ABSTRACT

Title of Thesis: HEMIPTERAN INSECTS AS MODELS FOR UNDERSTANDING SEGMENTATION

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Although segmentation is highly conserved in arthropods, diverse mechanisms underlie segmentation. Pair-rule genes (PRGs) are a group of genes controlling segmentation in *Drosophila melanogaster*, a holometabolous insect. While *Drosophila* are long-germ insects, most insects add segments sequentially. Studying the role of PRGs in sequentially-segmenting species will provide a deeper understanding in terms of developmental biology. Here, I studied two such insects: *Halyomorpha halys* and *Oncopeltus fasciatus*, hemimetabolous insects in a sister order to Holometabola. I annotated segmentation genes in the *Halyomorpha* genome and tested its response to RNA interference which I showed to be effective in this species for the first time. I further showed that three orthologs of *Drosophila* PRGs are present in the *Oncopeltus* genome and are expressed during stages at which segments are specified. Surprisingly, only one of these orthologs is expressed in a

PR-pattern, indicating that PRG expression and function have changed during insect evolution.

HEMIPTERAN INSECTS AS MODELS FOR UNDERSTANDING SEGMENTATION

by

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Dedication

To my parents, Xuexin and Yaping

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To my parents, thank you for your unconditional love and support. You allow me to explore the world and always are my strongest supporters, listening to me and guiding me through all the difficulties.

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List of Abbreviations

AEL: after egg laying

Antp: Antennapedia

A. mellifera: Apis mellifera

AP-axis: anterior-posterior axis

bcd: bicoid

bp: base pair

cact: cactus

cad: caudal

C. elegans: Caenorhabditis elegans

croc: crocodile

Dfd: Deformed

dl: dorsal

D. maculatus: Dermestes maculatus

D. melanogaster: Drosophila melanogaster

dpn: deadpan

dsRNA: double-strand RNA

en: engrailed

eve: even-skipped

FOX: forkhead box

ftz: *fushi tarazu*

ftz-f1: fushi tarazu factor 1

gt: giant

h: hairy

HOX: homeobox-containing

hb: hunchback

H. halys or *Hhal* or *Hh: Halyomorpha halys*

kni: knirps

knil: knirps-like

Kr: Kruppel

lz: lozenge

nos: nanos

odd: odd-skipped

O. fasciatus or *Ofas* or *Of: Oncopeltus fasciatus*

otd: orthodenticle

RH: Relative humidity

prd: paired

pRNAi: parental RNAi

PRGs: Pair-rule genes

RNAi: RNA interference

run: runt

SAZ: segment addition zone

Scr: Sex combs reduced

slp: sloppy paired

S. martitima or Smar: Strigamia maritima

T. castaneum: Tribolium castaneum

tll: tailless

TSA: Transcriptome Shotgun Assembly

Chapter 1: Background

1.1 Genetic hierarchy determines anterior-posterior axis segmentation in *Drosophila*

Genetic screens performed by Nusslein-Volhard and Wieschaus revealed zygotic mutations that caused segmentation defects in *Drosophila melanogaster* (*D. melanogaster*) (Nüsslein-Volhard & Wieschaus, 1980). Since then, a set of genes responsible for those specific defects were isolated and well investigated. A hierarchy of genes determine how anterior-posterior polarity is established in *Drosophila* embryos (Akam, 1987; Lewis, 1978; Nüsslein-Volhard & Wieschaus, 1980). This hierarchy is summarized in Figure 1. The hierarchy is initiated by maternal effect genes. Transcripts from maternal effect genes are localized at either the anterior or posterior end of the egg and form a gradient of protein in the egg. For example, *bicoid* transcript is localized in the anterior pole of egg, and the head is missing in *bicoid* mutants (Driever, Siegel, & Nusslein-Volhard, 1990). Gap genes read informational gradients set up by the maternal genes and determine broad regions in the embryo. The loss of several contiguous segments was observed in gap gene mutants. For example, in *Krüppel* mutants, absence of thoracic and anterior abdominal segments is observed (Nüsslein-Volhard & Wieschaus, 1980; Eric Wieschaus, Nusslein-Volhard, & Kluding, 1984). Pair-rule genes (PRGs) are expressed as a result of differing concentrations of gap and maternal proteins and are expressed in the primordia of alternate segmental units, which are absent in pair-rule mutant embryos. For example, seven even-numbered parasegments are missing in *fushi tarazu (ftz)* mutants (Wakimoto & Kaufman, 1981). Since segment polarity genes determine

parasegment boundaries and anterior-posterior polarity in each segment, segment polarity mutants show defects in every segment. Lastly, homeotic genes determine the unique identities of each segment. This type of mutation results in the transformation of one or more segments to the identity of another segment. For example, in *Antennapedia* mutants, *Antp* is ectopically expressed in the head, and antennae are transformed to T2 legs (Schneuwly, Klemenz, & Gehring, 1987). In general, the genes in this segmentation hierarchy are expressed in the primordia of

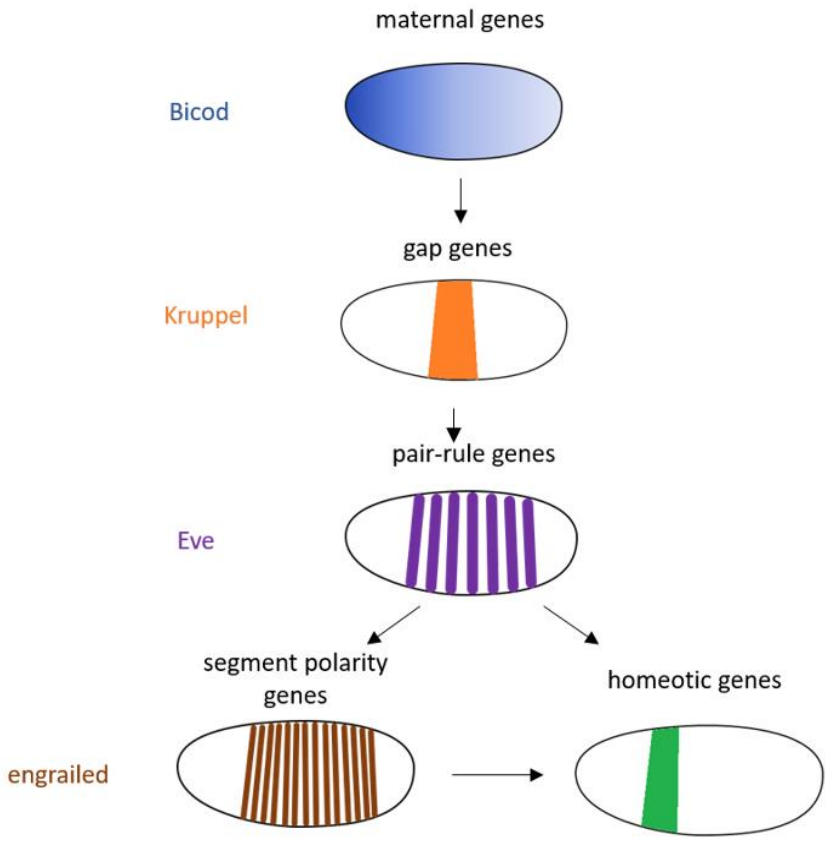

Figure 1. The genetic regulation of segmentation in *Drosophila*. Modified by Mengyao Chen from Molecular Biology (Alberts et al., 2002).

regions missing in corresponding mutants. For example, *fushi tarazu* (*ftz*) and *even-skipped* (*eve*) are expressed in seven transverse stripes in the primordia of the odd and

even numbered parasegments, respectively. Similarly, *engrailed* (*en*) is expressed in fourteen stripes in the primordia of the posterior compartment of each segment, which is missing in *en* mutants (Kornberg, 1981).

1.2 Long germ and Short germ in insect segmentation

Morphological segmentation is shared among insects. However, different insects use different segmentation modes to form these segments (Davis & Patel, 2002; P. Z. Liu & Kaufman, 2005b; Peel, Chipman, & Akam, 2005). *D. melanogaster* is a highly divergent insect with respect to segmentation in that all segments are specified more-or-less simultaneously at the blastoderm stage. This type of segmentation is known as the long-germ type. By contrast, for most insects, only the anterior segments are specified at the blastoderm stage. The more posterior segments are added sequentially along anteroposterior axis from the posterior growth zone. This is referred to as sequential segmentation. Sequential segmentation is further divided into two types: short-germ insects establish very few segments at the blastoderm stage, while intermediate-term insects, such as *Dermestes maculatus* (*D. maculatus*), establish head and thoracic segments in the germ anlage (Xiang, Forrest, & Pick, 2015). Long-germ insects usually have a large initial germ anlage, while short-germ insects have a small germ anlage that is related to their embryo size (Davis & Patel, 2002). Compared with short-germ insects, long-germ insects generally develop more quickly and utilize nurse cells during oogenesis (Davis & Patel, 2002). However, both short- and long-germs features have been reported in silk moth (*Bombyx mori)* embryogenesis. Even though its segmentation proceeds sequentially as short germ, it also shows some long-germ features such as large

embryonic anlage (Nakao, 2012).Insects with diverse modes of segmentation are widespread throughout insect taxa (Figure 2). Holometabola includes insects displaying all three different modes, while only one germ type has been observed so far in Hemiptera.

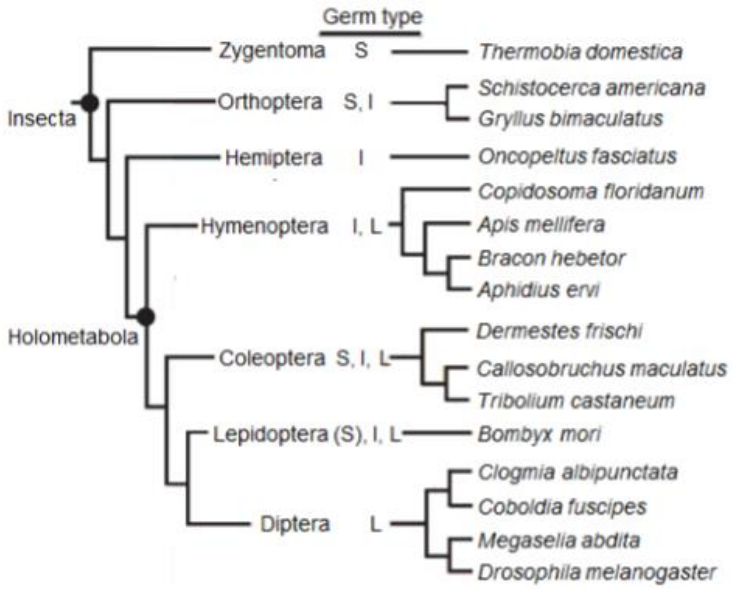

Figure 2. Different modes of segmentation in insect phylogeny. While short- and intermediate germ types have been reported throughout insect phylogeny, the long-germ mode is restricted to Holometabola. Figure is adapted from (Liu and Kaufman, 2005a).

1.3 RNA interference (RNAi) is a promising approach

When a double-stranded RNA (dsRNA) is introduced into a cell, it initiates a process that silences the corresponding mRNA (Huvenne & Smagghe, 2010). RNAi was first discovered in *Caenorhabditis elegans* (*C. elegans*) as a gene-silencing mechanism (Fire et al., 1998), and subsequently shown to function in fungi, plants, and animals, such as insects (Mello & Conte, 2004). This approach has been widely used in recent years to study gene function in several insect model systems. As a well-studied insect model, *D. melanogaster* was the first insect in which RNAi gene

knock down function was demonstrated. Kennerdell et al. (1998) showed that injection of dsRNA corresponding to the segmentation genes *ftz* or *eve* into *Drosophila* embryos resulted in segmentation defects (Kennerdell & Carthew, 1998). RNAi was also shown to work well in the flour beetle *Tribolium castaneum (T. castaneum)*: injection of *Deformed* (*Dfd*) dsRNA into *T. castaneum* embryos resulted in *Dfd* loss-off-function defects. Further studies showed that RNAi works in all life stages of *T. castaneum* (Brown, Mahaffey, Lorenzen, Denell, & Mahaffey, 1999; Bucher, Scholten, & Klingler, 2002; Tomoyasu & Denell, 2004). Bucher et al. (2002) noticed that injecting dsRNA into the haemocoel of female *T. castaneum* resulted in knock down of zygotic gene function in the offspring embryos. This phenomenon is called parental RNAi (pRNAi) and was first discovered *in C. elegans* (Fire et al., 1998). pRNAi also has been proven to be effective with other insects, such as the silk moth *Bombyx mori* and the milkweed bug *Oncopeltus fasciatus* (*O. fasciatus*) (P. Z. Liu & Kaufman, 2004; Quan, Kanda, & Tamura, 2002). Overall, many experiments have shown that delivering dsRNA into insects by injection is an efficient way to knock down genes. Thus, RNAi became a new application in functional genomics and the study of developmental evolution.

1.4 PRGs expression and function vary in insects

Although orthologs of the *Drosophila* PRGs are found in other insect species, accumulating evidence suggests that their function has varied during evolution. For example, *ftz* is expressed a classical Pair-rule (PR) pattern in *D. melanogaster* and *ftz* mutants present PR defects. However, although *ftz* is expressed in short-germ beetle *T. Castaneum* and long-germ insect honeybee *Apis mellifera (A. mellifera)* in PR

patterns, loss of *ftz* did not produce a PR defects in *T. Castaneum,* and in *A. mellifera, ftz* RNAi caused an absent anterior but with no posterior segmentation defects. (Nüsslein-Volhard & Wieschaus, 1980; Stuart, Brown, Beeman, & Denell, 1991; Wakimoto, Turner, & Kaufman, 1984; Wilson & Dearden, 2012). This indicates that even if gene orthologs are expressed in similar patterns, the gene function may still vary in different species. So far, the function of PRGs has been examined in only a limited number of insects. Shown in Table 1 is a short summary of information available about PRGs in insects with different segmentation modes, including *Drosophlia melanogaster* (longgerm, Diptera), *Bombyxi mori* (short germ; Lepidoptera), *Tribolium Castaneum* (short germ; Coleoptera), *Apis mellifera* (long germ; Hymenoptera) and *Gryllus bimaculatus* (intermediate germ, Orthoptera). From the summary table, we can see that PRGs show divergence in expression and function even in the limited number of species that have been examined to date.

				eve		ftz		prd		runt		odd		h		opa		slp		ftz-f1	
				Expr.	Func.	Expr.	Func.	Expr.	Func.	Expr.	Func.	Expr.	Func.	Expr.	Func.	Expr.	Func.	Expr.	Func.	Expr.	Func.
Insecta	Holometabola	Diptera	Drosophlia melanogaster (fruit fly)/long germ mode	PR-S	PR	PR	PR	PR-S	PR	PR-S	PR	PR-S	PR	PR	PR	U-S	PR	PR-S	PR	U	PR
		Lepidoptera	Bombyxi mori (domestic silkworm)/short germ mode	PR	T					PR-S	T	PR	T								
		Coleoptera	Tribolium Castaneum (red flour beetle)/short germ mode	PR-S	T	PR	N	PR-S	PR	PR	T	PR-S	T	PR-S	H	S	N	PR-S	PR	PR	PR
		Hymenoptera	Apis mellifera (honey bee)/long germ mode	PR-S	D	PR	D			PR-S	PR			PR	D						
	Hemimetabola	Orthoptera	Gryllus bimaculatus(two-spotted cricket)/intermediate germ mode	PR-S	T																

Table.1 Summary of PRG orthologs expression and function in insects. Abbreviations: Expr., expression; Func., function; PR, pair-rule pattern or defect; S, segmental pattern; U, ubiquitous expression; N, no stripes pattern or defect; T, truncated defect; D, disrupted segmentation; H, headless defect. Reference for information listed in this table:(Aranda, Marques-Souza, Bayer, & Tautz, 2008; S. J. Brown, Hilgenfeld, & Denell, 1994; Susan J. Brown & Denell, 1996; Choe & Brown, 2007; Choe, Miller, & Brown, 2006; Coulter et al., 1990; Eckert, Aranda, Wolff, & Tautz, 2004; Grossniklaus, Pearson, & Gehring, 1992; Heffer & Pick, 2013; Howard & Ingham, 1986; Kilchherr, Baumgartner, Bopp, Frei, & Noll, 1986; Klinger & Gergen, 1993; W. Liu, Yang, Jia, Miao, & Huang, 2008; Macdonald, Ingham, & Struhl, 1986; Mito et al., 2007; Nakao, 2012, 2015; Osborne & Dearden, 2005; Patel, Condron, & Zinn, 1994; Schröder, Jay, & Tautz, 1999; Sommer & Tautz, 1993; Stuart et al., 1991; Wakimoto et al., 1984; Wilson & Dearden, 2012; Yu et al., 1997)

Chapter 2: Establishment of *H. halys* colony and study of gene function with RNAi

2.1 Introduction

Most studies of arthropod evo-devo have utilized holometabolous insects, especially *Drosophila melanogaster*, and, more recently, *Tribolium castaneum* (*T. castaneum*) which are well-developed laboratory model systems with a wide array of resources available for communities of researchers (Brown et al., 2009; T. G. S. Consortium, 2008; M. M. Green, 2010; Mohr, Hu, Kim, Housden, & Perrimon, 2014; Pick, 2017; Stuart et al., 1991; Ugur, Chen, & Bellen, 2016) . However, focusing on only holometabolous insects may limit our ability to probe the origins of diversity within insects. Additional systems are required for comparative studies outside the holometabolous insects to understand the mechanisms controlling the development of insects that do not undergo metamorphosis.

The order Hemiptera, a sister group of Holometabola, which includes at least 54 families of the true bugs (Heteroptera), is the largest nonholometabolous insect order with more than 80,000 described species (Cryan & Urban, 2012; Weirauch & Schuh, 2011) . Hemiptera are hemimetabolous insects, sharing piercing, sucking mouth parts that cause harm to both plants and animals by virtue of direct physical damage, as well as by transmission of pathogens. The order includes major agricultural pests, such as aphids, stink bugs, and white flies, as well as pests of humans, such as bed bug and kissing bugs, vectors of serious human disease (reviewed (M, M, & E, 2015; Nunes-da-Fonseca, Berni, Tobias-Santos, Pane, & Araujo, 2017). Within the order Hemiptera, the milkweed bug *Oncopeltus fasciatus*

(*O. fasciatus*, Hemiptera: Lygaeidae) is emerging as a model species (see, for examples, Angelini & Kaufman, 2004, 2005; Chesebro, Hrycaj, Mahfooz, & Popadić, 2009; i5K Consortium, n.d.; J. Liu, Lemonds, Marden, & Popadić, 2016; Stahi & Chipman, 2016). Comparative studies within the hemimetabolous insects will provide more information to understand the basis of novelties, including for example, the large differences in host preference (plant feeders vs. human hosts), color patterns, and habitat choice seen within this clade.

With an estimated divergence from *O. fasciatus* of 244 MYA (Kumar, Stecher, Suleski, & Hedges, 2017), *Halyomorpha halys*, commonly known as the Brown Marmorated Stink Bug (*H. halys*, Hemiptera: Pentatomidae), represents a distant branch within Hemiptera, providing a useful phylogenetic point for comparative studies to *O. fasciatus. H. halys* is a polyphagous insect that feeds on tree fruits, vegetables, legumes, and ornamentals in the field and in nursery crops (reviewed in (Leskey et al., 2012; Nielsen & Hamilton, 2009). This is different from *O. fasciatus*, which is a highly specialized feeder. Another justification for studying *H. halys* is that it is a serious agricultural pest, that has caused significant damage in the Mid-Atlantic region of the U.S. in recent years (Khrimian et al., 2014; Leskey et al., 2012). With funding from USDA, a group of more than 60 scientists is working on finding pest management solutions for controlling *H. halys*. Studies of gene expression and gene function, as well as the ability to manipulate genes, will uncover information about the basic biology of *H. halys*, while providing novel means to make use of genetic approaches for pest control.

Since there were no available relevant molecular references for *H. halys* PRGs, all molecular methods needed to be established. I identified and manually annotated maternal genes, gap genes and all PRGs, with predicted intron and exon structure in a draft genome. I applied RNA interference (RNAi) successfully for this species, using the homeotic gene *Sex combs reduced* (*Scr*), demonstrating that molecular tests of gene function are feasible.

2.2 Methods and Materials

Insect husbandry and embryo collection

Former laboratory colonies of *H. halys* were initially reared as previously described (Taylor, Coffey, DeLay, & Dively, 2014). After several colonies collapsed using this approach, our lab switched to a rearing protocol kindly provided to us by Dr. Don Weber (Khrimian et al., 2014). We grew a new colony from ten egg masses provided by Dr. Weber's laboratory. In order to track the health of our colony and keep the most reproductive individuals together, different generations were kept separately. Briefly, eggs and first instar nymphs were kept in small petri dishes (5.5 cm in diameter) with wet cotton and pieces of fresh organic green beans. When nymphs reached the second instar, generally after 5 days, they were moved to clean plastic cylindrical containers (18.5 cm in diameter × 20.5 cm in height) with fresh organic green bean, sunflower and buckwheat seeds, and wet cotton. A piece of fine plastic mesh screen (32 × 32 mesh per inch) was also added to each adult cage as an egg-laying substrate. While *H. halys* egg masses stick to most surfaces fairly well, they can be easily peeled off of this plastic mesh. All *H. halys* cages were kept at 25 °C, RH of 55 ± 5%, with a 16 h light: 8 h dark photoperiod. Every other day, the

green beans were replaced in all cages and petri dishes. At this time, egg masses and any dead adults were removed from adult cages; the numbers of clutches and dead males and females were recorded so that cage population and female fecundity could be tracked. For timed egg collections, cages were checked every 4 h for newly laid eggs. The eggs were removed from the cages and kept in petri dishes under the same environmental conditions described above until the desired time points were reached. Under these conditions, the overall life cycle (egg to fertile adult) was ~ 1.5 months, with eggs hatching to the first nymphal stage in 5 days and each subsequent instar lasting between 5 and 10 days.

Identification of genes of interest

Prior to availability of genome or transcriptome data, degenerate PCR was carried out to isolate regions of *H. halys* orthologs of *Sex combs reduced* (*Hhal -Scr*). Primers were: ScrdegF 5′-CCRCARATHTAYCCRTGGATG-3′ and ScrdegR1 5′-CATRTGGYANGGNACRATRTTCAT-3′. Sequences were verified by comparison with transcriptome data. Assembled *H. halys* RNA-seq data (Ioannidis et al., 2014) in FASTA format were used to create a local *H. halys* BLAST database using the BLAST + package (Altschul, Gish, Miller, Myers, & Lipman, 1990). BLAST searches were carried out using the sequence of products generated by degenerate PCR, followed by TBLASTN using full-length *D. melanogaster* Scr protein sequences as the query sequences. Predicted *H. halys* genes were experimentally verified by reverse transcription PCR (RT-PCR) followed by Sanger sequencing. *Hhal-Scr*, GenBank: GBHT01003272.1 (Ioannidis et al., 2014).

Embryo fixation

To collect embryos, the plastic mesh egg-laying substrate was removed from the cage and embryos were simply peeled off the mesh and dropped into 2-ml centrifuge tubes, with ~ 20 embryos per tube. Embryos for in situ hybridization and immunostaining were aged to 18–72 h after egg laying (AEL). The fixation protocol was modified from that developed for *O. fasciatus*, kindly shared by Dr. Ariel Chipman's laboratory (Ben-David & Chipman, 2010). In brief, 600 µl of water was added to each tube of embryos which was placed in boiling water for 3 min and then placed on ice for 6 min. After the water was removed, 600 µl of heptane and 600 µl 4% paraformaldehyde (PFA) in PBS (0.137 M NaCl, 0.0027 M KCl, 0.0015 M KH2PO4, 0.008 M Na2HPO4) were added. Gentle shaking brought the embryos to the interface. Tubes were shaken vigorously on a Vortex mixer for 20 min. After shaking, the heptane and PFA were removed, and the embryos were rinsed once with heptane, then once with methanol. The embryos together with methanol were then put into wells on depression concave slides and the eggshells, which are rather thick in this species, were manually removed with forceps under a dissection microscope. The embryos were then passed through 75, 50, and 25% methanol/PBST (phosphate-buffered saline, 0.05% Tween® 20) gradient rinses for rehydration. The rehydrated embryos were fixed with 4% PFA in PBST for 90 min on a nutator. The fixed embryos were then washed three times with methanol and stored in methanol at − 20 °C for future use.

cDNA synthesis and RT-PCR

Embryos were collected every eight hours over a six-day period and maintained at 25°C until reaching the appropriate age. Enough RNAlater (Invitrogen) was added to cover embryos, and tubes were stored at -20°C until RNA extraction. RNA was extracted using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen). To synthesize cDNA for RT-PCR, RNA from three appropriately aged 0-12 h AEL collections was pooled for each time point (0-24 h, 24-48 h, 38-72 h, 72-96 h, and 96-120 h) to ensure that a diversity of ages within each time frame was represented. cDNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen). All primers were designed based on sequences in the *H. halys* draft genome. To check for primer specificity, cDNA from the five collections described above was mixed, yielding 0-120 h cDNA. Primers were used to amplify this mixed stage cDNA, and PCR products were sequenced (Genewiz).

Double-strand RNA (dsRNA) synthesis

Primers were designed to amplify a 327-bp region of *Hhal-Scr*, with T7 promoter sequences added to the 5′ end of both forward and reverse primers. Primer sequences: Hh-ScrFT7 5′-
TAATACGACTCACTATAGGGAGAGCAGGACCTGACTACGTCCTC-3′
and Hh-ScrRT7 5′-
AATACGACTCACTATAGGGAGATCCAGCTCCAGCGTCTGGTA-3′ (T7 promoter sequences underlined). PCR was carried out with cDNA that had been made from 0-to-6-day-old embryos using the manufacturer's recommended standard conditions (Reverse Transcription system, Promega). The PCR products were

purified and sent out for sequencing (Genewiz) to confirm that the correct gene was amplified. The purified PCR product (Gel Extraction Kit, Qiagen) was used as the template for in vitro transcription using the MEGAscript® T7 Transcription Kit (Life Technologies) following the manufacturer's recommendations. The final product was treated with DNase from the transcription kit to degrade the DNA template. In order to anneal the in vitro transcription product single-stranded RNAs, transcription products were heated to 94 °C for 5 min and slowly cooled in a PCR machine by decreasing the temperature 0.8 °C every minute until 45 °C was reached (TPersonal, Biometra). The annealed double-strand RNA was precipitated with 1/10 volume of 3 M sodium acetate (pH, 5.2) and 2× volume of ethanol and was then dissolved in 20–40 μl injection buffer (0.1 mM NaH2PO4, 5 mM KCl, pH 6.8), and stored at − 20 °C. The concentration of double-stranded RNA was measured with a NanoDrop spectrophotometer.

The dsRNA was injected into adult female *H. halys* using a Hamilton syringe and needle, as described previously for *O. fasciatus* (P. Z. Liu & Kaufman, 2004). *H. halys* females were anesthetized under CO2, and the Hamilton needle was inserted into the abdomen between the third and fourth abdominal sternites. Each female *H. halys* was injected with 2–6 μl Scr dsRNA at a concentration of 3 μg/μl. Four-to-ten individual females were injected in each experiment. After injection, the needle was held at the injection site for approximately 1 min to prevent leakage from the injection site. Injected females were kept separately for 1 day to allow for recovery before 2–3 males were added to each cage for mating. Eggs were collected and

allowed to hatch, and defects were assessed in both hatched nymphs and unhatched embryos.

2.3 Results

2.3.1 Establishment of *H. halys* laboratory colony

I started this colony with 10 egg masses from Dr. Weber's lab in USDA, which has reported keeping colonies in lab culture for at least 10 generations (Khrimian et al., 2014). Their *H. halys* adults were collected in soybean fields at the University of Maryland Beltsville Research Farm. For our colony, all rearing steps followed precisely the instruction of Dr. Weber's laboratory protocol (Khrimian et al., 2014). Briefly, eggs and first instar nymphs were kept in small petri dishes with wet cotton and pieces of fresh organic green beans. When nymphs reached the second instar, they were moved to clean hard plastic round containers (18.5 cm in diameter × 20.5 cm in height) with organic fresh green beans, sunflower and buckwheat seeds, and wet cotton. A piece of fine mesh screen (32x32 mesh per inch) was also added to each adult cage as an egg laying substrate. Different generations were kept separately with clear labels. All *H. halys* cages were kept at 25°C, RH of 55 ± 5%, with a 16 hour light: 8-hour dark photoperiod. Every other day, the green beans were replaced in all the cages and petri dishes. Egg masses and any dead adults were removed from adult cages. For timed egg collections, cages were checked every four hours for newly laid eggs. The eggs were removed from the cages and kept in petri dishes under the same environmental conditions described above until the desired time points were reached. However, after successfully keeping them for 4 generations, the colony collapsed without any sign. Note that prior attempts by other

lab members to maintain long-term colonies, using different rearing protocols, had also failed. In early 2017, I started a new colony with 40 field-collected adults from Sugarloaf Mountain in Maryland, collected with the Shrewsbury lab. The new colony smoothly reached the very beginning of the third generation under same conditions as former colonies. However, it collapsed again at the end of third generation around September 2017.

2.3.2. Annotation in *H. halys* draft genome

A set of genes controlling early embryonic patterning were identified in massive genetic screens in the model insect *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus 1980). Following the establishment of the basic body axes by maternal genes, three classes of segmentation genes – maternal, gap, pair-rule and segment polarity genes - sequentially subdivide the embryo into repeated metameric units along the anterior-posterior axis (AP-axis). Segment identity is then specified by a set of homeotic (Hox) genes, that assign different identities to specific segments, or groups of segments, along the AP-axis (Lewis 1978). Most of the genes in this regulatory hierarchy encode transcription factors that are highly conserved in insects, arthropods and in the case of *Hox* genes, metazoans more broadly (Carroll, Grenier et al. 2005). Despite conservation of gene sequences, the expression and function of many of these genes have diverged during evolution (Heffer, Shultz et al. 2010, Pick and Heffer 2012, Xiang, Reding et al. 2017). Here I have identified *H. halys* orthologs of maternal, gap, pair-rule genes, based on genome and transcriptome sequence. As mentioned above, in *D .melanogaste*r, maternal transcription factors organize the AP and dorsoventral axis of egg (Nusslein-Volhard, Frohnhofer et al.

1987, St. Johnston and Nusslein-Volhard 1992). Orthologs of four *D. melanogaste*r maternal genes were identified in the *H. halys* genome. Homologues of the *bicoid* (*bcd*) gene have not been isolated outside of higher Dipterans and no *bcd-like* gene was found in the *H. halys* genome. It is thought that the function of *bcd* in patterning of the anterior region of the embryo in *D. melanogaster* is carried out by other genes, such as *orthodenticle* (*otd*) and/or *hunchback* (*hb*) , in other species (Lynch and Desplan 2003). Orthologs of both genes were found in the *H. halys* genome (*otd*, Scaffold6 4:137302-406322, 55% identity to *Dmel-otd*). *Nanos* (*nos*) is required for posterior patterning in *D. melanogaster* and its sequence is characterized by two essential CCHC motifs (Curtis, Treiber et al. 1997). No match was found for *nos*, using *D. melanogaster* or *T. castaneum nos* nucleotide or amino acid sequence as query to search the *H. halys* genome directly. However, searches of the Transcriptome Shotgun Assembly (TSA) database using *D. melanogaster nos* amino acid sequence as query identified three highly significant matches: H. halys bmsbwbcontig71241 transcribed RNA sequence, Hhalys_USDA-ARS_IIBBL.73928 transcribed RNA sequence and Halyomorpha halys Hhalys_USDA-ARS_IIBBL.73927 transcribed RNA sequence. Queries of the *H. halys* genome with these three sequences identified overlapping matches to a single high significance (value < e-10) gene model in Scaffold1313. This gene was annotated as *Hhal-nanos*. Alignment with *nos* genes from other species shows that they share the two essential, and characteristic CCHC domains (Fig.3). Three other maternal genes: *caudal, dorsal, cactus*, were identified in the *H. halys* genome: *Hhal-cad* (Scaffold158) shares 73% identity with that of *Dmel cad*; *Hhal-dl* (Scaffold257) shares 61% identity with that of

Dmel-dl; and *Hhal-cact* (Scaffold257) shares 45% identity with that of *D.mel-cact*. The presence of these key maternal genes in the *H. halys* genome suggests that early stages of patterning use similar strategies to those seen in other insects.

Gap genes *giant* (*gt*), *hunchback* (*hb*), *Kruppel* (*Kr*), *knirps* (*kni*), and *tailless* (t*ll*), are the first zygotically-expressed segmentation genes in *D. melanogaster* (reviewed in Akam 1987, Gilbert 2010). The gap gene *kni* and its paralog, *knirps-like*

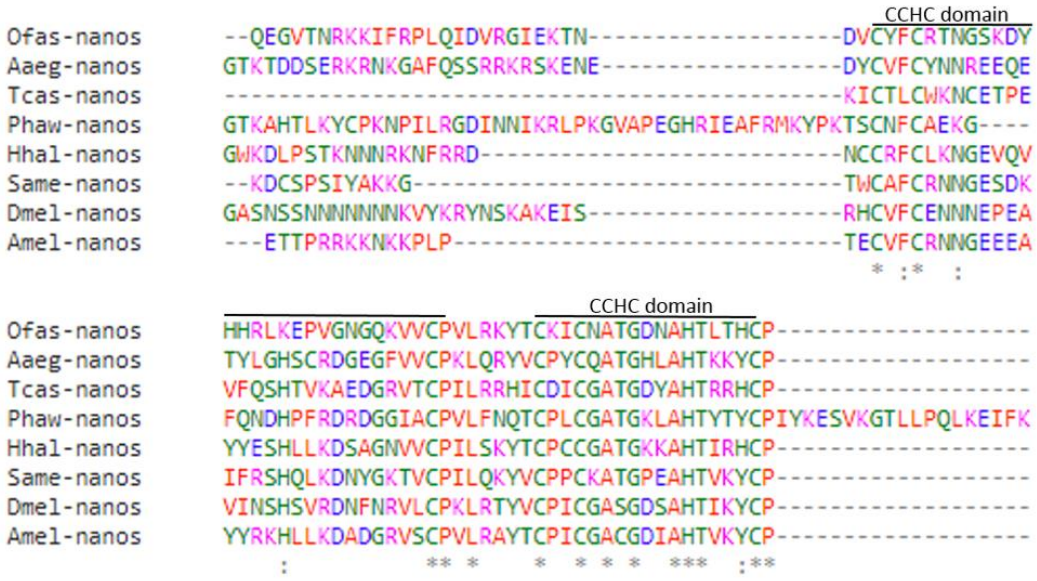

Figure 3. Sequence alignment of *nanos* genes from *H. halys* and other species. The black underlines indicate CCHC motif in nanos gene. Species abbreviations are as follows: *Aaeg: Aedes aegypti; Dmel: Drosophila melanogaster; Hhal: Halyomorpha halys; Ofas: O. fasciatus fasciatus; Phaw: Parhyale hawaiensis; Same: Schistocerca americana Tcas: Tribolium castaneum.*

(*knrl*), are the result of a duplication event in the lineage leading to insects, while duplication of *knrl* to produce *kni* appears to have occurred in brachyceran flies, which includes *D. melanogaster* (Naggan Perl, Schmid et al. 2013). Consistent with this, we were unable to identify *kni* in the *H. halys* genome or transcriptome.However, since the current *H. halys* genome is still divided among many scaffolds, it is possible that this gene is present. Orthologs of four gap genes were identified in the *H. halys*

genome: *gt*, *hb*, *Kr* and *tll*. *Hhal-gt* (Scaffold 474) shares 46% identity with that of *Tcas-gt*; *Hhal-hb* (Scaffold1234) shares 62% identity with that of *Dmel-hb*; *Hhal-Kr* (Scaffold 686) shares 68% identity with that of *Dmel-Kr*; and *Hhal-tll* (Scaffold 836) shares 77% identity with that of *Dmel-tll.* All genome annotations were supported by RNA-seq data (Ioannidis, Lu et al. 2014, Sparks, Shelby et al. 2014). All annotated maternal and gap genes are summarized in Table 2.

Gene type	Gene name	Location in H.halys draft genome
Maternal genes	*bicoid*	N/A
	cactus	Scaffold 257
	caudal	Scaffold 158
	dorsal	Scaffold 257
	nanos	Scaffold 1313
Gap genes	*giant*	Scaffold 474
	hunchback	Scaffold 1234
	Kruppel	Scaffold 686
	knirps	N/A
	tailless	Scaffold 836
Homeobox gene	*orthodenticle*	Scaffold 64

Table 2. Summary of annotation for *H. halys* orthologs of maternal and gap genes.
Abbreviations: N/A, no match found in genome.

2.3.3. Temporal expression of PRGs in *H. halys*

Orthologs of eight of the nine *D. melanogaster* PRGs (except *odd-paired*), referred to throughout as *Hhal*-PRG orthologs, gene structures were determined based on annotation in *H. halys* draft genome (Figure 4). Based on SYTOX green nuclear staining (not shown here), genes involved in segmentation should be expressed at 0-48 hours AEL, which includes blastoderm through germband elongation. RT-PCR spanning the first three days of *H. halys* development was used to determine whether *Hhal*-PRG orthologs are expressed at the right time to be involved in segmentation. For all experiments, *Hhal-actin* was simultaneously amplified as an internal positive

control (arrow in Figure 5). The relative gene expression levels over the first three days of *H. halys* development, from the pre-blastoderm stage through appendage formation, were determined. Under the conditions specified above, *H. halys* embryos usually hatch after 7 days, so this RT-PCR time course represents roughly 0-50% embryonic development. For each gene tested, only one major band was evident. Since *E75a* showed PR-like expression in *O. fasciatus* (Erezyilmaz, Kelstrup, & Riddiford, 2009), *Hhal-E75a* was included in this experiment.

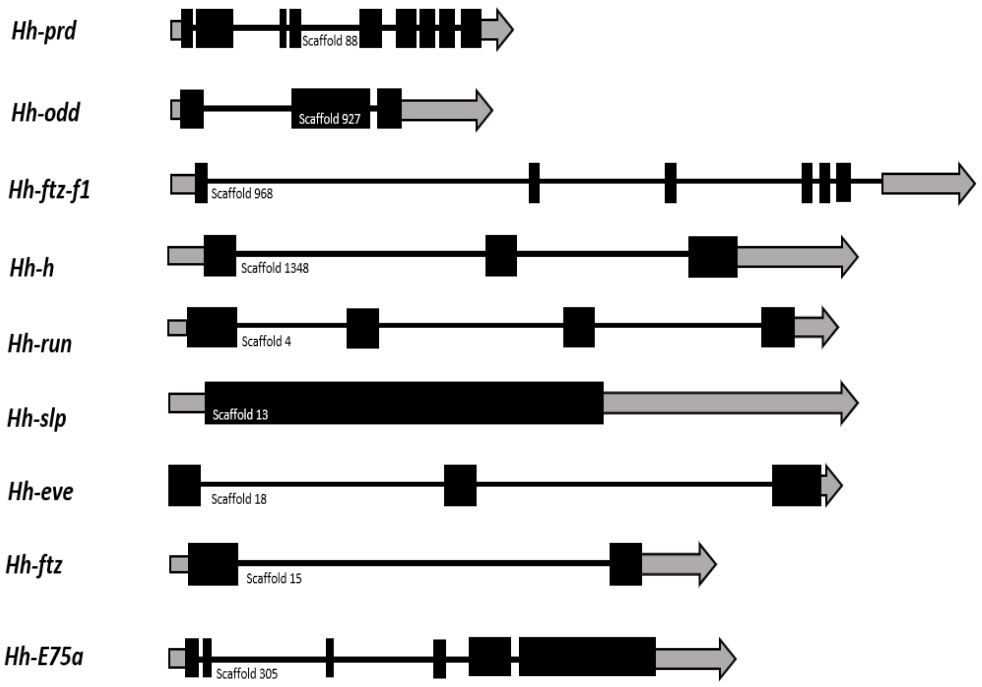

Figure 4. *Hhal*-PRG orthologs gene structures. Genes analyzed in this study are shown in this schematic. Black rectangles indicate coding regions. Gray bars indicate UTR. Lines indicates intros. Arrows designate the 3' end of the transcript.

Expression of *Hhal-eve,* was detected at 0-56 h AEL, with highest levels at 24-40 h AEL and slightly lower levels after this time point. *Hhal-odd, Hhal-run and Hhal-E75a* showed fairly consistent expression for all time points examined from the blastoderm stage through germband extension stage. Their continued expression after

germband extension suggest likely functions in additional roles in later *H. halys* embryonic development. *Hhal-ftz-f1* showed expression for 0-32h AEL but peaked at 32-40 h and 48-56 h AEL. *Hhal-ftz* was detected 16-40h AEL and showed weak expression after that. *Hhal-slp* expression was detected between 24 and 56h with highest levels at 32-40 h AEL. *Hhal-h* was only detected at 40-48 h AEL. In sum, although distinct profiles were seen for each gene, all orthologs examined were detected at 24-48 h AEL except *Hhal-h*. This suggests that *Hhal-h* does not have PR-

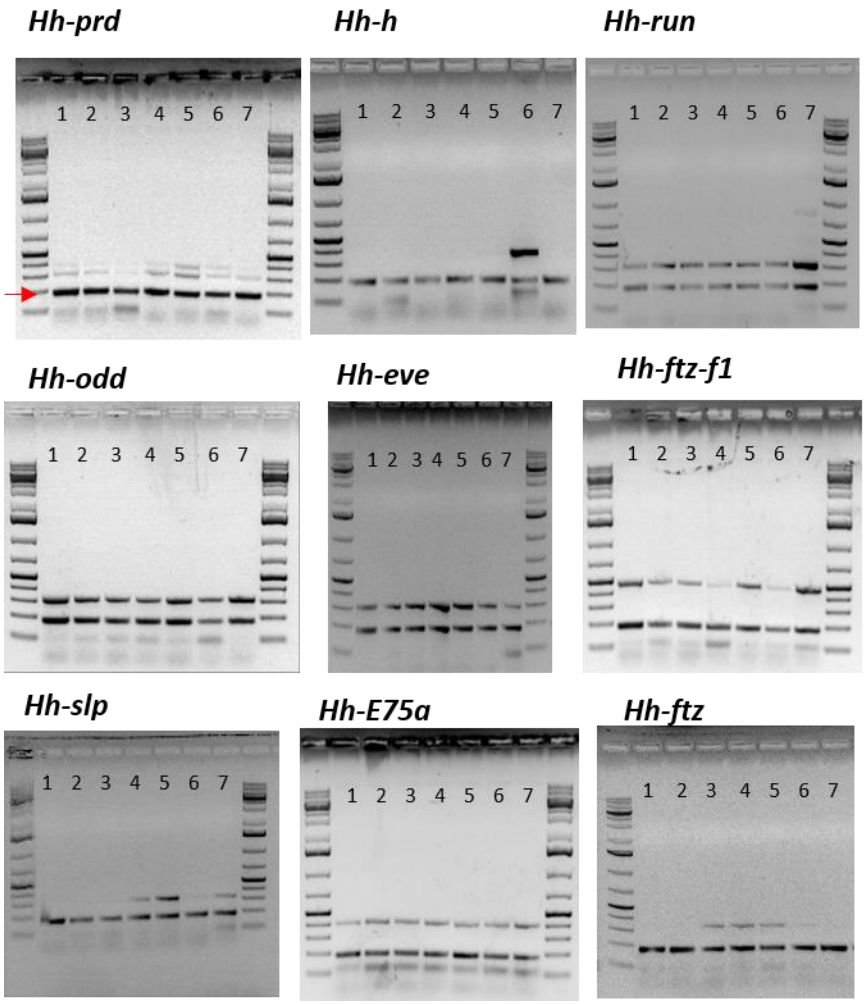

Figure 5. 8-hour RT-PCR for *H. halys* PRGs. (1) 0-8h AEL (2) 8-16h (3) 16-24h (4) 24-32h (5) 32-40h (6) 40-48h (7) 48-56h. *Hhal-actin* was used as control (~200 bp) marked as a red arrow.

like function as does *Dmel-h*. However, due to laboratory colonies collapsing, I could not collect enough material to support additional studies of *Hhal*-PRG ortholog expression and function.

2.3.4. RNA interference is effective in *H. halys*

RNAi is a useful method to knock down gene function in both plants and animals. To determine whether RNAi can be an effective tool for *H. halys*, I tested whether injection of dsRNA into adult females caused defects in offspring, so-called 'parental RNAi' (pRNAi), which has been shown to be effective in *T. castaneum*, *D. maculatus*, *O. fasciatus* and several other insect species (Bucher et al., 2002; P. Z. Liu & Kaufman, 2004; Xiang et al., 2015). To test in this method, I chose a gene whose perturbation would result in very specific morphological defects, as opposed to general lethality, to clearly assess the impacts of gene knockdown. For this purpose, I chose the homeotic gene *Sex combs reduced* (*Scr*). *Scr*, like other homeotic genes, is responsible for determining segmental identity in early embryos (Pattatucci, Otteson, & Kaufman, 1991). Inappropriate expression of homeotic genes results in transformation of one body part towards another body part and loss of homeotic function results in embryonic death (reviewed in Gehring, 1985). Knocking down *Scr* by RNAi was shown to have very clear and unique effects - the transformation of proboscis toward leg in *O. fasciatus* (Hughes & Kaufman, 2000) and reproduced by me (Figure 6), and in the American cockroach, *Periplaneta Americana* (Hrycaj, Chesebro, & Popadić, 2010). To test RNAi in *H. halys*, a 327 bp *Hhal-Scr* sequence, including 51 bp of the homeobox and 276 bp upstream, isolated by degenerate PCR, was extended based on transcriptome data to an 816 bp sequence including 76 bp of

5'UTR and a coding region of 246 amino acids that includes the YPWM motif and part of the homeodomain. The homeodomain is 100% identical to a partial *Scr* gene (185 amino acids) that was isolated from the southern green stink bug (*Nezara viridula*) (Tian et al., 2011). The partial homeodomain has a *Scr* signature sequence at the N-terminal arm of the homeodomain and is 100% identical to that of *D. melanogaster Scr*.

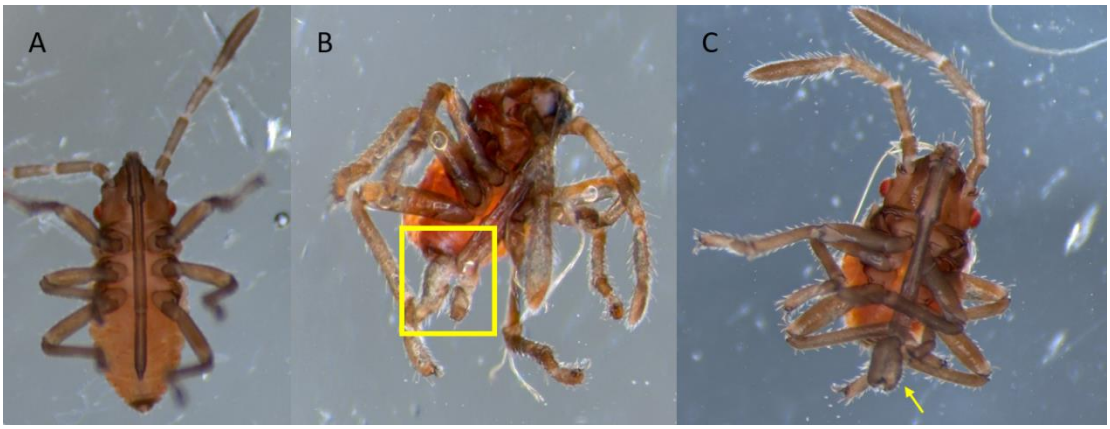

Figure 6. Knockdown of *Scr* in *O. fasciatus*. Scr was chosen as a standard to test RNAi in *H. halys* because of the clear phenotype observed in *O. fasciatus*. These results were repeated in our lab for comparison to *H. halys*. Photos of 1st instar nymphs are shown. A) Wild type. The proboscis has a needle-like shape with pointed tip; (B, C) Offspring of females injected with *Ofas-Scr* dsRNA. B) 1st instar nymph with severe effects has a bifurcated proboscis (yellow square); C) 1st instar nymph with less severe phenotype has a duplication at the end of the proboscis (yellow arrow).

As expected for RNAi, a range of defects was observed in *Hhal-Scr* pRNAi offspring (Lu, Chen, Reding, & Pick, 2017). In the most severely affected embryos, death prior to hatching was observed. To examine defects, embryos were dissected out of unhatched eggs from *Scr* dsRNA-injected females. Many of these eggs were not fully developed, suggesting lethal effects of loss of *Scr* function in early

embryogenesis. Some nearly hatched nymphs that were possible to remove from the egg cases lacked whole mouthparts (Figure 7B, C).

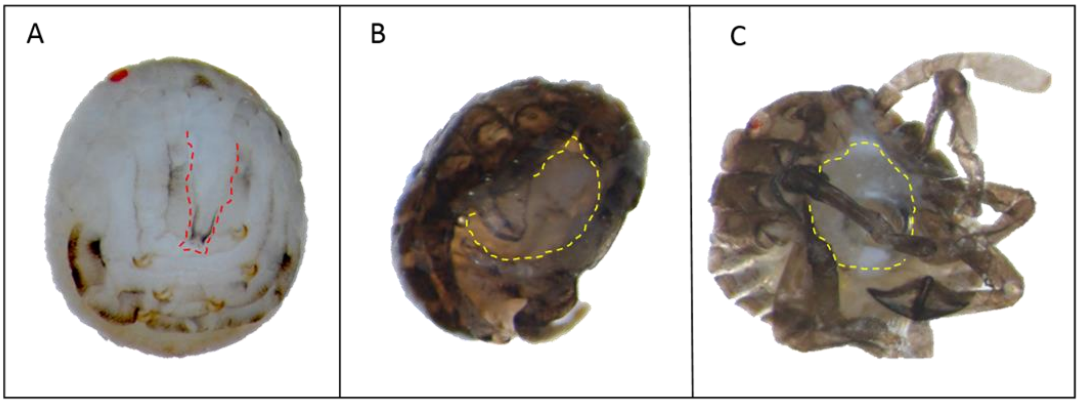

Figure 7. Severe defects in unhatched offspring after *Hhal-Scr* RNAi. Photographs of nymphs dissected out of egg cases are shown. A) Control, *gfp* pRNAi is fully developed with proboscis (red dashed line). Note that wild-type animals darken after they hatch. B, C) Different examples of *Hhal-Scr* pRNAi offspring with severely malformed or absent head structures (yellow dashed lines). These *Hhal-Scr* pRNAi embryos were presumably unable to hatch and therefore were still within the egg shell as they matured and darkened.

Additional offspring of *Scr* dsRNA-injected females were observed with abnormalities indicative of homeotic transformation of the mouthparts (Figure 8). The proboscis of wild type 1st instar nymphs is needle-like in shape and has a sharp tip (Figure 8A, black arrow). In the most severe cases, first instar nymphs hatched from eggs laid by *Scr* dsRNA-injected females had a bifurcated proboscis, and claws were seen at the tip of the proboscis (data not shown), suggestive of homeotic transformation towards leg (Figure 8B). Some 1st instar nymphs had less severe defects; for example, in some cases, instead of being bifurcated, the end of the proboscises expanded into a clubbed shape (Figure 8C). Others had shorter or bent and twisted proboscises, with partial duplication or bifurcation of distal structures (Figure 8D-E). Finally, other nymphs hatched with normal proboscises but with smaller and withered bodies.

Overall, while the defects observed were quite specific, the penetrance of defects varied in different experiments. In some cases, all hatched nymphs showed abnormal mouthparts. In other cases, while increased failure to hatch was observed, hatched larvae did not appear to have mouthpart abnormalities. In addition, in any one experiment, the severity of the defects attenuated as time went on, as was also observed for *O. fasciatus* and *D. maculatus* RNAi (Angelini & Kaufman, 2004; Xiang et al., 2015). For example, embryos laid within the first two weeks after injection showed the most severe phenotypes (bifurcated proboscises); slightly later,

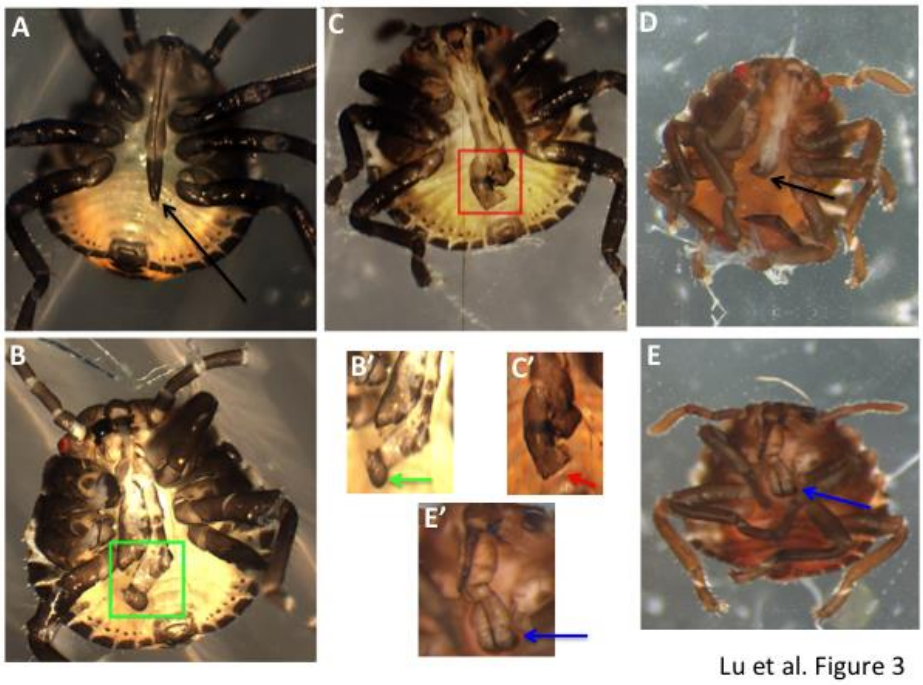

Figure 8. Knockdown of *Scr* by RNAi produces abnormal mouthparts in *H. halys*. Photographs of first instar nymphs are shown. A) Wild type. The proboscis has a needle-like shape with pointed tip (black arrow). B-E**)** Offspring of females injected with *Hhal-Scr* dsRNA (*Scr* pRNAi). B) First instar nymph with severe effects has a bifurcated proboscis (green square); B') tip of the proboscis from panel (B) (green arrow). C) First instar nymph has a bluntended proboscis (red square); C') tip of the proboscis from panel (C) (red arrow), D) another example of a transformed proboscis with bent, abnormal tip (black arrow). E) An example of a first instar nymph showing duplication and thickening at the tip of the proboscis (blue arrow). E') Enlargement of region from panel (E) (blue arrow).

embryos showed less severe phenotypes (clubbed shape proboscises); by the third week, embryos were all normal. In sum, RNAi was clearly effective in knocking down gene function in *H. halys*.

2.4 Discussion

As an invasive pest origin in Asia, *H. halys* has been found in 41 states in the USA and two Canadian provinces after first discovered in Allentown, PA in 1996 (Hoebeke & Carter, 2003). *H. halys* is a polyphagous insect and has caused significant agriculture damage. *H. halys* is also a nuisance for homes and businesses, seeking overwintering sites in any human-made structures in the fall. More importantly, *H. halys* is resistant to common insecticides, forcing farmers to use broad spectrum insecticides, which will cause outbreaks of secondary pests such as European red mites, wooly apple aphids and others, since their natural enemies are killed by these insecticides (Leskey et al., 2012). Since it has become a pest rapidly, spreading with human activities, scientists are looking for an environmentally friendly way to control it. Understanding the development of *H. halys* from a molecular biology perspective will provide inroads into novel approaches for management and monitoring of *H. halys* populations.

Here I presented methods used to analyze and annotate gene in draft genome, and to study gene function by RNAi in *H. halys*. The methods presented will allow other researchers in the field to expand studies of basic genetics and biology of *H. halys*. *p*RNAi with *Scr* showed high efficiency of gene knock-down. The establishment of RNAi in this species opens up the possibility of using this as a strategy for controlling *H. halys* in the field, an approach of increasing interest in

recent years (reviewed in (Joga, Zotti, Smagghe, & Christiaens, 2016; Zhang, Khan, Heckel, & Bock, 2017). To our knowledge, this is the first set of molecular methods to study development of a representative of the stink bug family Pentatomidae, a large family within the superfamily Pentatomoidea, which includes ~ 7000 species.

Although others have reported successful maintenance of *H. halys* in long-term laboratory cultures, our lab is still facing challenges. Thus, while additional studies of *H. halys* PRG expression and function are still being considered, this project has moved to lower priority as other ametabolous and hemimetabolous insects are more amenable to mass rearing in lab colonies. Since it is extremely difficult to collect large numbers of staged *H. halys* embryos, in situ hybridization methods to assess expression of *Hhal*-PRG orthologs was not attempted.

Chapter 3: PRG orthologs expression and function in *Oncopeltus fasciatus.*

3.1 Introduction

The discovery in *D. melanogaster* of a class of mutants lacking alternate body segment suggested that a two-segment-wide pre-pattern is an early step underlying segment formation in insects (Nüsslein-Volhard & Wieschaus, 1980; Wakimoto et al., 1984; E. Wieschaus, Nüsslein-Volhard, & Jürgens, 1984) . In *D. melanogaster*, most PRGs are expressed in seven stripes in blastoderm stage embryos in the primordia of regions missing in the corresponding mutant embryos. For example, *eve* and *ftz* are expressed in complementary seven-stripe patterns, each in the primordia of every other one of the 14 future parasegments (Lawrence, Johnston, Macdonald, & Struhl, 1987). So far, nine PRGs have been reported in *D. melanogaster*: *even-skipped (eve), paired (prd), runt (run), hairy (h), sloppy paired (slp), odd-skipped (odd), odd-paired (opa), ftz* and its cofactor *ftz-f1* (Nüsslein-Volhard & Wieschaus, 1980; Wakimoto et al., 1984; E. Wieschaus et al., 1984; Eric Wieschaus et al., 1984; Yu et al., 1997). Since much research has focused on this set of genes, their expression, function, genetic interactions and even transcriptional regulation has been well studied (Akam, 1987; Howard & Ingham, 1986; Ingham, 1988).

Different insects use different segmentation modes based on observation of insect embryogenesis and embryo morphologies. *D. melanogaster* are long-germ insects with all parasegments patterned simultaneously at the blastoderm stage. This mode of embryo development has only been found for some holometabolous species. For most insects, only head segments are specified at the end of blastoderm and

posterior segments are added sequentially after the blastoderm stage, from the posterior end of the germband, a region known as the segment addition zone (SAZ) (reviewed in (Davis & Patel, 2002; P. Z. Liu & Kaufman, 2005b). New questions have arisen to focus on how the PRGs are expressed in other species, especially in sequentially segmenting species. PR-like expression, defined as stripes of gene expression in the primordia of alternate segmental units, has been observed for orthologs of each of the nine *D. melanogaster* PRGs (PRG-orthologs) in various insects. To date, most studies of PRG-orthologs have been limited in Holometabola, and there has been little in-depth investigation of PRG expression outside of Holometabola in insects.

O. fasciatus belongs to the order Hemiptera, a sister group to the Holometabola (Misof et al., 2014), and is becoming a model species for non-holometabolan insects (Angelini & Kaufman, 2004, 2005; Chesebro et al., 2009; Stahi & Chipman, 2016). The gene encoding the nuclear receptor gene *E75a* was shown to have PR-expression and function in *O. fasciatus*, but is not known to play this role in any other species (Erezyilmaz et al., 2009). In *O. fasciatus, eve* is expressed in the SAZ and in stripes in every segment ("segmental expression") instead of PR-like expression (P. Z. Liu & Kaufman, 2005a) .Our lab set out to identify and examine the expression of all nine PRG-orthologs in *O. fasciatus*. My portion of the project focused on three PRGs: *runt (run), hairy (h), sloppy paired (slp).* I compared their expression to the only known *Ofas*-PRG, *E75a.* These results will provide a deeper understanding of how insect segmentation networks make use of a conserved set of genes in different ways in different species. It will reveal how *O.*

fasciatus and *D. melanogaster* use different sets of PR-type regulatory genes to establish an alternate-segment prepattern.

3.2 Methods and materials

Insect rearing

Oncopeltus fasciatus (Large milkweed bug), originally purchased from Carolina Biological and maintained in our lab for several years, were reared in 30 x 18 x 20 cm plastic cages with a piece of cotton for egg laying. All adults and nymphs on a diet of water and sunflower seeds at 24±1°C, 50% RH, with a photoperiod of 16-hour light: 8-hour dark.

Embryo collection

At the beginning of an embryo collection period, a piece of cotton was placed on top of a small section of paper towel and added to each adult *O. fasciatus* cage; the towel barrier ensured that no older embryos already in the cage would be collected. At the end of the collection period, the cotton was removed from the cage and kept at 24°C until reaching the appropriate age. Just prior to fixation, the cotton was carefully picked apart, allowing the embryos to fall on to a piece of white paper below. Approximately 100 µl of embryos were then transferred to each microcentrifuge tube.

cDNA synthesis and RT-PCR

Embryos were collected every eight hours over a five-day period and maintained at 24°C until reaching the appropriate age. Enough RNAlater (Invitrogen) was added to cover embryos, and tubes were stored at -20°C until RNA extraction.

RNA was extracted using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen). To synthesize cDNA for RT-PCR, RNA from three appropriately aged 0-8 h AEL collections was pooled for each time point (0-24 h, 24-48 h, 38-72 h, 72-96 h, and 96-120 h) to ensure that a diversity of ages within each time frame was represented. cDNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen). All primers were designed based on sequences in the *O. fasciatus* draft genome. To check for primer specificity, cDNA from the five collections described above was mixed, yielding 0-120 h cDNA. Primers were used to amplify this mixed stage cDNA, and PCR products were sequenced (Genewiz).

Embryo fixation, in situ hybridization, and SYTOX staining

Embryos were fixed as described by Liu and Kaufman (P. Z. Liu & Kaufman, 2004) with modifications made by Ben-David and Chipman (Ben-David & Chipman, 2010) and by our lab. Briefly, tubes containing ~ 100 µl embryos in ~600 µl water were briefly and gently spun down until submerged. Tubes of submerged embryos were placed in a boiling water bath for 3 min, then chilled on ice for 6 min. Tubes were then laid on their sides and shaken for 20 min at 250 rpm in 1:1 heptane:12% paraformaldehyde. The paraformaldehyde was then removed and replaced with 600 µL methanol. Tubes were then gently shaken by hand. Heptane and methanol were removed, and embryos were washed three times in methanol. At this point, embryos can be stored in methanol and completed the remaining steps on the day of use; otherwise, all steps were performed in one day. Embryos were transferred stepwise from methanol to PBST, and eggshells were manually removed by forces. Embryos

were washed in 4% paraformaldehyde/PBST for 1.5 h and used immediately or stored

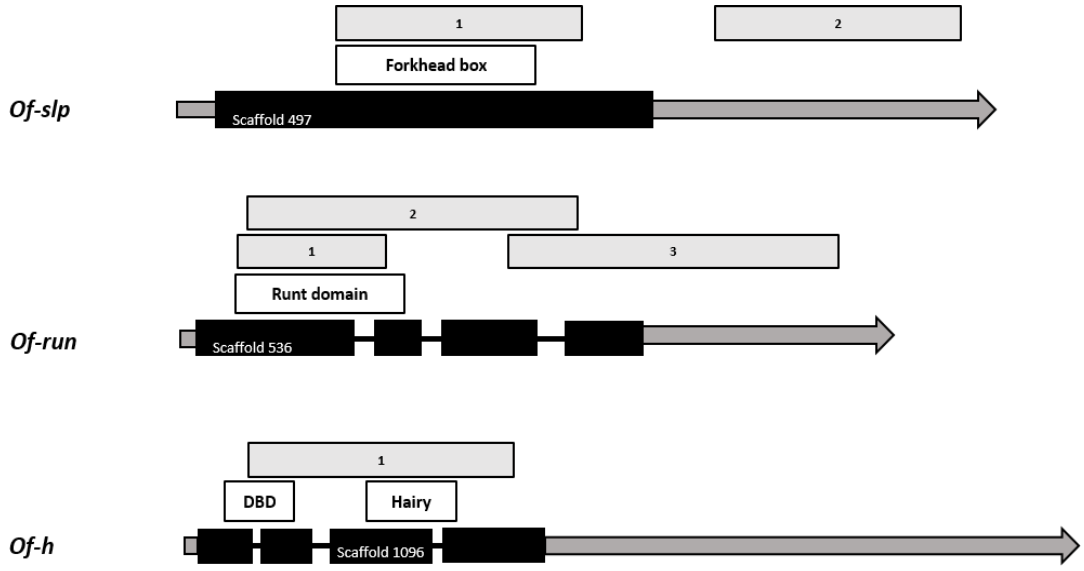

Figure 9. Gene structures of three *Ofas*-PRG orthologs. Genes analyzed in this study are shown in schematic. Black rectangles indicate coding regions. Dark gray bars indicate UTR. Arrows designate the 3' end of the transcript. Lines indicates introns. Labeled white bars above structures show the location of signature domains, as indicated. Light gray bars above structures indicate different probes used for in situ hybridization.

in methanol at -20°C.

Antisense RNA probes were synthesized using digoxigenin or fluorescein RNA labeling mix (Roche) and DNA templates generated by PCR amplification of staged cDNA with reverse primers containing the T7 promoter sequence. The T7 RNA transcription reaction was carried out at 37°C for 2h, followed by precipitation in cold ethanol and LiCl. Since original probes were designed to hybridize to conserved domains, I designed additional probes for *run* and *slp* that were gene-specific to ensure that results were not obscured by cross-hybridization with related genes. Gene-specificity was confirmed by conducting a BLAST search of the *O. fasciatus* genome using the probe sequence as the query; if only the sequence of the

target gene matched the probe sequence, I considered the probe gene-specific. I confirmed with *run* and *slp* that the gene-specific probes (labeled probe 2 or 3 in Fig. 9) gave the same results as the less specific probes (labeled probe 1 in Figure 9). In situ hybridization was carried out as previously described either by Ben-David and Chipman (Ben-David & Chipman, 2010) or Liu & Kaufman (P. Liu & Kaufman, 2009), with the addition of a 1 h 5% BSA blocking step prior to blocking with 10% sheep serum. Either BCIP/NBT or BCIP/INT was used as the chromogenic substrates for alkaline phosphatase. Nuclear stains were carried out by rocking fixed embryos in 1:1000 SYTOX Green (Invitrogen) in PBST for 30 mins at room temperature. Embryos were then washed three times in PBST.

Phylogenetic analyses

To conduct phylogenetic analyses of candidate ortholog sequences, alignments of orthologous sequences were generated using MUSCLE (Edgar, 2004). Alignments were then trimmed using Aliview (Larsson, 2014). Finally, phylogenetic analysis was carried out in TOPALi v2.5 using the Bayesian algorithm MrBayes (Milne et al., 2009; Ronquist & Huelsenbeck, 2003). Additional formatting of trees was performed in MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

3.3 Results

3.3.1 Isolation of *O. fasciatus* orthologs of *Drosophila* pair-rule genes.

In order to isolate three candidate orthologs of *D. melanogaster* PRGs: *sloppy-paired*, *runt* and *hairy*, I searched the *O. fasciatus* draft genome with *D. melanogaster*

amino acid sequence as queries. All scaffolds that matched with high significance to the query sequence were investigated (e-values usually on the order of 1e-10 or less). Once several gene candidates were identified, multiple sequence alignments, reciprocal BLAST searches, and phylogenetic analyses were conducted to identify the most likely ortholog. Full or partial gene sequences were then isolated by PCR and confirmed by sequencing.

Drosophila have four *Runx* family members, all of which share a conserved Runt domain and a C-terminal VWRPY motif, necessary for interactions with the corepressor Groucho (Aronson, Fisher, Blechman, Caudy, & Gergen, 1997; Bao & Friedrich, 2008). Searches for *runt (run)* in the *O. fasciatus* genome yielded three candidate sequences containing Runt domains, likely orthologs of *run, lozenge (lz), runxA,* and *runxB.* To determine which of these sequences is the most likely ortholog of *Dmel-run*, phylogenetic analysis using Runt family orthologs from a variety of arthropod species was carried out (Fig. 10). One candidate sequence (scaffold 536) groups with other *run* orthologs, contains the Groucho-interacting motif, and was designates *Ofas-run*. The other candidate sequence on Scaffold 9348 groups with other *runx* orthologs. The candidate sequence on Scaffold 758 was determined to be *lz.* The Runt domain from this sequence is 61% identical to that of *Dmel-runt.*

Similar to *run*, *hairy(h)* orthologs contain a C-terminal motif (WRPW) which is necessary for interaction with Groucho (Jiménez, Paroush, & Ish-Horowicz, 1997). Searches in the *O. fasciatus* genome yielded two candidate sequences, both of which contained WRPW motifs and highly conserved Hairy-Orange DNA-binding domains. By phylogenetic analysis with other *h* and related gene *deadpan (dpn)* orthologs, I

designated the candidate sequence from scaffold 1096 as *Ofas-h* (Fig. 11). The region of this sequence is 68% identical to that of *Dmel-h*.

sloppy paired (slp) is part of the FoxG subfamily of a fairly large, highly conserved family of *forkhead box* (*Fox*) genes (Grossniklaus, Pearson, & Gehring, 1992; Lee & Frasch, 2004). Searches for *slp* in the *O. fasciatus* genome yielded four results with highly conserved forkhead domains; *slp* and other forkhead family members contain this type of DNA-binding domain. Phylogenetic analysis was carried out with other *slp* and related gene *crocodile (croc)* orthologs to determine the most likely *Ofas-slp* sequence. The candidate sequence from scaffold 497 grouped with other *slp* orthologs; thus, it was designated as *Ofas-slp* (Fig. 12). This sequence matches the partial *slp* sequence found previously by Liu and Patel and shares 65% and 56% amino acid identity in the forkhead domain with *Dmel-slp1* and *Dmel-slp2*, respectively(P. Z. Liu & Patel, 2010).

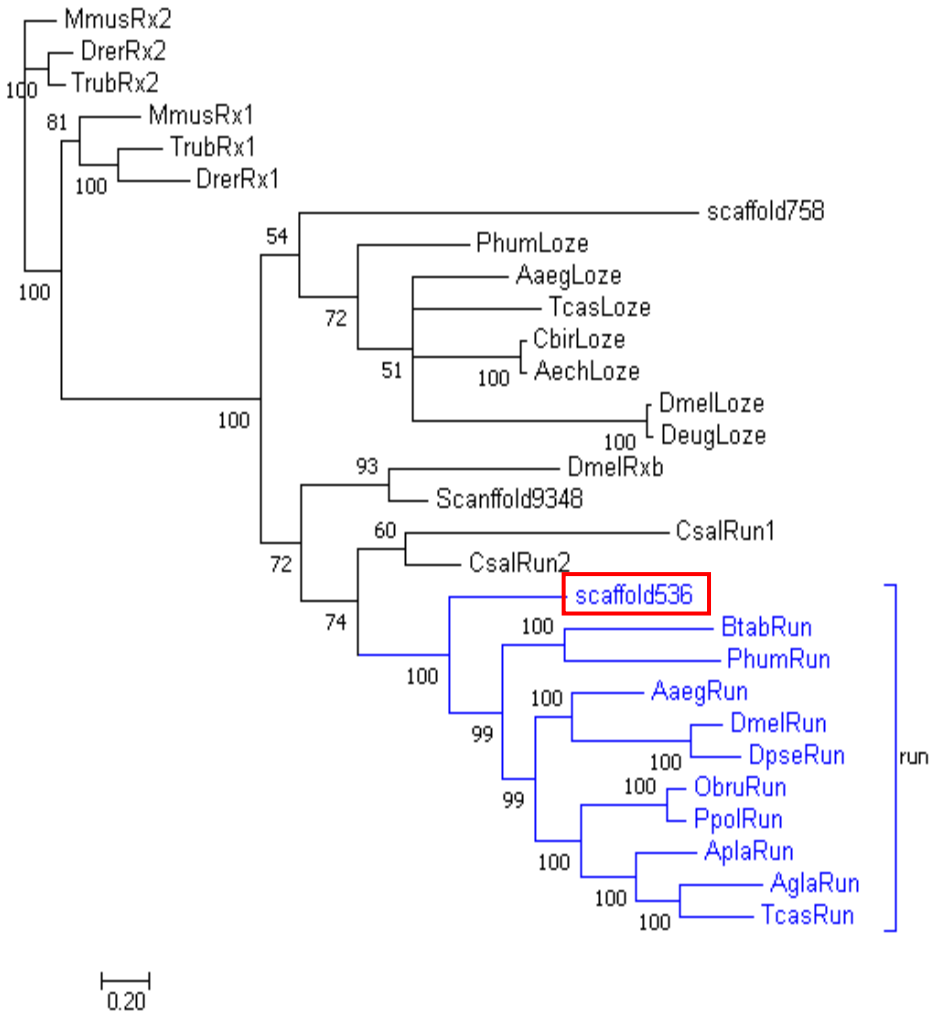

Figure 10. Phylogenetic analyses of *O. fasciatus run* orthologs. Phylogenetic analyses of Runt protein sequences. Bootstrap values (1,000 replicates) are shown adjacent to branches. Scaffold 536 is marked with red rectangle. Abbreviations: *Aaeg: Aedes aegypti; Aech: Acromyrmex echinatior; Agla: Anoplophora glabripennis; Apla; Agrilus planipennis; Btab: Bemisia tabaci; Cbir: Cerapachys biroi; Csal: Cupiennius salei; Deug: Drosophila eugracilis; Dmel: Drosophila melanogaster; Dpse: Drosophila pseudoobscura pseudoobscura; Drer: Danio rerio; Mmus: Mus musculus; Obru: Operophtera brumata; Phum: Pediculus humanus corporis; Ppol: Papilio polytes; Trub: Takifugu rubripes; Tcas: Tribolium castaneum.*

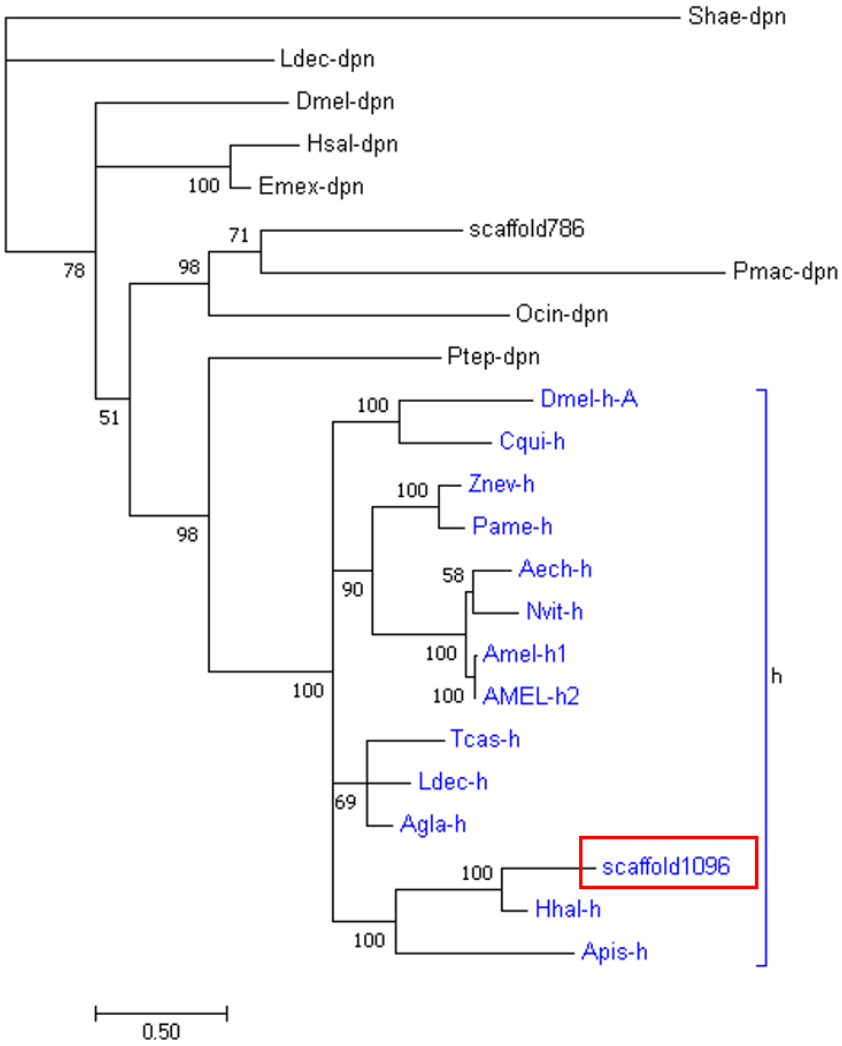

Figure 11. Phylogenetic analyses of *O. fasciatus h* orthologs. Phylogenetic analyses of hairy protein sequences. Bootstrap values (1,000 replicates) are shown adjacent to branches. Scaffold 1096 is marked with red rectangle. Abbreviations: *Aech: Acromyrmex echinatior; Agla: Anoplophora glabripennis; Amel: Apis mellifera; Apis: Acyrthosiphon pisum; Apla; Agrilus planipennis; Cqui: Culex quinquefasciatus; Deug: Drosophila eugracilis; Dmel: Drosophila melanogaster; Dpse: Drosophila pseudoobscura pseudoobscura; Drer: Danio rerio; Hhal: Halyomorpha halys; Hsal: Harpegnathos saltator; Emex: Eufriesea mexicana; Ldec:Leptinotarsa decemlineata; Mmus: Mus musculus; Nvit: Nasonia vitripennis; Obru: Operophtera brumata; Ocin: Orchesella cincta; Pame: Periplaneta americana; Pmac: Papilio machaon; Ptep: Parasteatoda tepidariorum; Shea: Schistosoma haematobium; Tcas: Tribolium castaneum; Znev: Zootermopsis nevadensis.*

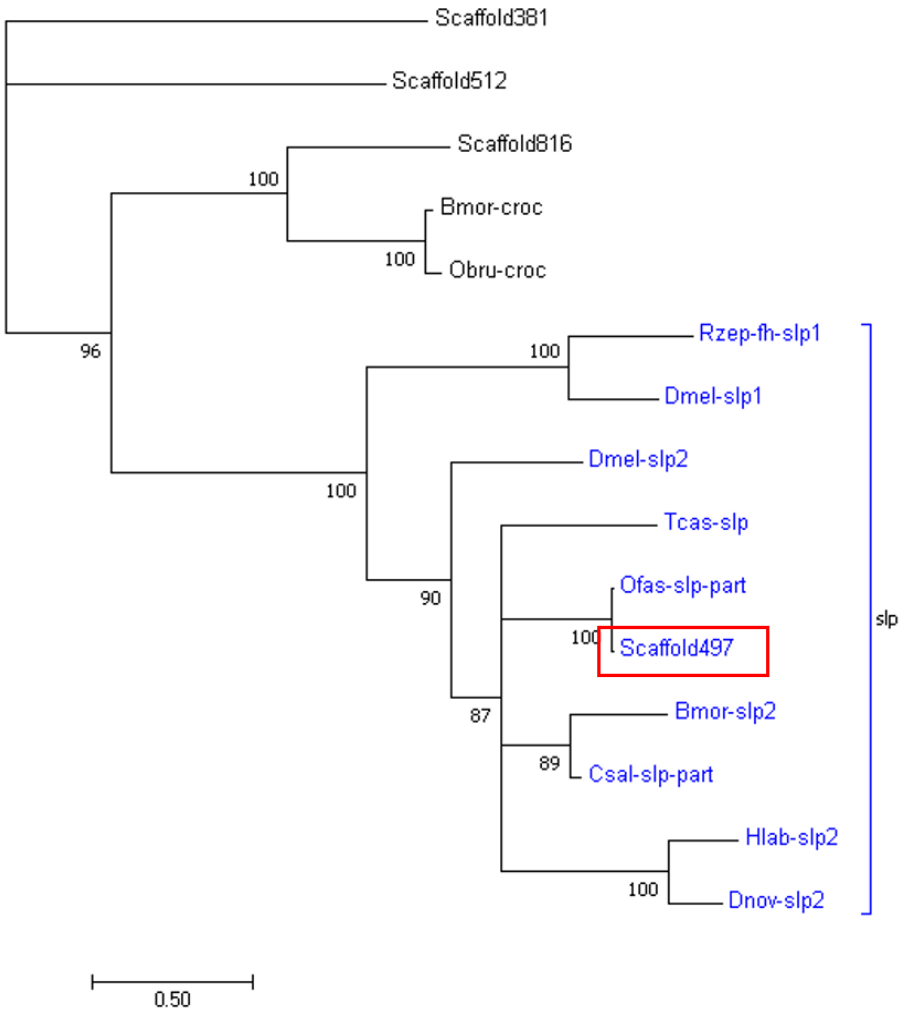

Figure 12. Phylogenetic analyses of *O. fasciatus slp* orthologs. Phylogenetic analyses of sloppy paired protein sequences. Bootstrap values (1,000 replicates) are shown adjacent to branches. Scaffold 497 is marked with red rectangle. Abbreviations: *Bmor: Bombyx mori; Csal: Cupiennius salei; Dmel: Drosophila melanogaster; Dnov: Dufourea novaeangliae; Hlab:Habropoda laboriosa; Obru: Operophtera brumata; Rzep: Rhagoletis zephyria; Tcas: Tribolium castaneum.*

3.3.2 Temporal expression of three PRGs in *O. fasciatus.*

Based on SYTOX green nuclear staining (Figure 13), genes involved in segmentation should be expressed at 24-48 hours AEL, which includes late blastoderm through early germband elongation. RT-PCR spanning the first five days of *O. fasciatus* development was used to determine whether *Ofas*-PRG orthologs are expressed at the right time to be involved in segmentation. For all experiments, *Ofas-actin* was simultaneously amplified as an internal positive control (arrow in Figure 14) and relative gene expression levels over the first five days of *O. fasciatus* development, from the pre-blastoderm stage through appendage formation, were determined. At the conditions specified above, *O. fasciatus* embryos usually

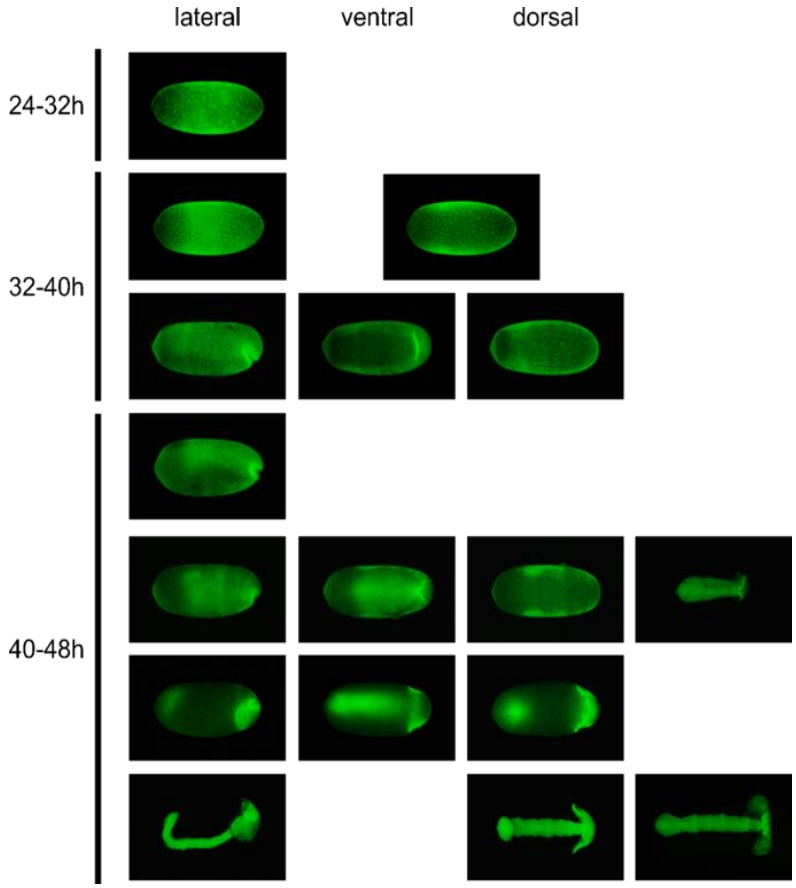

Figure 13. Development of early *O. fascatus* embryos. Embryos stained with SYTOX by Katie Reding.

hatch after about 6 days, so this RT-PCR time course represents roughly 0-80% embryonic development. For each gene tested, only one major band was evident. *Ofas-slp* was most highly expressed from 24-72h AEL, from the blastoderm stage through germband extension (Figure 7), but *Ofas-slp* expression continued, although at apparently lower levels, through the fifth day of embryonic development. *Ofas-run* was also expressed highest 24-48h AEL, with attenuated expression for two more days following (Figure 12) *Ofas-h* appeared to be expressed with consistent expression detected 24-120h AEL (Figure 14). Their continued expression after germband extension suggests it likely functions in additional roles in later *O. fasciatus* embryonic development.

In sum, all three orthologs examined were detected at 24-48 h AEL, consistent with roles in segmentation, although distinct profiles were seen for each gene.

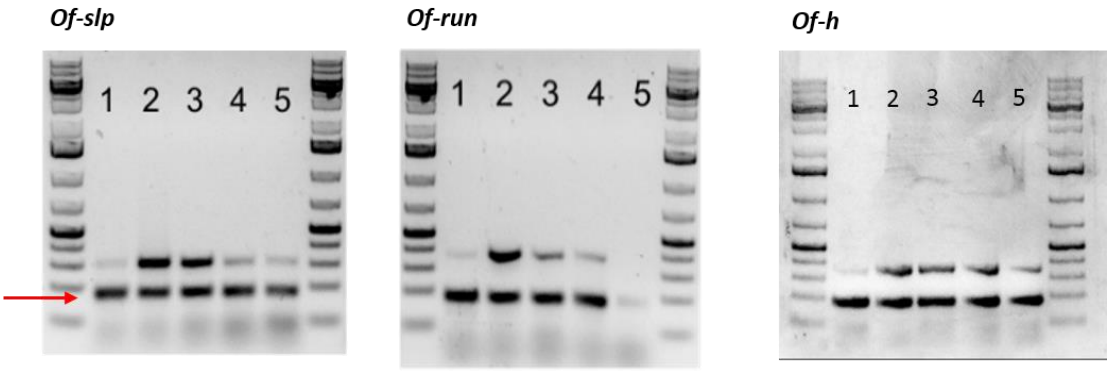

Figure 14. Temporal expression of three *O. fasciatus* PRGs. (1) 0-24h AEL; (2) 24-48h; (3) 48-72h; (4) 72-96h; (5) 96-120h. *Ofas-actin* was used as control (~200 bp) marked as a red arrow.

3.3.3 *Ofas-run* is expressed in a pair-rule-like manner during embryonic development

To determine whether *Ofas*-PRG-orthologs are expressed in PR-like spatial patterns, i.e., in stripes in the primordia of every-other body segment, whole-mount in situ hybridization was carried out. *Ofas-E75a*, expressed in PR-stripes (Erezyilmaz et al., 2009) serves as a positive control. *Ofas-E75a* was initially expressed in two stripes straddling the middle of the blastoderm-stage embryo, the anterior stripe being broad and diffuse and the posterior stripe appearing much narrower (Fig. 15a). Slightly later, three stripes were observed; this third stripe was added from the posterior, at first diffuse and later narrowing (Fig. 15b). Later, a fourth stripe was observed just before gastrulation. As gastrulation proceeded, all four blastoderm-stage stripes moved toward the posterior. In early germbands, a broad stripe was observed in the SAZ with a narrower stripe just anterior (Fig. 15d). In later germbands, a pair of stripes was seen in the anterior portion of the SAZ, with another weak stripe present in the segmental primordia (Erezyilmaz et al., 2009).

Of all the *Of*as-PRG orthologs examined, *Ofas-run* expression was the most PR-like (Fig. 15 e-m'). *Ofas-run* was first detected in blastoderm-stage embryos with a broad domain covering roughly half posterior egg length. Slightly later, it turned into two diffuse stripes in the center of the embryo, as well as a posterior 'cap' (arrowhead, Fig. 13e), similar to run expression in beetles (Choe, Miller, & Brown, 2006; Xiang, Reding, Heffer, & Pick, 2017). At the beginning of gastrulation, stripes 1 and 2 split, while a third primary stripe resolved from the posterior cap generating five stripes prior to gastrulation (Fig. 15f, g). The presence of two thick stripes, each

of which split, is reminiscent of PR-gene expression for *prd* in *Drosophila*, and *eve, h,* and *prd* in *D. maculatus* (Kilchherr, Baumgartner, Bopp, Frei, & Noll, 1986; Xiang et al., 2017). However, this pattern differs from *Ofas-E75a* which retains a strict, alternate segment, PR-like expression, with stripes never splitting. To compare expression of these genes, embryos were bisected, and halves were examined for either *Ofas-run* or *Ofas-E75a* expression. At late blastoderm, when two *Ofas-E75a* stripes were detectable, four *run* stripes were present (Fig. 15h).

During germband elongation, the cap of *Ofas-run* expression in the posterior SAZ persisted. As abdominal segments were added, dynamic SAZ expression was evident which is similar to *Ofas-E75a* SAZ expression pattern (Fig. 15 i-m'). In some germbands, only one stripe was observed in the anterior SAZ, and *Ofas-run* expression in the posterior SAZ clearly extended to the posterior edge of the germband. In others, two stripes were evident in the anterior SAZ and a broad stripe, appearing to span the width of two segments, was observed just posterior (Fig. 15 m' bracket), while expression in the germband's posterior extreme had cleared. Expression was also seen in later germbands in the head lobes, as well as in segmentally reiterated pairs of dots around the central midline in the thoracic and abdominal segments (Fig. 15m). *run* expression in limb primordia was also observed in the spider mite *Tetranychus urticae* (Dearden, Donly, & Grbić, 2002). In sum, *Ofas-run* appears to initiate expression in a PR-like register in blastoderm embryos, with stripes splitting to generate a segmentally reiterated pattern. *Ofas-run* is also expressed dynamically in the SAZ, similar to run expression in other sequentially segmenting species. I classify *Ofas-run* expression as PR-like because of the initial

broad stripes that split at blastoderm, as well as the appearance of stripes two-segments wide in the germband. However, a clear set of PR alternate-segment stripes, as seen for *Ofas-E75a*, was not observed for *Ofas-run*.

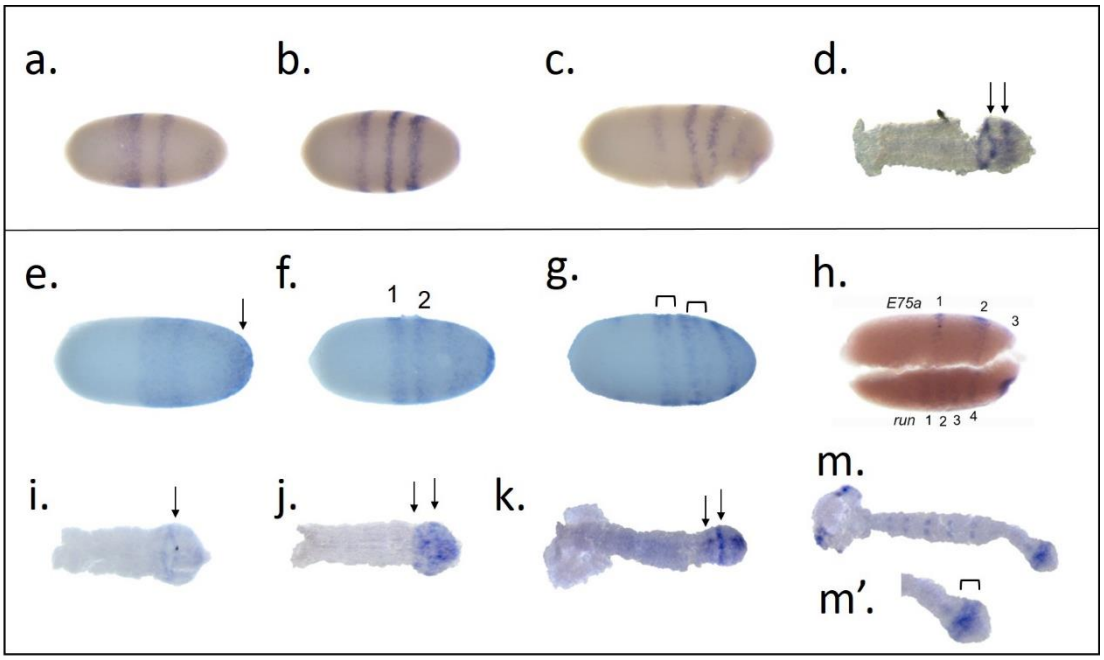

Figure 15. *Ofas-E75a* and *Ofas-run* are expressed in pair-rule-like patterns. a-d) *E75a* expression, e-m) *run* expression. a-e) *E75a* was first observed as two stripes in the blastoderm. By germband invagination, four stripes were observed; e) *run* was first observed in two centralized stripes and a posterior cap (arrow); f. g) The two anterior most stripes appear to split (brackets) while the posterior cap resolves into a stripe; then five stripes observed at the onset of germband invagination; h) Three stripes of *E75a* visible in an embryo with four *run* stripes it is possible that the posterior most stripes have already invaginated at this point (*E75a* stripe 4, *run* stripe 5); i-m') Dynamic SAZ expression was observed, including in some cases pairs of stripes (arrowheads) arising from the SAZ, where two-segment-wide stripes (brackets) were observed, similar to *E75a*. Embryos are oriented anterior left. (a -c, h performed by Katie Reding)

3.3.4 Persistent segmental expression *Ofas–slp*

Ofas-slp was expressed in a stripy pattern at blastoderm. In early blastoderm-stage embryos, a broad domain covering roughly 0-40% egg length was observed (Fig. 16a). This pattern appears to be complementary to early *Ofas-eve* expression (P.

Z. Liu & Kaufman, 2005a). In slightly later embryos, two stripes had emerged at the posterior boundary of this broad expression domain as the most anterior expression had started to clear (Fig. c). This anterior expression had completely cleared by later blastoderm-stages, leaving two close stripes at ~30% egg length (Fig. 14f). A total of seven stripes were observed in the blastoderm (Fig. 16e).At late blastoderm stage, *Ofas-slp* was similar to what was seen for the segmental-polarity gene *engrailed* (P. Z. Liu & Kaufman, 2004). *Ofas-slp* expression appeared in each segment anterior to each *en* stripe, as shown by double-staining early germbands (Fig. 16f). In later germbands, the stripes of *Ofas-slp* expression were observed to narrow somewhat, leaving a gap between *slp* and *en* stripes. In germband stages, *Ofas-slp* expression

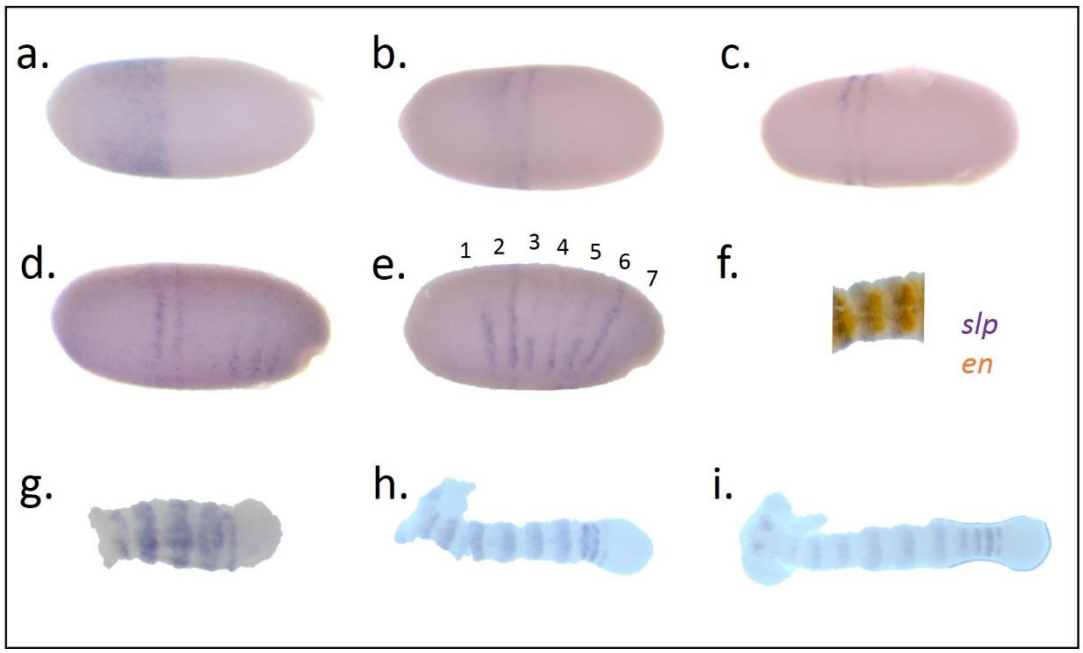

Figure 16. *Ofas-slp* is expressed in persistent segmental stripes. a) *slp* expression first observed in the anterior half of the embryo; b. c) two anteriormost stripes arose first; d. e) a total of seven *slp* stripes observed at blastoderm; f) double stain with *slp* (purple) and *en* (orange); g-i) *slp* was seen in each mature segment of the germband during elongation and was notably absent from the SAZ. Embryos are oriented anterior left.

stripes were added sequentially from in the segment addition zone (SAZ), but no expression was seen in SAZ itself.

3.3.5 *Ofas-h* has unique features

Compared with other PRG-orthologs, *Ofas-h* expression was first detected in later time stage. *Ofas-h* expression showed in late blastoderm-stage embryos in three faint stripes which is very similar with three-stripe pattern of *Ofas-E75a* (invagination pore in Fig.15 a marked by arrow) but these stripes were never observed to split. These stripes are likely the earliest manifestation of the germband expression seen slightly later. In germband stage, stripes of *Ofas-h* expression were seen in five segments and posterior to each *en* stripe and gaps along the central midline were observed in each stripe (Fig. 17b). It appears that this segmental expression is limited to the same five segments. Notably, expression of *Ofas-h* appeared to lag behind that of the other genes examined, as it was observed in older, more mature segments, rather than in the younger segments being generated from the SAZ, suggesting coordinate activation of *Ofas-h* stripes by gene(s) already expressed segmentally (Fig. 17b c). In later germbands, the old five stripes disappeared, and new stripes were seen in the abdominal segments, in addition to expression in the antennal segments (Fig. 17d marked by arrows and asterisks). In fully extended germbands, expression in the abdomen faded away, and new expression was seen in the labial through T3 segments (Fig. 17e). Later, two or three dots in the thoracic appendage primordia were observed in a partially retracted germband (Fig. 17e).

Together, these experiments showed that the three PRG-orthologs, *Ofas-slp, -run* – and *–h* are expressed in early embryos at the time expected for segment

specification. The expression pattern for *Ofas-slp* suggests that it does not function as a PRG, since it is not expressed in alternate segments, but rather that it may function as a segment polarity gene, similar to *en*. The expression patterns of *Ofas-run* is consistent with PR-like roles. *Ofas-h* appeared to lag behind other PRG-orthologs at blastoderm stage, which suggests that it might have other specific functions in later stages.

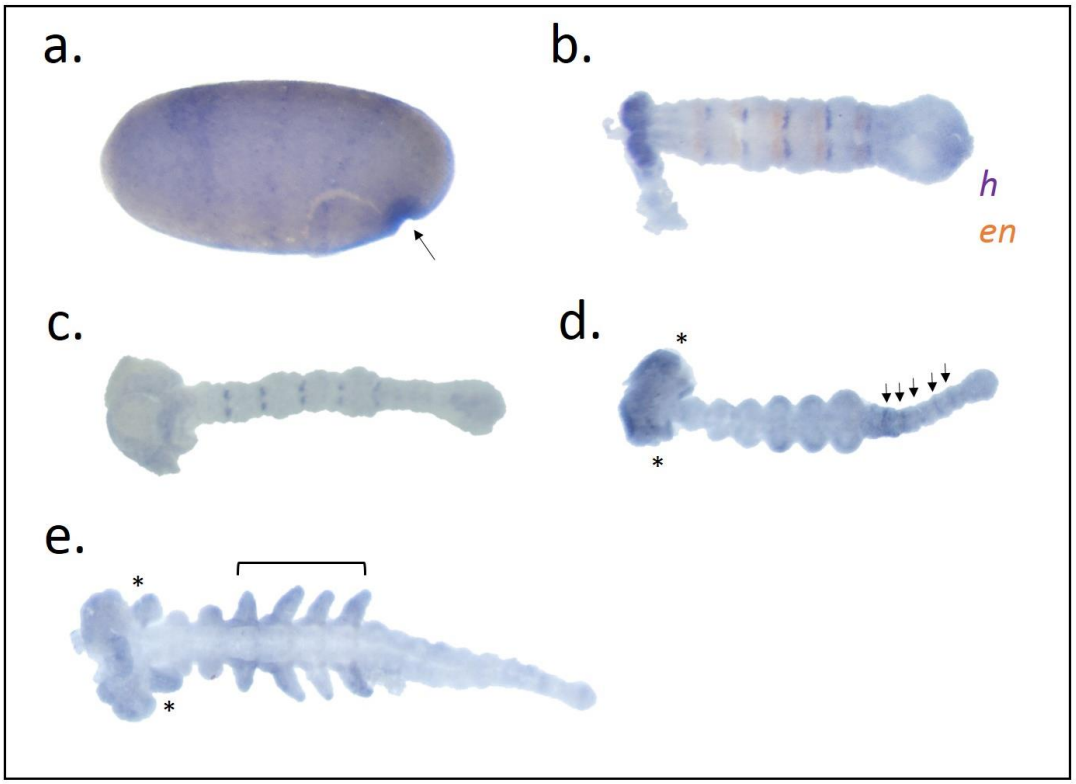

Figure 17. *Ofas-h* is expressed segmentally. a) *h* was first observed in three stripes at late blastoderm; b) double stain with *h* (purple) and *en* (orange) in an early germband; c. d)five segmental stripes of *Of-h* were observed through germband elongation; e) in later germbands, *h* expression was observed in the limb primordia. Embryos are oriented anterior left.

3.4 Discussion

To date, several insects have been used as a model system for studying segmentation genes. Most of them belong to Holomotabola, such as long-germ mode *D. melanogaster* and short-germ mode *T. castaneum.* PRGs, which first establish periodicity in the early embryo, are required for formation of body segments. PRGs have highly conserved gene sequences, shared among different species, but their expression patterns often differ. Investigations in non-holometabolous insects will help researchers to understand the diversity of segmentation gene regulatory mechanisms. *O. fasciatus*, representing the diverse clade of Hemiptera, a sister group to Holometabola, became an ideal option for studying PRG orthologs.

My results indicate that *Ofas-run* is not expressed in a striped pattern in alternate segments in the blastoderm like *Ofas-E75a* expression, but rather in two broad stripes that then split in a manner like *Dmel-prd.* In contrast, *E75a,* which has no PR-like expression in *D. melanogaster,* was expressed in the primordia of alternate body segments in *O. fasciatus* (Erezyilmaz et al., 2009). *Ofas-slp* was observed in a segmental pattern at blastoderm and was later seen as persistent stripes in each mature segment of the germband but was absent from the SAZ. *Ofas-h* was observed in five stripes which appear to be more spatially restricted in the mature germband. *Ofas-h* was present in sets of stripes, either in anterior or in posterior segments, just posterior to *Ofas-en* in germbands. The temporal expression of *Ofas-h,* along with restricted spatial localization of the stripes suggests it does not function as a PRG. Future studies will determine the functions of all of these genes.

Chapter 4: Conclusion and future directions

For my master's Thesis, I successfully annotated maternal, gap and PRG-genes in the *H. halys* draft genome and determined the temporal expression of *Hhal*-PRG orthologs. I also tested feasibility of RNAi in *H.haly* using the homeotic gene *Scr*. I showed that pRNAi-mediated knockdown of *H.hal-Scr* resulted in a dramatic transformation of proboscis toward leg. I also annotated and analyzed three *Ofas*-PRG orthologs and determined their temporal and spatial expression. The results showed that these gene are expressed in three distinct spatial patterns. Only *Ofas-run* is expressed in a PR-like pattern that is similar to *Ofas-E75a*, which is known to have PR-like function in *O. fasciatus* (Erezyilmaz et al., 2009). *Of-slp* is expressed segmentally and *Of-h* has a unique pattern compared with other genes.

Orthologs of *Dmel*-PRGs are expressed in PR-like patterns in a number of holometabolous insects (Aranda, Marques-Souza, Bayer, & Tautz, 2008; Choe et al., 2006; Rosenberg, Brent, Payre, & Desplan, 2014; Wilson & Dearden, 2012; Xiang et al., 2017). Orthologs of *Dmel-run, -slp* and *–h* are expressed PR-like patterns in *T. castaneum* (Aranda et al., 2008; Choe & Brown, 2007). In earlier-branching arthropods, the centipede *Strigamia maritima* (*S.martitima*), orthologs of *run,* and *h* are expressed in stripes that appear in alternate segments (Chipman & Akam, 2008; J. Green & Akam, 2013), while *Smar-slp* is expressed in a segmental pattern (J. Green & Akam, 2013).

Combined with other *Ofas*-PRG orthologs expression results from other members of the Pick lab (manuscript submitted), it appears that most *Ofas*-PRG orthologs are expressed segmentally. Their expression appears to be similar to the

pattern of segment polarity genes in *Drosophila*. Within this group, different temporal and spatial classes of expression were observed. *Ofas-eve, -odd,* and *–prd* were observed in six segmental stripes in the blastoderm. These genes were expressed at a segmental register in a transition zone between the posterior SAZ and the segmented germband. *Ofas-opa* has a similar expression to *Ofas-slp*. *Ofas-ftz-f1* was only observed in one or two stripes anterior to the SAZ in the mature germband.

Based on this, one hypothesis suggests that the ancestor of the PRG-orthologs of all arthropods showed PR-expression, but PR-like expression was lost in the lineage leading to *Oncopeltus.* This may have been enabled by their replacement by other transcription factors, such as *E75a* (Erezyilmaz et al., 2009). Another hypothesis is that segmental expression of PRG-orthologs is ancestral and PR-like expression was gained twice independently in the lineages to Myriapods and Holometabola. While the later hypothesis is less parsimonious, more data are needed to determine whether PRG-orthologs play homologous roles in holometabolous insects and in distant arthropods. If a PR-function was indeed ancestral, this role was lost for many PRG-orthologs in hemimetabolous lineages. In either case, these results suggest extensive rewiring of segmentation networks in arthropods. It would be interesting to carry out the same study on segmentation patterning in earlier branching insects, such as *Thermobia domestica*. Future comparative analysis will provide insight into the novel and derived regulatory mechanisms in this large clade.

Appendix I. List of Primers

Part I. Primers for degenerate PCR

ScrdegF CCRCARATHTAYCCRTGGATG

ScrdegR1 CATRTGGYANGGNACRATRTTCAT

Part II. Primers for amplifying templates for dsRNA synthesis

gfpF taatacgactcactatagggagaTTCACTGGAGTTGTCCCAAT

gfpR taatacgactcactatagggagaCCCAGCAGCTGTTACAAACT

OfdsRNAScr5'Ft7 taatacgactcactatagggTCAATGAACGTCATCCCGTA

OfdsRNAScr5'Rt7 taatacgactcactatagggTCAAGTATACACAGGTACGCT

Hh-ScrFT7 taatacgactcactatagggAGAGCAGGACCTGACTACGTCCTC

Hh-ScrRT7 aatacgactcactatagggAGATCCAGCTCCAGCGTCTGGTA

Part III. Primers for RT-PCR

Hhal-E75a

Hh-E75a-F-1 CAGATGAAGAGGACGGACATCATC

Hh-E75a-R-1 TGGCACCATAAGAATGAGTTCGTTC

Hhal-ftz-f1

Hh-FF1-F-1 TCGAAGCGGTTCGAGCGGATAGGATG

Hh-FF1-R-1 GAGCCCTCATAGCTGAAGCTCTTCTGG

Hhal-odd

Hh-odd-F-1 TCAGAGATCACAGGTATATCCACAG

Hh-odd-R-1 GGCTTTGCCTTAGTCTCGAATAAACG

Hhal-ftz

Hh-ftzF-2 CGCCAATCAAGGAGGAAGAC

Hh-ftzR-2 TGTCGTGTACGTTTGGTTCC

Hhal-eve

Hh-eveF-1 GGAATCGCCCACCAGAACAG

Hh-eveR-1 GCGACGCCGCTATTGTTGGAT

Hhal-run

Hh-runF-1 ACTCTGGGCGGTTTTTCTCT

Hh-runR-1 CTGTTGGTCGGTTTGGTCTT

Hhal-hairy

Hh-hairyF-1 CAGATCCAGCGATTGTCAAC

Hh-hairyR-1 GACAATCCCTACCACGGCCT

Hhal-slp

Hh-slpF-1 AGCATCGAGGAACAGGTTAG

Hh-slpR-1 GACACATTTGGCTACTCACAC

Hhal-actin

Hh-actin-F-1 ATGGTCGGTATGGGCCAAAA

Hh-actin-R-1 GTGCTCCTCTGGGGCAACTCG

Hhal-prd

Hh-prd-F-2 GCTGCGTCTCCAAGATACTC

Hh-prd-R-2 GGCTCAGAGTCGCAATCGGAA

Ofas-actin

F_Of_actin ATGGTCGGTATGGGACAGAA

R_Of_actin TGTTCTTCAGGGGCAACTCT

Ofas-run

Of-runt amplify ATGCATCTCCCAGGTGTGAC

Of-runt 1R ATCTGGTGAGGTGACGATCC

Of-runt 1R T7

TAATACGACTCACTATAGGGAGAATCTGGTGAGGTGACGATCC

Of-runt R2 GGTAGAAGTGGTAGCGGTGG

Of-runt R2T7 taatacgactcactatagggagaGGTAGAAGTGGTAGCGGTGG

Of-run-2-F CACTACATGACTGGTGGTGG

Of-run-3-R CTGAGTCGTCAAAAGATACAATATACTTTCTC

Of-run-3-RT7

taatacgactcactatagggagaCTGAGTCGTCAAAAGATACAATATACTTTCTC

Ofas-slp

Of-slp F2 CTTACAGCTACAACGCCCTC

Of-slp R2 CATGGACCTCTTGAAGGCG

Of-slp3UTR-F1 GTGGTTGTAGTCGAGGTGAAA

Of-slp3UTR-R1 TGAGGAATGTGACGACTTTAGG

Ofas-h

Of-hairyF3 GCCTCAATGAGCTCAAGTCC

Of-hairyR3 GCTTGGCAAGGAACAAGAAG

Part IV. Primers for 3'RACE

Ofas-run

Of-runt-outer1 CTCGGCCCTCTGGGATTCTCTCTTCTGC

Of-runt-inner1 CGGCCATAGTGACTCCCGGATCTGAGC

Ofas-slp

Of-slp-outer1 GGAGGACGTGTTCATCGGCGGTACGAC

Of-slp-inner1 GCCTCCCTGCTACGCCGACCTCTAC

Appendix II. List of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Round Plastic Container - 289C	PIONEERPLASTICS	289C	For *H.halys* colonies maintenance.
Amber Lumite Screen, 32 X 32 Mesh, 530 Micron	BioQuip	7250Q	For *H.halys* colonies egg laying
FILTER PAPER 9CM BLK PK100	Ahlstrom	28342-010	
Organic green beans	Mom's market		
Organic Sunflower Seeds Hulled (Raw, No Shell)	Sincerely Nuts		
Insect cage (size medium, 30.5x19x20.3 cm)	Exo Terra	PT2260	For *O. fasciatus* colonies maintenance. Can use larger or smaller cage if needed.
Petri dish	VWR	89038-968	
Cotton ball	Fisher	22-456-883	
Megascript T7 transcription kit	Fisher	AM1334	
Model 801 Syringe (10 µl)	Hamilton	7642-01	
Needle (32-gauge)	Hamilton	7762-05	
Food coloring (green)	McCormick		For dsRNA injection
Fixation Solution (Pampel's)	BioQuip Products, Inc.	1184C	Toxic, needs to be handled in fume hood

Forcep (DUMONT #5)	Fine Science Tools	11252-30	
Dissecting microscopy for phenotypic visualization	Zeiss	SteREO Discover. V12	
DIC microscopy	Zeiss	AXIO Imager. M1	
Dissecting microscopy	Olympus	SZ40	

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