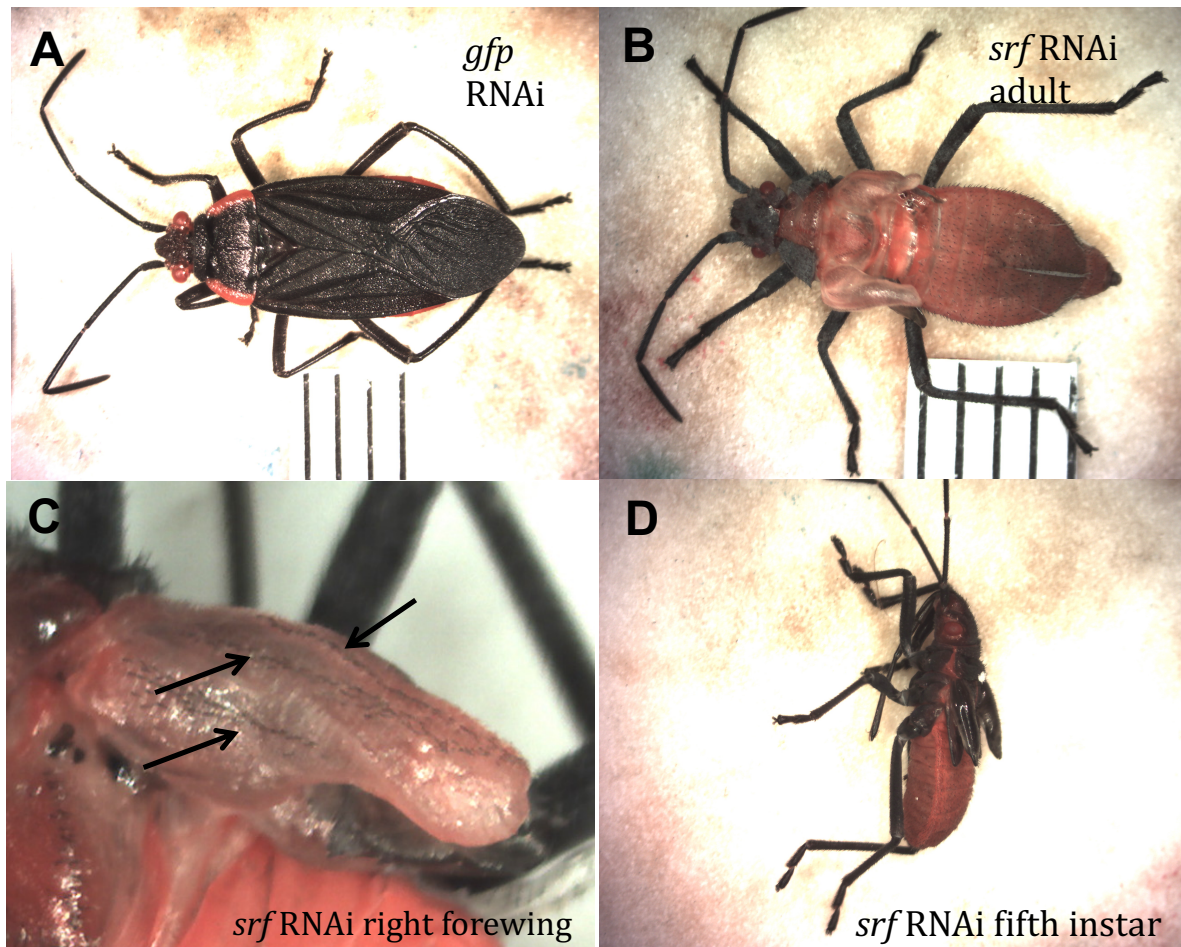


Figure 8. Serum response factor knockdown in *Jadera haematoloma* results in an inability to complete adult molt and inflated wings and wing pads in treated adults and fifth instar nymphs. (A) Normally developed adult *J. haematoloma* subjected to *gfp* RNAi as control. (B) Adult subjected to *srf* RNAi. Cuticle has been removed from the forewings to highlight their inflation. (C) Close up image of an adult forewing from a *srf* RNAi individual. Arrows highlight bristle patterning emulating venation, suggesting that vein identity is established before fourth instar when RNAi was performed. (D) A fifth instar *J. haematoloma* nymph displayed laterally to demonstrate inflation of wing pads resulting from *srf* RNAi. (*srf*, Serum response factor; *gfp*, green fluorescent protein. *Gfp* is exogenous to *J. haematoloma*)

Wildtype *J. haematoloma* develop bristles along their longitudinal veins. Therefore, the presence of bristles approximating vein patterning in *srf* knockdowns suggests the cells

that produced bristles have vein identity, and that identity was established prior to the fourth instar. The lack of ectopic venation or bristles elsewhere in the wing further supports this notion. Wing inflation observed during both the fifth instar and adult molts indicates that *srf* expression is required for maintaining adhesion between the dorsal and ventral epithelia of the wing from the at latest the fourth instar to adulthood. The reduced wing size in attempted adult morphs reveals that *srf* may be required for normal proliferation of intervein cells. These conclusions suggest that *srf* plays a similar role in maintaining dorsal-ventral adhesion in both *J. haematoloma* and *D. melanogaster*, but of its role in determining provein identity occurs earlier in *J. haematoloma*.

(4) EPIDERMAL GROWTH FACTOR SIGNALING – EGFR

In *Drosophila*, interrupting EGF-signaling results in a loss of wing vein development (Diaz-Benjumea and Garcia-Bellido, 1990). I hypothesized that *Egfr* knockdown in *O. fasciatus* would result in loss of wing veins as well. However, no bugs that completed adult molt displayed atypical phenotypes in their wings or elsewhere (Figure 2B). This result was unexpected due to drastic wing phenotypes observed with EGF-signaling disruption in *D. melanogaster*. When considering *O. fasciatus* wing development, the lack of an atypical phenotype with *EGFR* knockdown may indicate that EGF signaling is not active in the developing wing tissue during the fourth instar. It is possible that EGF signaling in provein cells takes place at earlier instars, a stark difference from *D. melanogaster* where EGF signaling is crucial in the middle of the final larval instar. This difference in timing follows our previous observations in *sal*, *omb*, and *srf* knockdowns, as each of those genes is known to interact directly or indirectly with EGF pathway members in determining provein

identity. The lack of an abnormal phenotype from this knockdown therefore supports the theory that the timings of wing development differ between Hemiptera and Diptera. However, It should be noted that a lower concentration of *EGFR* dsRNA was used in this knockdown due to concerns over potential off-target effects or high lethality caused by the overall importance of EGF signaling. The lowered concentration of *EGFR dsRNA* used may have not been enough to inhibit *EGFR* translation. The experiment should be repeated with a higher concentration and qRT-PCR analysis of mRNA prevalence for verification.

CONCLUSIONS

(1) Vein Development and Vein Homology Between D. melanogaster and Hemiptera

The goal of this study was to investigate how genetic processes governing wing and vein development in exopterygote species compare to what is known about wing and vein development in endopterygotes. Taken as a whole, the lack of ectopic vein development in any of the RNAi knockdowns performed in both *O. fasciatus* and *J. haematoloma* point toward provein identity being established prior to the fourth instar, when knockdowns were administered, in both species. This conclusion is strongly supported by our observation of bristles approximating normal vein patterning in otherwise highly deformed wings resulting from *srf* knockdown. Such a result suggests that vein cell identity is established prior to the fourth instar, and the provein cells maintain that identity even through disruption of normal wing development. It is possible that the genes I examined play no role in provein specification, but this conclusion is unlikely due to wings being a plesiomorphic trait for both Diptera and Hemiptera and the relative homology between the wings of all pterygotes. None of our findings were able to establish homologies between

specific veins of the *D. melanogaster* wing and veins in either of the two hemipteran species studied.

There is reason to suspect that the action of various genes in vein development and maintenance differ between species. The strongest support for this hypothesis comes from *sal* knockdowns. Removing *sal* expression resulted in dramatically altered forewing pigmentation in both *J. haematoloma* and *O. fasciatus* (Figures 6 and 7). Similar phenotypes have been observed in both *O. fasciatus* and multiple *Drosophila* species through vein disruption (Liu et al., 2014; True et al., 1999). It can therefore be concluded that the atypical pigmentation phenotypes resulting from *sal* knockdown in both our study species are due to improper vein development rather than any direct role that *sal* may play in melanization. This idea is further supported by the apparent buildup of black pigment observed in *O. fasciatus* proximal forewings and nota, observations suggesting pigment precursors are being properly produced but are not transported distally. To the best of our knowledge similar experiments with *spalt* have not been performed in *D. melanogaster* or other species, leaving open the possibility that *spalt* plays a similar role in vein development beyond the Hemiptera.

(2) Intervein and Wing Development

Our results suggest *srf* is responsible for maintaining intervein cell adhesion at least as early as the fifth instar in *J. haematoloma*, similar to how it functions in *D. melanogaster* intervein cells. Similar blistered phenotypes to those observed in *J. haematoloma* have long been documented in both *Tribolium* and *Drosophila* (Montagne et al., 1996; Tomoyasu et al., 2009). Therefore, it can be concluded that the function of *srf* as a marker and maintainer of

intervein spaces in the developing wing is conserved between endopterygotes and exopterygotes.

(3) Future directions

To further investigate wing and vein development in Hemiptera, it will be necessary to repeat these experiments at earlier life stages. Earlier knockdown could be accomplished simply by injecting earlier, maternal RNAi, in which a female is injected with dsRNA then mated so that her offspring are exposed to dsRNA starting at fertilization, or through RNAi by feeding. However, I have never had great success getting bugs to adulthood when treating them with early RNAi.

Visualizing gene expression in primordial wing tissues of both *O. fasciatus* and *J. haematoloma* through whole mount *in situ* hybridization (WMISH) or antibody staining will allow more specific comparisons between species. Additionally, this approach would potentially allow a more detailed study of vein homology by allowing comparison of gene expression in specific veins of the hemipteran species to known expression patterns in *D. melanogaster*. I have attempted to perform WMISH for *O. fasciatus Dll* in fifth instar primordial wing tissue, but acquiring tissue that is receptive to staining has proved difficult.

Further study of wing development in non-drosophilan insects is necessary to determine if the mechanisms that have been studied so thoroughly in *D. melanogaster* are valid outside of Diptera.

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