SEX-SPECIFIC GENE INTERACTIONS IN THE PATTERNING OF

ONCOPELTUS FASCIATUS GENITALIA

By

Ariel C. Aspiras

Submitted to the

Faculty of the College of Arts and Sciences

of American University

in Partial Fulfillment of

the Requirements for the Degree

of Masters of Science

In

Biology

Chair:

David R. Angelini, Ph.D.

David B. Carlini, Ph.D.

Victoria Connaughton, Ph.D.

Dean of the College of Arts and Sciences

4 November 2011
Date

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Dean of the [Name of Your School]

[Date This Page Will Be Signed]
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DEDICATION

To my Guardians Michael and Eileen Harte, and my mother Rose Aspiras, for being my biggest fans.

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ABSTRACT

Genitalia play an important role in the life histories of insects, as in other animals. These structures are a unique developmental system to explore as they are rapidly evolving sexually dimorphic structures derived from multiple segment primordia. Despite the importance of insect genitalia, descriptions of their genetic patterning have been limited to fruit flies. In this study, we report the functions, interactions and regulation of appendage patterning genes (e.g. *homothorax*, *dachshund*, and *Distal-less*) in the milkweed bug *Oncopeltus fasciatus*. Female *O. fasciatus* have a multi-jointed ovipositor while male *O. fasciatus* have a genital capsule consisting of large gonocoxopodites and claspers. *O. fasciatus* required appendage-patterning genes for development of the male claspers, but not the proximal gonocoxopodite, suggesting a non-appendicular origin for this structure. The posterior Hox genes (*abdominal-A* and *Abdominal-B*) were required for proper genital development in *O. fasciatus*, and regulated *Distal-less* and *homothorax* similarly in both sexes. Appendage patterning regulation of *Distal-less* and *dachshund* was different between males and females. Knockdown of *intersex* produced a partial female-to-male transformation of abdominal and genital

anatomy, and also resulted in abrogation of female-specific regulation of these genes. These results provide developmental genetic support for specific anatomical hypotheses of serial homology. Importantly, these gene functions and interactions describe the developmental patterning of sexually dimorphic structures that have been critical to the diversification of this species-rich insect group.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. David R. Angelini, for all of his support and guidance, my committee members Dr. David Carlini and Dr. Vicki Connaughton, for all of their advice. In addition, I would like thank Jenny Knauss and Rashmi Prasad for their assistance with realtime PCR, and Nikita Donti for her assistance in the measurement of specimens. Last but not least, I would like to thank my parents Michael and Eileen Harte and Rose Aspiras for the much needed emotional support.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF ILLUSTRATIONS

Figure

CHAPTER 1

BACKGROUND

In different tissues or organs, differentiation may share many aspects; however especially at later stages distinct cues direct cells to adopt different fates. Serial homology and sexual dimorphism are two phenomena that highlight this issue. Serial homology describes the relationship between similar morphological structures originating from the same body plan sharing some aspects of development (Angelini et.al., in review), while sexual dimorphism denotes the inherent differences between sexes. In both cases, organs in separate sexes or at different axial body locations share many aspects of development, but diverge in key ways toward distinct phenotypes. Insects exhibit serial homology of body segments and appendages, as well as sometimes dramatic sexual dimorphism. Little is known about the nature of the gene interactions and patterning processes that lead to these distinct developmental end points.

Genitalia

Genitalia play an important role in the life history of most animals. They evolve rapidly and in some cases are the sole divergent morphological characters between closely related species (reviewed by Eberhard, 2011). Due to the dramatic differences between some male genital structures, the so-called "lock and key" hypothesis proposed that genitalia function as a mechanical barrier to out-cross hybridizations, thus directly

contributing to speciation (Shapiro and Porter, 1989). However, more recent theories favor post-copulatory sexual selection, particularly sexually antagonistic evolution and cryptic female choice, as mechanisms that may accelerate genital divergence between populations (Eberhard, 2011).

Insect genitalia are complex structures, with elements derived from the internal reproductive organs, posterior abdominal segments, and appendages, which may be elaborated or reduced in different groups. Male genitalia consist of the copulatory organ, and in some groups males possess external claspers (Fig. 1A-C, E). The anatomy of female ovipositors varies greatly among insect groups (Chapman, 1998; Scudder, 1961), but can be divided into two main types, which we will refer to as terminal and subterminal. Terminal ovipositors are modified from the posterior-most region of the abdomen, which telescopes out to deposit eggs on substrate and may be retracted when at rest (Fig. 1G). Coleoptera, Diptera, and some Lepidoptera have terminal ovipositors. Subterminal ovipositors are derived from the appendages of abdominal segments 8 and 9 (A8-A9) and are typically used to deposit eggs on or in specific plant or animal hosts (Fig. 1D-E). Thysanura, some Odonata, Orthoptera, some Thysanoptera, Hemiptera and Hymenoptera possess subterminal ovipositors. These orders include many dramatic examples of ovipositor specialization. For example, ichneumonid wasps use an elongated ovipositor to parasitize caterpillars and other insect larvae (Abbott, 1934; Boring et al., 2009), and the ovipositors of cicadas (Hemiptera) are capable of boring through wood.

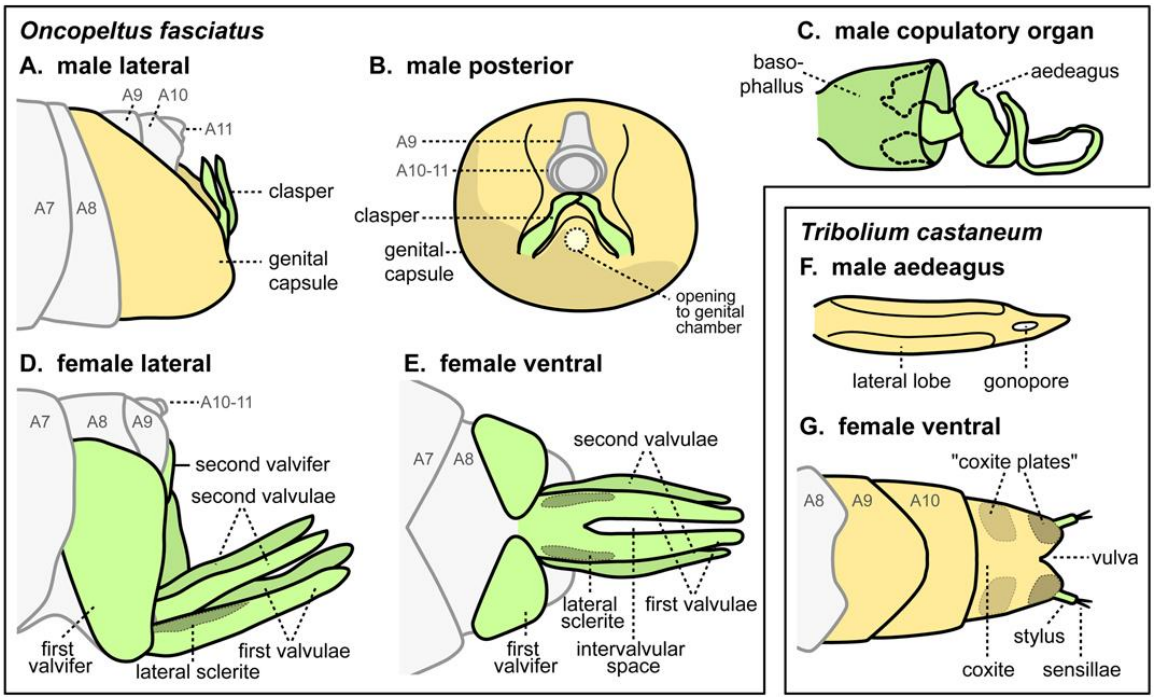

Figure 1 Diagram of Genitalia in *O. fasciatus* (A-E) and *T. castaneum* (F-G). Lateral (A) and posterior (B) sketches of the genitalia of male *O. fasciatus* show the prominent genital capsule, with articulating claspers. The copulatory organ emerges from an opening (dotted circle) obscured by a posterior ridge. The copulatory organ (C) consists of a distal aedegaus which is coiled when at rest, but inflates and extends during copulation. The basophallus is scleritized and rigid and houses the coiled aedeagus. Here, the organ is shown partially extended. Female *O. fasciatus* have a subterminal ovipositor consisting of two pairs of appendages, each with proximal valvifers and distal valvulae, shown here in lateral (D) and ventral views (E). The elements of the ovipositor are drawn as if sprayed out for clarity. The genitalia of T. castaneum males consist of the aedeagus (F), which is normally withdrawn internally. Female T. castaneum have a terminal ovipositor, consisting of 3 segments (G). The most posterior (or distal) is the coxite, which ends in the vulva. Styli flank the genital opening. Green indicates structures for which this study supports an appendicular origin. Yellow indicates non-appendicular genital structures, and adjacent non-genital structures are gray. Panels D and E are modified from Bonhag and Wick (1953). Panel G is modified from Sokoloff (1972).

Despite the biological importance of insect genitalia, their formative genetic patterning has only been examined in *Drosophila melanogaster* (Estrada and Sanchez-Herrero, 2001; Foronda et al., 2006; Gorfinkiel et al., 1999; Gorfinkiel et al., 2003;

Sánchez et al., 2001; Sánchez and Guerrero, 2001), which have relatively simple external genitalia. Genital patterning poses several unique developmental questions. Genitalia are one of only two appendages to appear at adulthood in hemimetabolous insects (wings being the second). Additionally, the genitalia are formed by contributions from several posterior body segments. In *D. melanogaster* A8-A10 contribute to the genital imaginal disc, with A8 and A9 developing into the genitalia and A10 developing into the analia. Furthermore, genitalia are a critical system in which to understand how patterning differs in sexually dimorphic structures.

In many insect groups, the genitalia of both sexes include putatively appendage-derived structures, such as male claspers and lance-like female ovipositors, which have been considered serially homologous to the ventral appendages: the antennae, mouthparts and legs (Boxshall, 2004; Minelli, 2002; Rosa-Molinar and Burke, 2002; Snodgrass, 1935). In *Oncopeltus fasciatus*, male and female genitalia differ greatly (Bonhag and Wick, 1953). The male genital capsule resembles two coxae fused medially and projecting to the posterior (Fig. 1A-B). Assuming this homology, anatomists have termed these the gonocoxopodites. Heavily scleritized claspers articulate from the posterior of the genital capsule. Between the claspers, behind a scleritized ridge, is an opening where the copulatory organ emerges during mating. In *O. fasciatus* this organ has a scleritized basophallus and a flexible distal aedeagus that coils into the basophallus when not inflated for copulation (Fig. 1C). Serial homologies between specific segments of the genitalia and other appendages have been uncertain. Since the male external genitalia have a single proximal-distal (PD) PDaxis, they are considered to consist of a single pair

of appendage primordia. In comparison, the female ovipositor of *O. fasciatus* is composed of two subterminal pairs of articulated structures, presumably two appendage pairs. Each appendage has a proximal valvifer and distal valvula (Fig. 1D-E). The first and second valvulae remain tightly associated and fused medially in the functional ovipositor.

Drosophila Development

In other appendage types, such as legs, development first begins with the specification of appendage tissue during embryogenesis. In the *D. melanogaster* embryo, appendage primordia are specified at segment boundaries by wingless activation and inhibited from dorsal and ventral directions by *decapentaplegic* and *epidermal growth factor (EGF),* respectively. These interactions localize expression of *Distal-less (Dll)* to the cells of the appendage primordia, which will give rise to the imaginal discs (Fig. 2) (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Second, Hox gene expression within the appendage primordia confers appendage identity and regulates appendage patterning to direct segment-specific appendage anatomies. In *D. melanogaster* , *Antennapedia* is required for leg identity (Struhl, 1982). The posterior Hox genes *abdominal-A (abd-A)* and *Abdominal-B (Abd-B)* specify identity in the abdomen and terminalia. *Abd-B* expression in the genital imaginal disc directs development of this tissue into the genitalia and analia in both sexes, while *abd-A* represses *Dll* expression in the abdomen and in the genital disc. *abd-A* appears to only be required in the female genitalia of flies, which is mostly derived from the more anterior

A8 segment where *abd-A* is expressed. Third, appendage-patterning genes establish regional domains along the limb (Wu and Cohen, 1999). The canonical leg-patterning genes, *homothorax* (*hth*), *dachshund* (*dac*) and *Distal-less* (*Dll*), have conserved roles in arthropod leg development. These genes promote growth and/or regional identity along the PD axis of the legs in *O. fasciatus* (Fig. 3) (Angelini and Kaufman, 2004; Angelini and Kaufman, 2005), T. castaneum (Angelini et al., in review; Beermann et al., 2001; Suzuki et al., 2009) and other arthropods (Ronco et al., 2008; Schoppmeier and Damen, 2001). Studies exploring the function and expression of these genes in other appendages, such as the antennae and mouthparts, have provided insights into insect appendage development and serial homology (Angelini and Kaufman, 2004; Casares and Mann, 1998; Dong et al., 2000; Morata, 2001; Ronco et al., 2008). For sexually dimorphic characters like genitalia, additional input from the somatic sex determination pathway is also required. In *D. melanogaster* two critical factors in this pathway are *doublesex* (*dsx*), which has sex-specific splicing variants, and *intersex* (*ix*), which encodes a cofactor for the female isoform of *dsx* that interacts with the Mediator complex. Both genes are structurally conserved among insects, compared to those acting earlier in the sex determination pathway. Functional conservation of *ix* has been demonstrated through rescue of the *D. melanogaster* *ix* null mutant with orthologous *ix* sequences from other Diptera and Lepidoptera (Siegal and Baker 2005; Cavaliere et al. 2009; Arunkumar and Nagaraju 2011).

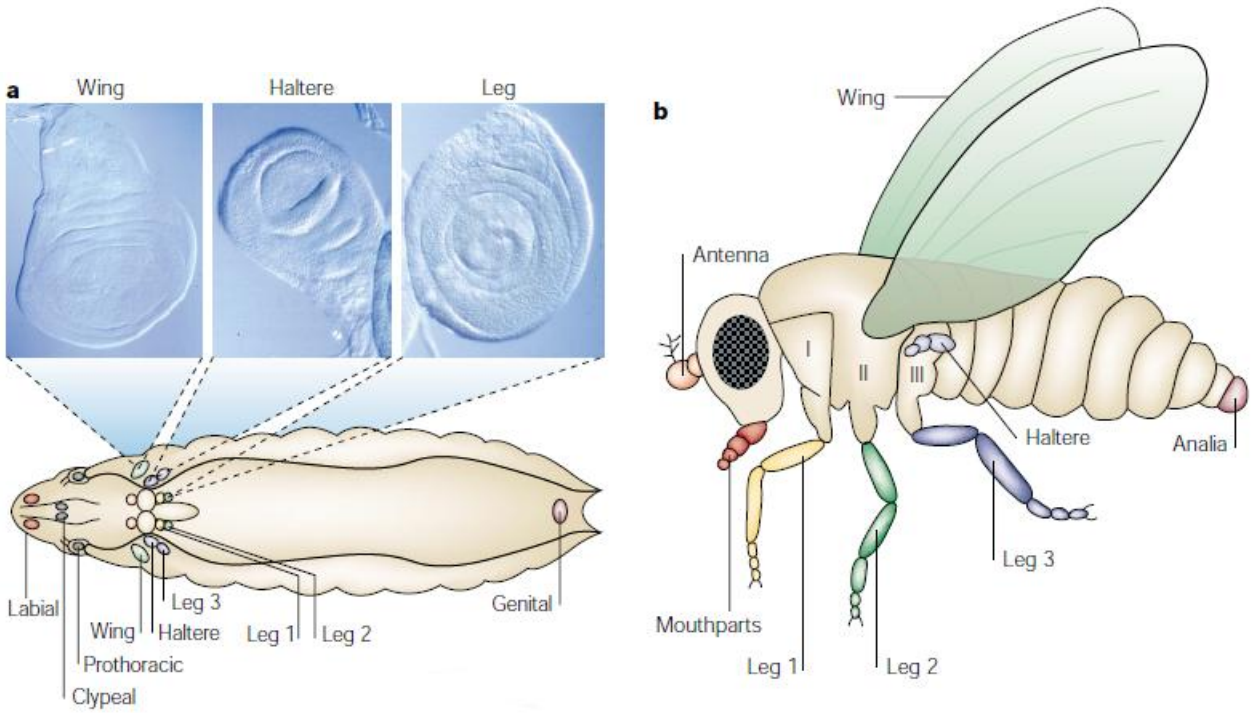

Figure 2 Diagrams of *D. melanogaster* Imaginal Discs (A) and Corresponding Adult Appendage (B). Notice 8 bilateral pairs of imaginal discs and one unpaired genital disc at the posterior of the larvae (A). The discs composed of tissue set aside during embryogenesis and patterned during larval development (A). The imaginal discs correspond to serially homologous appendages found in the adult fruit fly (B). Figure from Morata 2001.

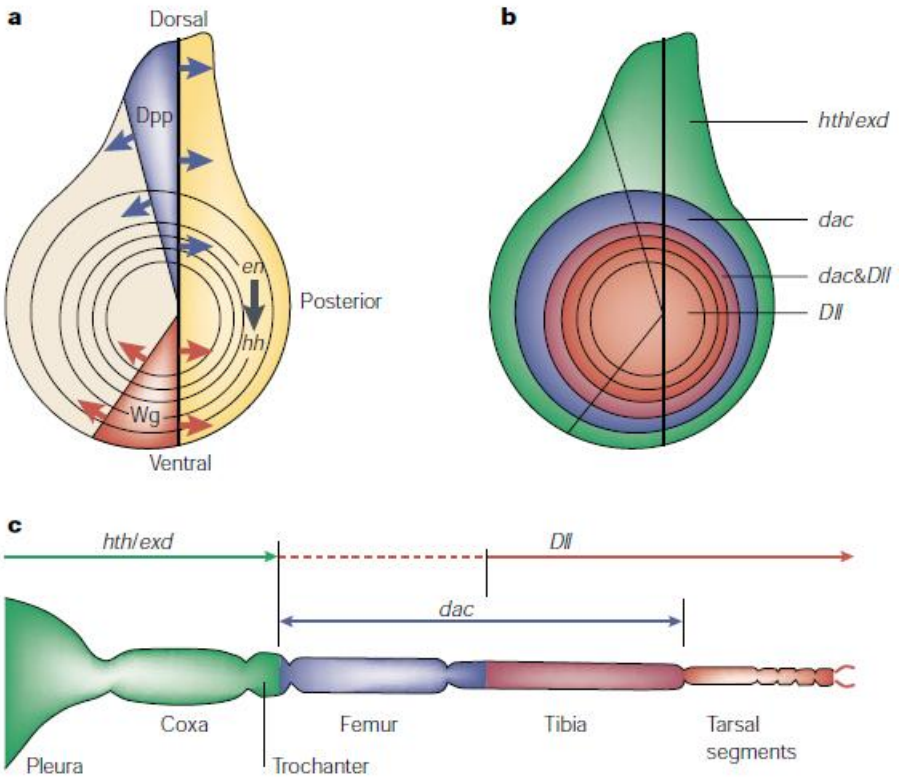

Figure 3 Anterior-Posterior (A-P) and Proximal-Distal (P-D) Specification of *D. melanogaster* Leg. Segmentation genes *engrailed (en)* and *hedgehog(hh),* activate *decapentaplegic (dpp)* dorsally and *wingless (wg)* ventrally, both of which diffuse and pattern A-P identity (A). *homothorax (hth), dachshund (dac),* and *Distal-less (Dll)* are expressed in concentric rings which demarcate the P-D identity respectively in the adult appendage (B-C). Figure from Morata 2001.

CHAPTER 2

OBJECTIVES AND SIGNIFICANCE

I propose to investigate the genetic underpinnings of genital development of the milkweed bug, *Oncopeltus fasciatus*, through a loss-of-function analysis via RNA interference (RNAi) using a canididate gene approach and a quantitative examination of the interactions between the genes using quantitative real-time reverse transcription polymerase chain reaction (qPCR) in order to address current questions in developmental biology.

Question 1

How does hemimetabolous insect appendage development differ from holometabolous insect development? (Fig. 3) The majority of what we know about appendage development stems from *Drosophila*, a holometablous insect, which form their limbs using imagninal discs during their larval stages. In contrast to holometablous insects which undergo a complete metamorphosis where the juveniles are morphologically distinct from adults, more basal insects, like hemipterans (*O. fasciatus*), where the juveniles possess most adult structures (except wings and genitalia), use limb buds during their embryogenesis and continue to remake their limbs through each juvenal instar.

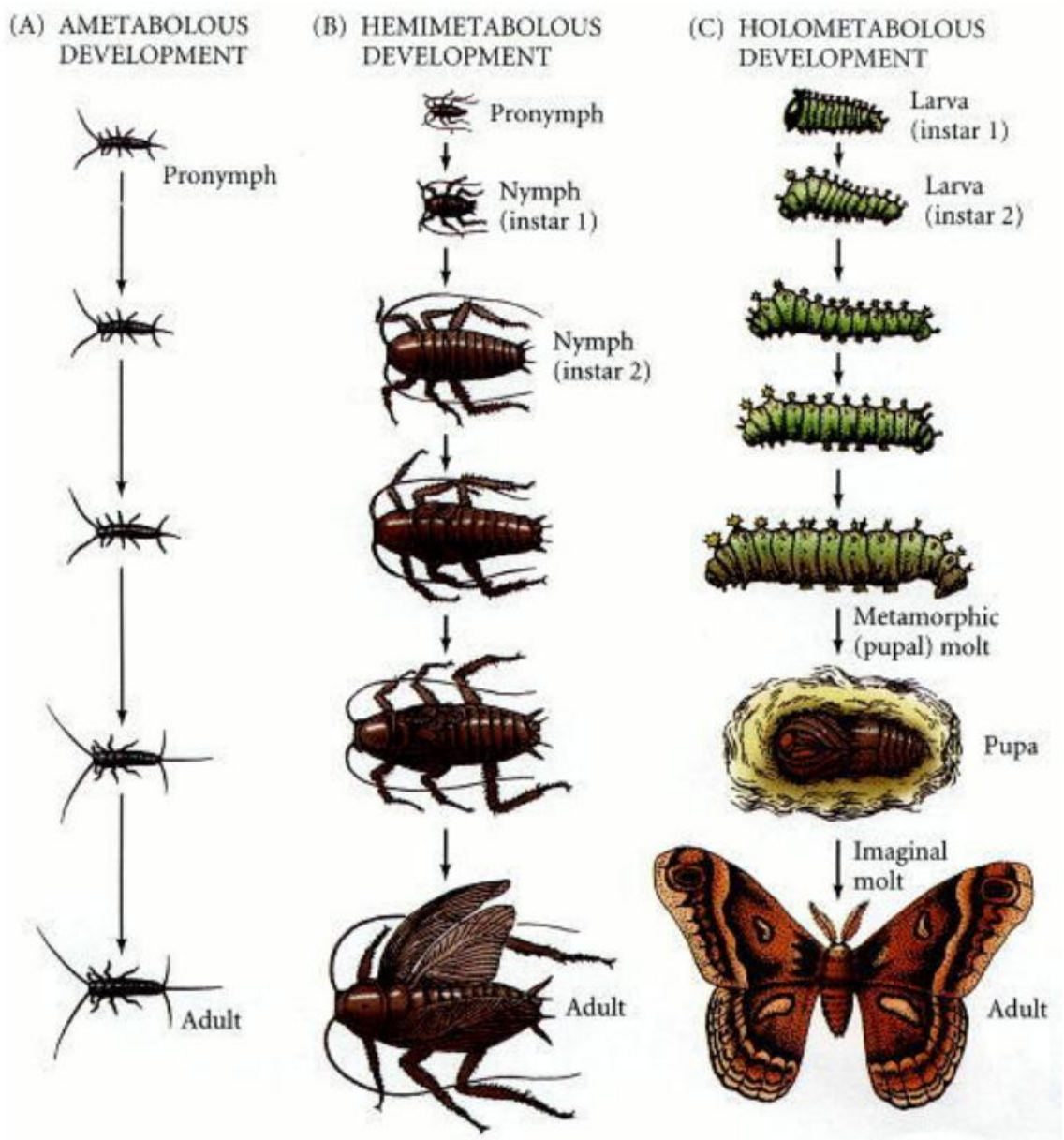

Figure 4 Different Modes of Insect Development. Silverfish undergo a pronymph phase (A), after which the insect resemble the adults characteristic of ametabolous (direct) development. Cockroaches (B), a hemimetabolous (gradually developing) insect, have five nymphal stages where the juveniles have most of the adult morphological structures (except wings and genitalia). Butterflies (C) proceed through five larval (juvenile) stages and a pupal stage (cocoon), both of which are morphologically disparate from the adult form. Figure from Gilbert 2000.

Question 2

How does *O. fasciatus* genital development depart from *O. fasciatus* antenna, leg, and mouthpart development? Genitalia in hemipterans appear only after adult eclosure, while the other appendages are reformed throughout juvenile development. Furthermore, antennae and legs derive from primordia spanning a single segment, while genitalia are derived from segments A8-11 (at least as described in *Drosophila* reviewed by Sánchez and Guerrero, 2001) Thus, this question addresses how development is modulated both in heterochrony and heterotopy to form a serially homologous appendage.

Question 3

How does *O. fasciatus* make sexually dimorphic genitalia? Insects differ from vertebrate sexual differentiation in that they do not utilize hormones to make sex-specific characters (reviewed by Sanchez 2008). Rather each cell undergoes sexual differentiation, occasionally leading to gynandromorphs or mosaic inter-sexual insects. As genitalia may be serially homologous to legs, there may be some overlap in the genes patterning both sexes. Determining the degree of the overlap may lead to not only a new insight on rapid genital evolution but also a deeper understanding of how development modulates gene expression, interaction, and function to form distinct appendages.

CHAPTER 3

MATERIALS AND METHODS

Oncopeltus Fasciatus

Milkweed bugs (Hemiptera: Lygaeidae) are true bugs, approximately 9-18 mm long, with a long beak used for sucking nutrients from seeds. *O. fasciatus,* as is true with all hemimetabolous insects, undergo gradual development where their five nymphal (juvenile) stages possess most adult structure excluding wings and genitalia. In each nymphal molt, appendages are reformed. In the wild, they are usually found on milkweed plants in order to feed on their seeds and oviposit (lay their eggs) in the fluff found in the pods. The milkweed seeds contain poisons which cause *O. fasciatus* to taste bitter to their predators. Besides the ease of culture and genetic manipulation via RNAi, *O. fasciatus* is an ideal candidate to study and compare genital development as Hemiptera is a closely-related out-group to Holometabola, which contains *D. melanogaster* , the only other insect model where genital patterning has been explored. This relationship is an advantage which allows easy identification and cloning of candidate genes, while maintaining enough phylogenetic distance to provide meaningful comparisons. Additionally, the ovary and embryonic transcriptome was recently published by Ewen-Campen et al. (2011), which allows for rapid cloning of new genes.

Insect Culture

Wildtype cultures of *O. fasciatus* were obtained from Carolina Biological Supply Company. Milkweed bugs were maintained according to Hughes and Kaufman (2000). To briefly summarize, milkweed bugs were housed in clear plastic cages with spring water and organic sunflower seeds. Adult cultures were provided cotton balls to lay their clutches, which were removed weekly to start new synchronous colonies.

Selection and Cloning of Candidate Genes

Candidate genes were identified for study based on the literature of leg and genital disc development in *D. melanogaster* and comparative data from other arthropods (Table 1). Most candidate genes used in this study have been previously cloned (Angelini and Kaufman, 2004; Angelini et al., 2005). *Oncopeltus fasciatus intersex* (*ix*) was cloned using exact primers (forward: GTAGGTTATTGTGAGTGTTGAGGTTG; reverse: GGTCTGTAGAAAGGAGGAACTTTTGA) designed from *ix* transcript sequence generated by Ewen-Campen and colleagues (2011) and available online (http://www.extavourlab.com/resources/). The amplified fragment was cloned using standard methods and sequenced to confirm its identity. To briefly describe cloning, the fragment is annealed to TOPO4 vectors and chemically competant *E.coli* were heat shocked to facilitate plasmid adsorption. This *O. fasciatus ix* sequence was deposited in GenBank (Accession JN368475).

Table 1. Candidate Genes Used in This Study. Genes were identified from the literature and clones were obtained based on the referenced studies.

gene	symbol	protein class	GenBank	reference
abdominal-A	*abd-A*	homeobox TXF	AY627361	Liu & Kaufman, 2004
Abdominal-B	*Abd-B*	homeobox TXF	AY627362	″
Distal-less	*Dll*	homeobox TXF	AY584472	Angelini & Kaufman, 2004
dachshund	*dac*	Ski/Sno-related TXF	AY584473	″
homothorax	*hth*	homeobox TXF	AY584474	″
intersex	*ix*	Mediator subunit	JN368475	Ewen-Campen et al., 2011; this study

Preparation of Double-stranded RNA and RNA Interference

Phenotypes were generated in adult insects using juvenile RNA interference. Knockdown of gene activity was verified using quantitative realtime RT-PCR. To synthesize double-strand RNA, a template DNA was amplified from a cloned gene fragment, using exact primers with the T7 promoter sequence added at the 5´ end. Double-stranded RNA (dsRNA) was transcribed using the MegaScript Transcription Kit (Applied Biosystems) with T7 RNA polymerase, then treated with DNase I to remove plasmid DNA. The product was annealed by cooling and purified by precipitation in ammonium acetate and ethanol. After resuspension in nuclease-free water, dsRNA concentrations were determined through triplicate measurements on a nanoscale spectrophotometer (GE Life Sciences NanoVue) and diluted to 4 μg/μl with 0.05% McCormick green food coloring, 0.01 mM $NaPO_4$, and 5 mM KCl.

Injection of *O. fasciatus* was done in fourth instar nymphs, anesthetized using CO_2 or with a 4-minute exposure to diethyl ether vapor. A penultimate instar injection allowed for RNAi mediated knock-down in the ultimate (5^{th}) instar prior to ecdysis.

Using a front-loaded pulled-glass capillary needle, approximately 1 µl of 4 µg/µl dsRNA was injected at the base of the right metathoracic coxa. This location facilitated easy delivery into the hemolymph and no defects were observed at the site after ecdysis.

Measurement of Gene Expression

The extent of gene knockdown was determined using quantitative realtime RT-PCR (qPCR) amplification of target gene sequences. For validation of RNAi, expression was compared between gene-specific and nonspecific control dsRNA treatments (*GFP*). Total RNA was isolated using the PureLink RNA Mini Kit (Life Technologies) from the abdominal tissue (A4-A11) of individual adult *O. fasciatus*. Isolated RNA was stored at -80˚C. For all *O. fasciatus* treatments, at least 3 biological replicates were included. Total RNA concentrations were determined by triplicate measures on a nanoscale spectrophotometer and diluted to 100 ng/µl immediately prior to assays. Total RNA was used as template in reverse transcription / SYBR Green realtime PCR reactions (Quanta BioSciences).

For each gene, exact primers (Table2) were designed using the Primer3 algorithm (Rozen and Skaletsky, 2000), avoiding conserved functional domains and dsRNA regions. Dissociation curves for each reaction were used to verify that only single products were amplified. To produce quantitative template standards, clones were linearized and transcribed *in vitro* from T7 promoters to produce single-stranded RNA. This RNA was treated with DNase I to remove template DNA and purified by precipitation in ammonium acetate and ethanol. Immediately before qPCR assays, the

RNA concentration was determined in triplicate (as described above) and the molar quantity was calculated based on the size of the RNA. Dilution series were then prepared fresh for each plate at concentrations of 10^3, 10^5, and 10^7 RNA molecules to serve as a standard curve (Pfaffl, 2004). The degree of knockdown in RNAi specimens is given in Table 3 with statistical significance based on Tukey's honest significant difference (HSD) test ($p < 0.05$).

Table 2: Primers Used for dsRNA Synthesis and qPCR

gene	primer name	DNA oligo sequence	position	product size
dsRNA synthesis				
EGFP	T7-GFP-f1	taatacgactcactataggg GCTGTTCACCGGGGTGGTGC	21	600
	T7-GFP-r1	taatacgactcactataggg GCGGACTGGGTGCTCAGGTA	580	
hth	T7-Of'*hth*-f1	taatacgactcactataggg GCTACATCAGCTGCTTGAAG	18	240
	T7-Of'*hth*-r1	taatacgactcactataggg GTGGCCGGGGTAGGAGAGCG	217	
	T7-Of'*hth*-f2	taatacgactcactataggg CGCGGCAAGGAGAGGTGTTT	361	240
	T7-Of'*hth*-r2	taatacgactcactataggg ACAACCAAGCTCTGAGGATA	560	
dac	T7-Of'*dac*-f1	taatacgactcactataggg GTCAGGATCCTCCGGGGTCT	1	240
	T7-Of'*dac*-r1	taatacgactcactataggg AGACCCCGGAGGATCCTGAC	200	
	T7-Of'*dac*-f2	taatacgactcactataggg CCTGTACTGAACCTCTCGAA	601	240
	T7-Of'*dac*-r2	taatacgactcactataggg GAATAGTTGAGGGCGTGTGG	800	
Dll	T7-Of'*Dll*-f1	taatacgactcactataggg CAGAATCCCTACAACCCCGT	1	240
	T7-Of'*Dll*-r1	taatacgactcactataggg ACGTCTTTAGGCGGAGAAGG	200	
	T7-Of'*Dll*-f2	taatacgactcactataggg TAAACACCGATTTTTGGAGG	201	240
	T7-Of'*Dll*-r2	taatacgactcactataggg CTGCCAGCTCGGCTCTCTCG	400	
abd-A	T7-Of'abdA-f1	taatacgactcactataggg CGAGCAGGCGAGGAGGGAAC	26	160
	T7-Of'abdA-r1a	taatacgactcactataggg TGTGGGGGTCGTGTATCTGG	145	
Abd-B	T7-Of'AbdB-f1	taatacgactcactataggg GAGGTGGGAGCTGGCGAGGA	37	226
	T7-Of'AbdB-r1	taatacgactcactataggg GGTACCATCTGATGCGGAGG	222	
	T7-Of'AbdB-f2	taatacgactcactataggg CATCACCCTCCCAGTGGAGC	224	247
	T7-Of'AbdB-r2	taatacgactcactataggg TGAGAACGAGTGGAGGTTCT	430	
ix	T7-Of'*ix*-f1	taatacgactcactataggg AGAGTCCCTTGTTGCTACTC	246	241
	T7-Of'*ix*-r1	taatacgactcactataggg GAGCTCTGACTCATGCATTC	446	
qPCR				
abd-A	Of'abdA-qf3	AGATGATGGGCTCGCTGGAC	146	79
	Of'abdA-qr3	GTAGCCTATGTCTGCACTTTGGTGA	224	
Abd-B	Of'AbdB-qf5	CCTCTTCAACGCCTACGTCTCTA	23	124
	Of'AbdB-qr5	CTGCGTGTTCTTCTTGTTCTTCA	146	
hth	Of'*hth*-qf3	AGCACCTCAGCTTGACCACTC	274	88
	Of'*hth*-qr3	GTCTGAAGCTGCCCTCGCTT	361	
dac	Of'*dac*-qf8	AACCCACAACTACAACATCACCTCAT	385	129

	Of'*dac*-qr8	TTCTCGCAGTCTTTCCAAATGTTTTA	513	
Dll	Of'*Dll*-qf5	GAGGATGTAGGAGCATTCACCGAAC	217	88
	Of'*Dll*-qr5	GCTTCCTCATCTTCTTGCCCTTTC	304	
ix	Of'*ix*-qf2	CTAGCAGTCAGCGCTATCTATCTTTG	446	97
	Of'*ix*-qr2	AGACACTGTGGGTAGGTAAGTGTGAG	542	

To determine gene interactions, potential gene regulators are knocked down using juvenile RNAi and target gene expression is measured using qPCR. If regulator gene knockdown via RNAi increases targe gene expression relative to GFP RNAi controls, then the regulator gene or its downstream targets is an inhibitor of the target gene. If the target gene expression is decreased, then the regulator gene or its downstream targets is an activator of the target gene.

Characterization of RNAi Effects

Tables 3 summarize the phenotypic penetrance and rates of gene knockdown for RNAi. Some treatments did not have a statistically significant knockdown in expression; however phenotypic penetrance was high Tables 3 and phenocopied gene-specific loss-of-function defects in other appendages, similar to those observed previously (Angelini and Kaufman, 2005; Angelini et al., 2009). At the time of injection, juveniles lack obvious sex-specific characters; therefore sex was scored after adult eclosure. Nonspecific GFP dsRNA treatment had no effects on genital development.

Table 3. Summary Results for Juvenile *O. fasciatus* RNAi. Asterisks describe significant knock-down relative to nonspecific GFP controls (TukeyHSD, p-value <0.05).

dsRNA	number scored	penetrance	target gene knockdown
GFP	27 ♂	(0)	
	34 ♀	(0)	
abd-A	32 ♂	84% (27)	37 *

	48	♀	100%	(48)	27	*
Abd-B	48	♂	88%	(42)	33	
	51	♀	96%	(49)	68	*
Dll	24	♂	92%	(22)	55	*
	32	♀	97%	(31)	61	*
dac	37	♂	97%	(36)	20	*
	35	♀	91%	(32)	47	*
hth	42	♂	74%	(31)	74	*
	39	♀	85%	(33)	70	*
ix	24	♂	100%	(24)	20	
	28	♀	100%	(28)	3%	
ix, Dll	8	♂	100%	(8)		
	12	♀	100%	(12)		
ix, dac	6	♂	100%	(6)		
	13	♀	100%	(13)		
ix, hth	13	♂	100%	(13)		
	11	♀	100%	(11)		

Specimens of *O. fasciatus* were stored in 70% ethanol within 12 hours of adult eclosure. Hox and *ix* dsRNA treated specimens were unable to completely shed the nymphal cuticle, so the loose abdominal exuvia was removed by hand to improve visualization of the genital morphology. Internal anatomy was examined after dissection, and copulatory organs were mounted in Aqua Poly/Mount (Polysciences, Inc.) prior to imaging. A representative sample (8 to 10) of each sex for *O. fasciatus* dsRNA treatments were imaged using an Olympus SZX16 dissecting microscope equipped with an Hamamatsu C8484 high-resolution digital camera.

Genital measurements of *O. fasciatus* were made from digital images using ImageJ (Abramoff et al., 2004). For male specimens, distances were measured from the ventral edge of the genital capsule to the dorsal analia, as well as from base of a clasper to its tip (Fig. 5A,H). For female specimens the length of the first (Fig. 6I) and second

valvulae (Fig. 6J) were measured. The distance across the head, between the innermost edges of the eyes (ocular distance), was used to normalize for overall body size. However, no effects of ocular distance were found, therefore figures report absolute measurements. Treatment effects were tested using one-way ANOVA and post-hoc Tukey's HSD tests were used to identify treatments that differed significantly from nonspecific *GFP* dsRNA controls. In all cases, the determination of signifiacance in nonparametric tests (Kruskal-Wallis and Wilcoxon rank sum tests) agreed with ANOVA (treatment effect) and the Tukey's HSD (pairwise difference) tests. All statistical tests were conducted in R (Ihaka and Gentleman, 1996).

CHAPTER 4

RESULTS

RNA Interference of Appendage-patterning Genes Produced Growth and Patterning Defects in the Genitalia of *O. fasciatus*

In the male genitalia, *Dll* knockdown reduced the length of the claspers (Fig. 5B). This reduction in length was statistically significant (Fig. 5H; Tukey's HSD, $p = 0.0014$). In female *Dll* RNAi specimens, the ovipositor was also reduced (Fig. 6B). Both the first and second valvulae were significantly shorter (Fig. 6J-K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p = 0.039$ for valvula 2). Reduction was more severe in the first valvulae, the more anterior of the two pairs. *Dll* juvenile RNAi produced allometric reductions of the mouthparts, as well as fusions of the tarsi (not shown).

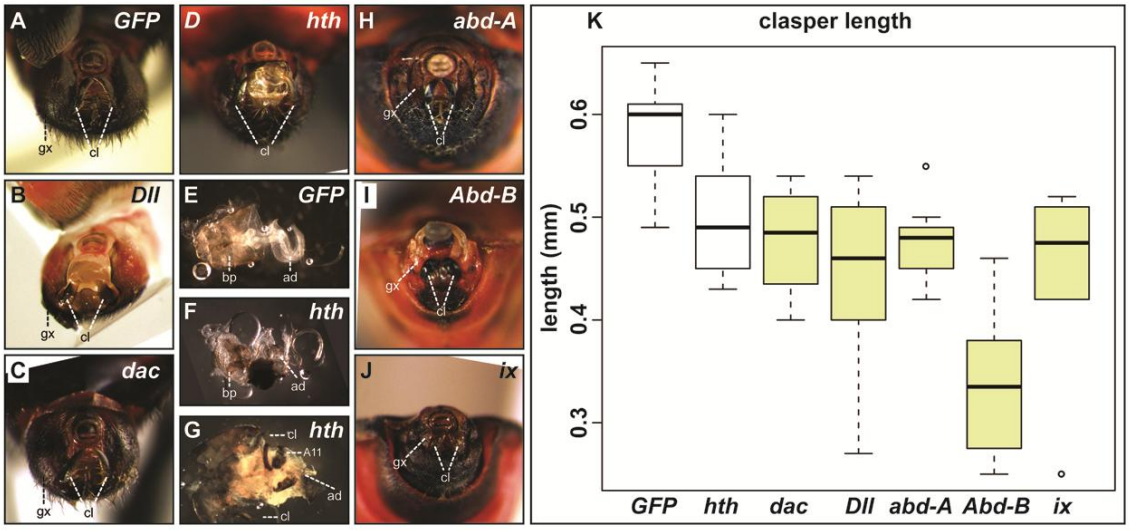

Figure 5 The Male Genital Capsule of *O. fasciatus*. (A) Nonspecific GFP control dsRNA treatments were indistinguishable from unmanipulated males. (B) *Dll* RNAi in males reduced length of the clasper. (C) *dac* RNAi caused reduced claspers. (D) In *hth* RNAi

the male copulatory organ (the white tissue) was exposed and malformed. (E) The copulatory organ in control dsRNA males consists of a proximal phallobase and distal aedeagus, which coils up and is stored in the distal part of the phallobase. (F) In mildly affected *hth* RNAi specimens the copulatory organ was reduced overall. (G) In more severe *hth* knockdown males, the copulatory organ lacked an obvious phallobase and a malformed aedeagus protruded externally as in this dissected genital capsule. (H) *abd-A* RNAi caused reduction of the male genitalia. (F) *Abd-B* knockdown caused the most dramatic reduction in male genitalia of all the genes examined. (G) Knockdown of *ix* caused reduction of the male genitalia. (H) Box plots of male clasper length from dsRNA treatments. The dark line represents the median value, the box shows the 25th to the 75th quartiles; dotted lines give outer quartile range; circles show outliers. Treatments differing significantly from nonspecific GFP controls are colored and marked by asterisks (Tukey's HSD, $p < 0.05$). Abbreviations: ad, aedeagus; cl, clasper; gx, gonocoxopodite; pb, phallobase.

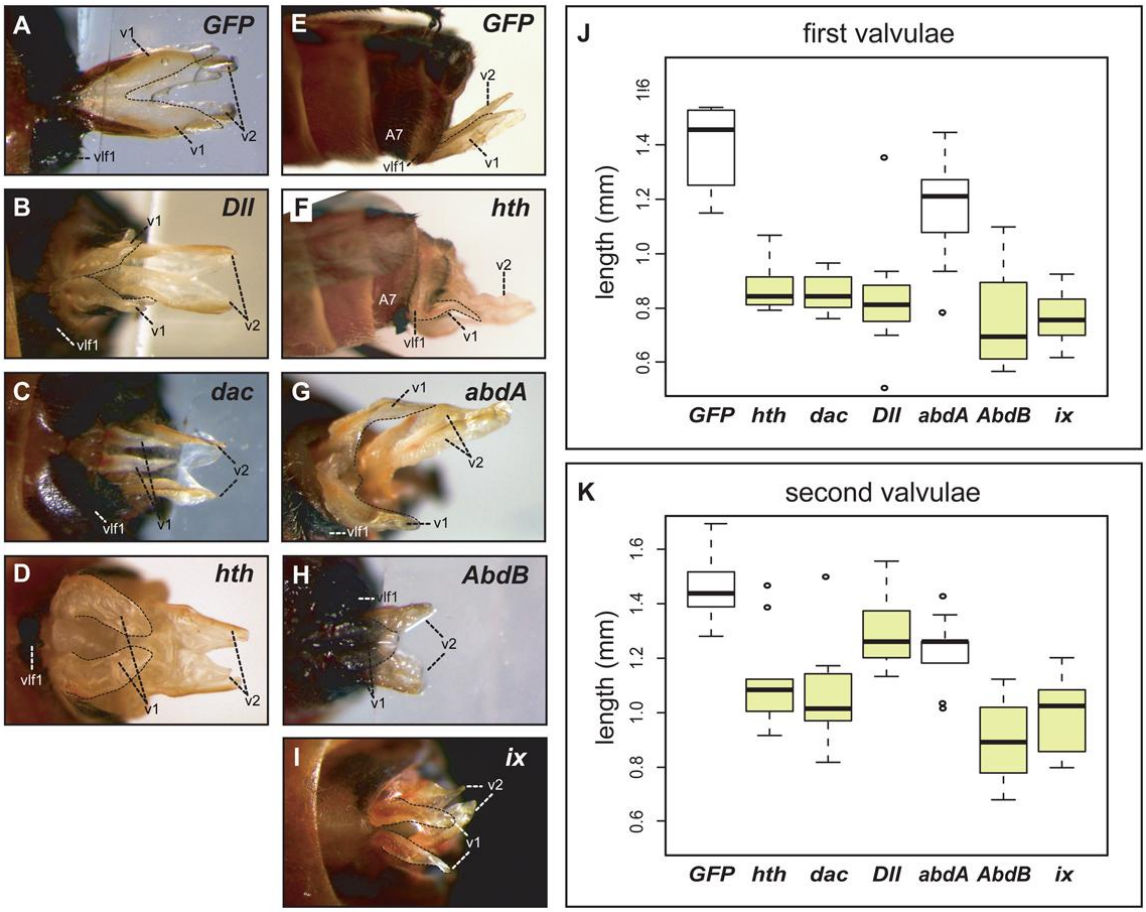

Figure 6 The Ovipositor of *O. fasciatus*. (A) Nonspecific GFP control dsRNA treatments were indistinguishable from unmanipulated females. The view is from a ventral

perspective; anterior is to the left. (B) In *Dll* RNAi females both appendage pairs of the ovipositor were significantly shorter. However, first valvulae were dramatically shorter than second valvulae. (C) *dac* knock-down females have reduced valvulae and a failure of medial fusion in the first valvulae. (D) In *hth* RNAi, all valvulae were reduced and first valvulae did not fuse medially. (E) A lateral view of the GFP control ovipositor shows the proximal valvifers. (F) Valvifers were enlarged by *hth* RNAi. (G) *abd-A* RNAi produced a transformation of the anterior first valvulae towards the structure of the second valvulae. These structures failed to interlock into a functional ovipositor. (H) The knockdown of *Abd-B* caused dramatic reduction in all components of the female genitalia. (I) *ix* RNAi caused a reduction of female genitalia, a failure of left and right appendages to fuse, and changes in pigmentation and scleritization that suggest a partial female-to-male transformation. Box plots showing the length of first valvulae (J) and second valvulae (K) from each dsRNA treatment. Range and significance are indicated as in Figure 5H. Abbreviations: v1, first valvulae; v2, second valvulae; vlf1, first valvifer.

The clasper length of the *dac* RNAi specimens was significantly shorter than with control dsRNA treatment (Fig. 5C,H; Tukey's HSD, $p = 0.027$). *dac* knock-down in females had a dramatic effect on the genitalia (Fig. 6C). Valvulae were significantly reduced (Fig. 6J-K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p = 1.0 \times 10^{-7}$ for valvula 2). Additionally, the left and right first valvulae did not fuse at the midline, and the membranous tissue of the second valvulae was disorganized and did not fold properly (Fig. 6C). Juvenile *dac* RNAi also caused defects in the stylets, which typically did not extend out of the head to their normal length (not shown). Internal reproductive anatomy was not obviously effected by *dac* depletion, including the single medial spermatheca and its duct.

In male *hth* RNAi, clasper length was not significantly reduced. However, these males developed with reduced and malformed copulatory organs (Fig. 5D-G). This reduction affected both the phallobase and aedeagus (proximal and distal structures; Fig.

5F). The dorsal-ventral depth of the genital capsule was also measured for *Dll*, *dac* and *hth* RNAi treatments, however none of these differed significantly from measurements of *GFP* dsRNA control males (not shown). In females, *hth* RNAi caused the proximal valvifers to become enlarged (Fig. 6E-F), however the valvulae were significantly reduced (Fig. 6D,J-K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p = 8.2\times10^{-6}$ for valvula 2). Additionally, the first valvulae failed to fuse medially. *hth* juvenile knock-down also affected the stylets, which failed to lay neatly in the rostrum (not shown).

RNA Interference of Posterior Hox Genes

The posterior Hox genes were required for normal development of the genitalia in *O. fasciatus*. *abdominal-A* knockdown significantly reduced male genital characters including the distance between clasper bases (Fig. 5H) and clasper length (Fig. 5K; $p = 0.015$). The genital capsule was also significantly reduced in size (Tukey's HSD, $p < 10^{-16}$) and did not project out from the abdomen as in unmanipulated bugs. Knockdown of *abd-A* in females did not reduce the length of valvulae (Fig. 6J-K). However, the valvulae of *abd-A* depleted females did not nest normally with one another. When at rest, the valvulae of female *O. fasciatus* fold up and are covered by the first valvifers. However, the valvulae of *abd-A* knockdown females were positioned abnormally and did not retract. This defect may result from homeotic transformation of the first valvulae towards the structure of the second valvulae (Fig. 6G). Additionally in *abd-A* RNAi, pigmentation of the anterior abdomen (A2-A8) was missing in both sexes. In females the sex-specific A4 sternal process was also absent. These effects resemble embryonic *abd-A* RNAi

phenotypes (Angelini et al., 2005) and seem to suggest the expansion of posterior *Abd-B* activity into more anterior abdominal segments in the absence of normal *abd-A* expression.

Abdominal-B knockdown significantly reduced both male and female genitalia. In males, the gonocoxopodite (Tukey's HSD, $p = 4.2\times10^{-5}$) and claspers were significantly reduced (Fig. 5I,K; Tukey's HSD, $p < 10^{-16}$). Abdominal body segments A7-A10 lacked the normal black pigmentation, although A11 remained pigmented. In females, both first and second valvulae were reduced (Fig. 6H,J-K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p < 10^{-16}$ for valvula 2). Moreover, the proximal valvifers were drastically reduced such that they were typically not visible beneath the overlying A7 sternite (Fig. 6H). In females, A7 lost its normal black pigmentation, although the valvifers remained black.. In both sexes, the abdomen was significantly longer in *Abd-B* knockdown, relative to controls (Tukey's HSD, $p = 0.0059$).

Interactions Among Appendage-patterning Genes in the Pre-adult Abdomen

The expression of several genes was determined in the posterior abdomen of newly molted *O. fasciatus* adults after fourth instar RNAi treatment. This allows examination of indirect genetic interactions among genes in this region of the body (summarized in Fig. 9). In the abdomen of females, *Dll* expression was significantly reduced with the knockdown of either *dac* or *hth* (Fig. 7D), implying these genes normally promote activation of *Dll* expression. In males *Dll* expression was independent of *dac* and *hth* (Fig. 5A). A similar sex-specific difference in regulation was found for

dac. Expression of *dac* was significantly reduced in *hth* RNAi females (Fig. 7E). In males, *hth* RNAi appeared to increase *dac* transcript levels, but the effect was not significant (Fig. 7B; p = 0.49). Regulation of *hth* in the posterior abdomen was independent of *dac* and *Dll*, in both sexes (Fig. 7C,F).

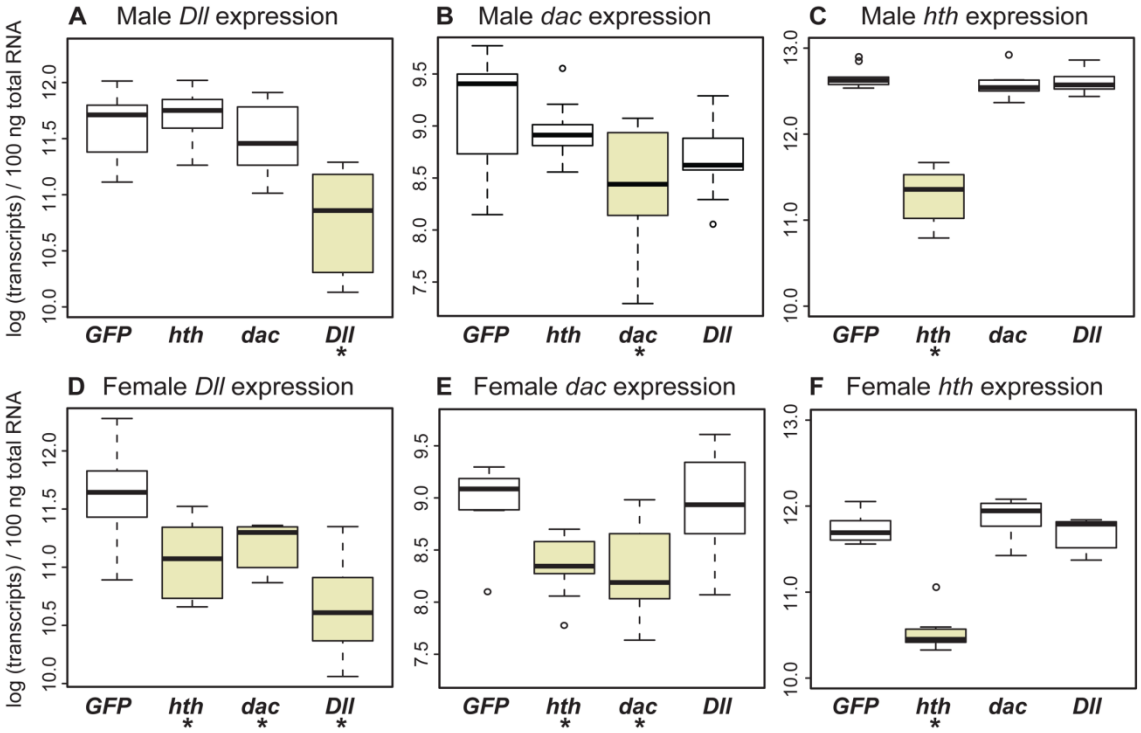

Figure 7 Interactions Among Appendage-patterning Genes in Abdominal Tissue During Adult Development of Each Sex. Transcript numbers of *Dll* (A,D), *dac* (B,E) and *hth* (C,F) are shown (log scale) from 1 ng of total RNA isolated from RNAi specimens. Genes targeted for RNAi were reduced in expression. Significant genetic interactions were also detected (D,E) and appear to be sex specific. Range and significance are indicated as in Figure 5H

Because of the important role of the Hox genes in patterning the posterior abdomen, we also examined regulation of the appendage-patterning genes in this region by the Hox genes (Fig. 8). *Dll* expression was significantly reduced in posterior

abdominal tissue in *abd-A* RNAi males (Fig. 8A; Tukey's HSD, $p = 5.1\times10^{-4}$). This suggests that *abd-A* normally activates *Dll* expression in *O. fasciatus* males. Reduction of *Dll* expression in *abd-A* RNAi females was suggested, but not significant (Tukey's HSD, $p = 0.38$). There results were surprising, since in *O. fasciatus* embryos *Dll* is repressed by *abd-A* in the abdomen (Angelini et al., 2005), and in *D. melanogaster* , *Dll* is repressed by *abd-A* in the female genital disc (Foronda et al., 2006). In pre-adult *O. fasciatus* abdominal tissue, *Dll* expression was significantly reduced by *Abd-B* knockdown in both sexes (Fig. 8A,C), suggesting positive regulation. *dac* expression was significantly elevated by *abd-A* RNAi in females (Fig. 8D), indicating negative regulation. *hth* expression was not regulated in a sex-specific manner and increased as a result of depletion of either *abd-A* or *Abd-B* (Fig. 8C,G), suggesting that the Hox genes repress *hth* expression.

intersex RNA Interference

intersex was selected for functional analysis in *O. fasciatus* as a candidate component of the somatic sex determination pathway. In *D. melanogaster* , *ix* is expressed in both male and female genital imaginal discs, but its activity is only required in females where it acts as a co-factor for Dsx^{F} (Garrett-Engele et al., 2002). Depletion of *ix* in juvenile *O. fasciatus* produced defects in both sexes, reducing the size of both male and female genitalia. In male *ix* specimens, clasper length was significantly reduced (Fig. 5J-K; Tukey's HSD, $p = 7.5\times10^{-4}$) and the size of the genital capsule was somewhat

reduced (p = 0.07). In females, both genital appendage pairs were significantly reduced (Fig. 6I-K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p = 4.0\times10^{-7}$ for valvula 2). The medial membranous tissue of the intervalvular space was missing, and the valvulae were more rigidly scleritized and heavily pigmented than in control females, resembling the morphology of the male claspers. In addition to this partial sex-reversal phenotype in the genitalia, *ix* knockdown females lack the sexually dimorphic A4 sternal process. These defects suggest that females have undergone a partial transformation towards male identity. Female-to-male sex reversal is also the main effect of *ix* mutations in *D. melanogaster* (Garrett-Engele et al., 2002).

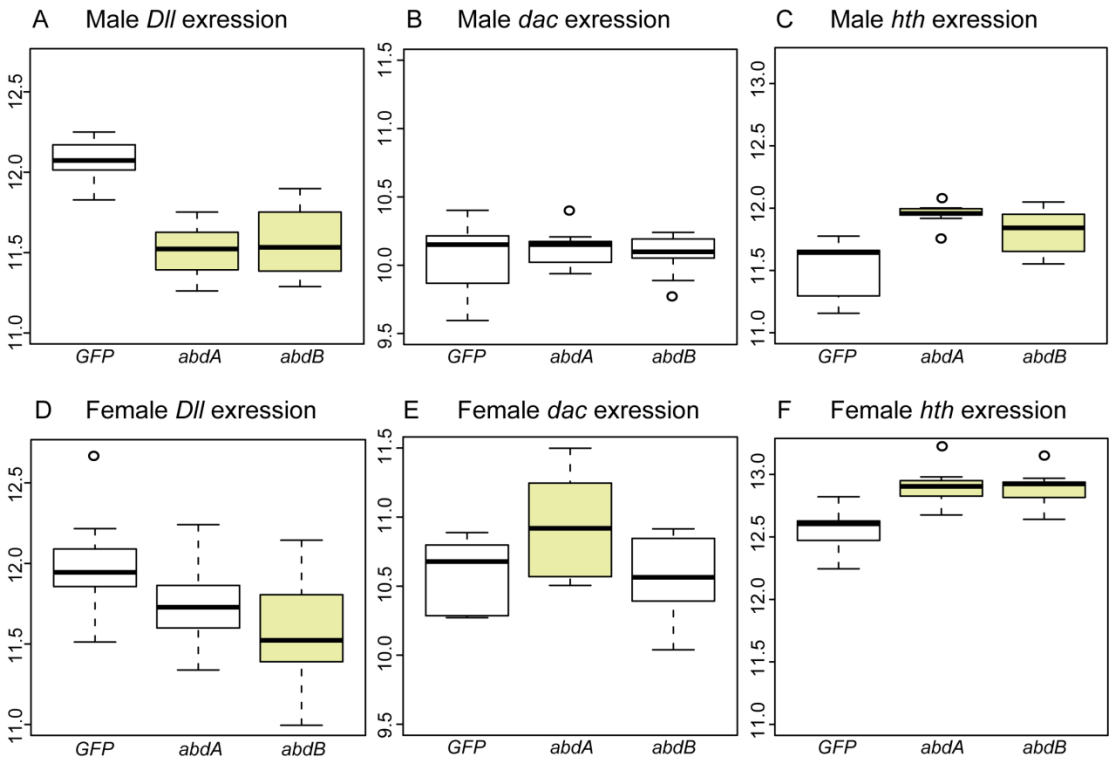

Figure 8 Regulation of Appendage-patterning Genes by Posterior Hox Genes. Transcript numbers of *Dll* (A,D), *dac* (B,E) and *hth* (C,F) are shown (log scale) from GFP, *abd-A* and *Abd-B* RNAi specimens. *Dll* is activated by both Hox genes in both sexes (A,D), although the interaction is not significant in females. *abd-A* displays negative regulation of *dac* in females (E). Both Hox genes were negative regulators of *hth* in both sexes (C,F). Range and significance are indicated as in Figure 5H.

Sex-dependence of Gene Interactions

To verify that the observed sex-specific gene regulation is a result of the sex of an individual, we used RNAi to manipulate the somatic sexual differentiation of milkweed bugs. As mentioned previously, *ix* RNAi partially transformed females towards male-like secondary sexual characteristics in the abdomen and genitalia (Fig. 6I). This supports the hypothesis that *O. fasciatus ix* is required for the development of female-specific phenotypes as a member of the somatic sex determination pathway, similar to the role of *ix* in *D. melanogaster* . Thus *ix*-depleted females should display male-like genetic interactions. Therefore, the regulation of *Dll* and *dac* by other appendage patterning genes was examined in an *ix*-depleted environment. In females with unmanipulated *ix* activity, but depleted for *dac* and *hth*, *Dll* transcripts were reduced, suggesting positive regulation (Fig. 9B). However these interactions were absent in male abdominal tissue (Fig. 9A). In an *ix* RNAi background, females displayed a male-like pattern of *Dll* regulation (Fig. 9C-D), with insignificant differences in expression under *dac* and *hth* RNAi. In females *dac* was also regulated by *hth*, with reduced *dac* expression under *hth* RNAi (Fig. 9F). This interaction was absent in males (Fig. 9E). With concomitant

knockdown of *ix* and *hth*, *dac* transcript levels were not significantly different from *ix* depletion alone (Fig. 9H), similar to the result from males (Fig. 9G). These results implicate the somatic sex determination pathway in the regulation of *Dll* and *dac* in the developing posterior adult abdomen and confirm that regulation of these genes is sex-specific in the posterior abdomen of developing adults.

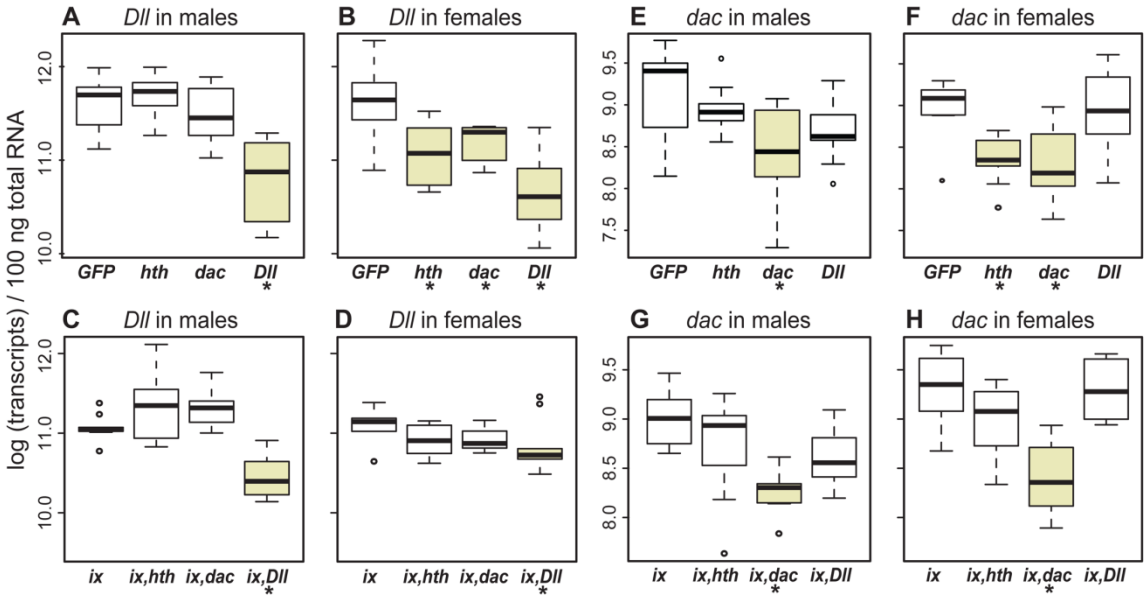

Figure 9 *intersex* is Required for Female-specific Gene Regulation. (A) Male *Dll* expression was independent of *hth* and *dac*. (B) Female *Dll* expression was reduced in *hth* and *dac* RNAi. (C) *ix* RNAi had no effect on *Dll* regulation in males, but (D) abrogated female-specific *Dll* regulation by *hth* and *dac*. (E) Expression of *dac* in males was unaffected by RNAi targeting *hth* and *Dll*. (F) In females, *dac* expression was reduced with *hth* knockdown. (G) In an *ix*-depleted background, male regulation of *dac* remains unchanged. (H) However, in females *dac* expression returned to control levels in *ix*, *hth* double RNAi. Therefore, reduction of *ix* activity altered regulation of *Dll* and *dac* from female to male patterns. Range and significance are indicated as in Figure 5H.

CHAPTER 5

DISCUSSION

Genitalia are Abdominal Appendages—at Least in Part

Developmental genetics may be examined as one biological level that informs considerations of homology (Wagner, 2007), and we use this data here to evaluate classical hypotheses for serial homology of genitalia and other appendages (color-coded in Fig. 1). Appendage specification and patterning has been characterized in many insect lineages, through studies of "leg patterning" genes and Hox genes. However, outside of *D. melanogaster* , no functional studies have yet examined the development of anatomically diverse genitalia. *Dll* is a marker of appendage identity and it is known to function in distal outgrowths from the body in many animals (Panganiban et al., 1997). *Dll* expression has been described in the embryonic appendages of diverse insects (Panganiban et al., 1994). The requirement for *Dll* in development of the *O. fasciatus* claspers (Fig. 5B,K) and ovipositors of *O. fasciatus* (Fig. 6C,J-K) is evidence in support of the longstanding hypothesis of homology between these genitalia and the other serially homologous appendages (i.e. antenna, mouthparts and legs). The involvement of the leg patterning genes in genital development, together with previous anatomical studies (Bonhag and Wick, 1953; Marks, 1951; Sokoloff, 1972; Snodgrass, 1935; Truxal, 1952; Tschinkel and Doyen, 1980), provides compelling evidence of the appendicular origin of

the genitalia. Additionally, *Dll* RNAi defects in both the first and second valvulae of *O. fasciatus* suggest that the subterminal ovipositor is composed of two pairs of appendage primordia. The failure of left and right valvulae to fuse under most RNAi treatments (Fig. 6B-E,H) also implies that the primordia grow independently and later fuse to produce the adult ovipositor.

The serial homology of the heteropteran genital capsule has been debated by anatomists (Bonhag and Wick, 1953; Marks, 1951; Snodgrass, 1935; Truxal, 1952). The gonocoxopodite of male *O. fasciatus* appears to be unaffected by depletion of *Dll, dac,* or *hth*. This tissue was not resistant to RNAi and developed defects with Hox knockdown. One interpretation is that the gonocoxopodite is derived from appendage primordia but has lost the requirement for activity of these genes. Because no known appendage-derivative develops without the requirement of at least one of these genes, we favor the interpretation that the male gonocoxopodite is not derived from appendicular tissue and is not homologous to the coxa of the leg, as its name implies. Instead our data are consistent with the hypothesis that the gonocoxopodites derive from the abdominal sternum (Marks, 1951; Snodgrass, 1935; Truxal, 1952).

Differences in Male and Female Genital Patterning in *O. fasciatus*

Development of the *O. fasciatus* ovipositor requires *Dll, dac* and *hth*. Similar reductions of the valvulae were caused by the depletion of each gene (Fig. 6J-K), although *hth* was also required for proximal valvifer development. In the valvulae it is likely that the similarity in phenotypes (the overlapping level of PD defect) is due in part

to the positive regulatory interactions among these genes in the female pre-adult abdomen (Fig. 10B).

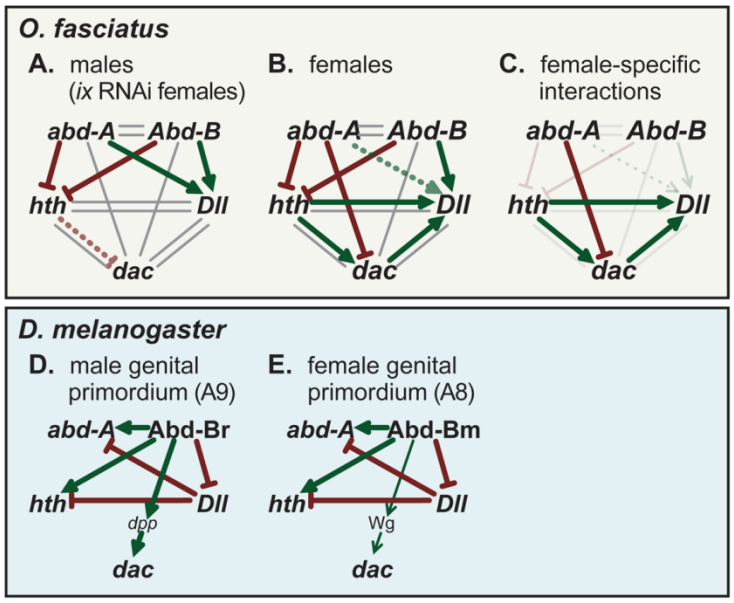

Figure 10 Summary of Regulatory Interactions Detected in the Pre-adult Posterior Abdomen of *O. fasciatus* Males (A) and Females (B). (C) Sex-specific interactions are highlighted. Interactions of these genes in the male (D) and female (E) genital imaginal disc of D. melanogaster. Green arrows indicate activation; red blunted lines indicate repression. Dotted lines represent relationships that are suggestive but not statistically significant ($0.05 < p < 0.1$). Light gray lines indicated tested relationships for which no interaction was detected ($p > 0.1$).

Interestingly, the functions of both the appendage patterning genes and Hox genes vary between the sexes. Knockdown of *dac* and *hth* had a much greater affect on female genitalia than on males. Valvulae were consistently reduced and failed to fuse medially under *dac* and *hth* RNAi (Fig. 6C-F,J-K). In contrast, the male claspers were not significantly reduced in *hth* treatments, and although the length was significantly reduced by *dac* RNAi (Fig. 5K), the overall morphology of the claspers remained unaffected (Fig.

5C). We also found evidence for regulation of *Dll* by *dac* and *hth* in females (Figs. 7A,C). Part of the sensitivity of females to knockdown of *dac* and *hth* may stem from activation of *Dll* in this sex by these other appendage patterning genes. This difference in *Dll* regulation implies that the somatic sex determination pathway also has regulatory input on *Dll*. Transcriptional profiling of *D. melanogaster* genital discs also identified downstream targets of *dsx,* which were expressed in a sex-specific manner (Chatterjee et al., 2011). *ix* knockdown confirmed that female-specific activity of the somatic sex determination cascade has a role in *Dll* regulation, given that sex-specific *Dll* regulation was abrogated with *ix* RNAi (Fig. 9B,D).

Another interesting case of sex-specific gene function was found among the posterior Hox genes. *abd-A* knock-down reduced male genitalia (Fig. 5H,K) while transforming the segmental identity of the anterior female valvulae (Fig. 6G). In contrast, *Abd-B* RNAi produced similar phenotypes in both sexes, severely reducing the genitalia (Figs. 2I; 3H). This difference implies that *Abd-B* acts in a sex-independent manner, or that its activity occurs before genital primordia are still sexually committed, no later than the fourth instar. The female-specific A4 sternal process is observable in fifth instars, implying that somatic sex specification occurs around the time of the penultimate molt. In contrast, the more sex-specific phenotypes of *abd-A* RNAi imply that this gene acts after or at the same time as sex determination in the genital primordia.

Comparisons Between the Development of the Genitalia and Other Appendages

The genitalia are unique appendages in several ways: they are sexually dimorphic, composed of multiple appendage pairs, and evolve rapidly. The canonical appendage patterning genes (*Dll*, *dac* and *hth*) were first described in leg development, where their expression and interactions are largely exclusionary (Abu-Shaar and Mann, 1998; Angelini and Kaufman, 2004). However, in other appendages, such as the antennae and mouthparts, extensive overlap and positive interactions have been reported (Casares and Mann, 2001; Dong et al., 2000; Dong et al., 2001; Morata, 2001; Ronco et al., 2008).

One interesting similarity in gene function among appendages comes from the function of *hth*. In *O. fasciatus* males, *hth* RNAi caused the development of an everted and malformed copulatory organ (Fig. 5D,G). The requirement of *hth* may suggest that the proximal appendicular primordia do contribute to this normally internalized structure. The heteropteran mandibular and maxillary appendages also develop as internalized structures, the retortiform organs, before everting at hatching (and at each molt) to produce functional feeding stylets (Newcomer, 1948). These structures also require *hth* activity, and its depletion by RNAi causes an everted and malformed phenotype (Angelini and Kaufman, 2004) similar to that seen for the copulatory organ. Both the stylets and the copulatory organ have also been proposed as appendage derivatives (Minelli, 2002; Snodgrass, 1921), and *hth* is necessary to direct internalization during the development of both structures.

Comparison of Genital Development and Patterning Among Insects

While hemimetabolous and holometabolous insects differ greatly in the ontogeny of adult appendages, genital development is similar in both groups in that primordia are internalized and do not complete differentiation until the imaginal molt. The genital imaginal disc of *D. melanogaster* is comprised of cells originating from four body segments, A8-A11 (reviewed by Sánchez and Guerrero, 2001). Females have vaginal plates flanking the vulva, while the male genitalia consist of an aedeagus and lateral claspers. Genetic analyses have shown that development of some of these structures requires appendage-patterning genes. *Dll* is expressed in the developing vaginal plate, male claspers, and anal plate; however, Gorfinkiel et al. (1999) found that that *Dll* is not required in all these structures. *Dll* mutants have mild defects, with reduced anal plates in both sexes and disorganized vaginal plates in females. The genitalia of *D. melanogaster* have a larger role for *dac*. Mutations in *dac* eliminate large portions of the male clasper and cause a fusion of the spermathecal ducts in females. Both male and female genital primordia in flies express *dac* in sex-specific patterns. In females the *dac* expression pattern is regulated by the female *Dsx* isoform in conjunction with activation through Wingless (Wg) singaling and repression from Dpp, while in males in the male-specific *Dsx* isoform causes repression from Wg signaling, altough Dpp activates *dac* without influence from Dsx^{M} (Keisman and Baker, 2001; Sanchez et al., 2001). Cell clones lacking *hth* activity in the developing genitalia caused defects in the vaginal plates and occasionally the male claspers (Estrada and Sanchez-Herrero, 2001). *Abd-B* is required to specify identity in the genital disc, and *Abd-B* loss-of-function results in up-regulated *Dll*

and *dac*, causing transformation to leg or rarely to antenna (Estrada and Sanchez-Herrero, 2001). In *D. melanogaster* , *abd-A* inhibits *Dll* and is inhibited by *Abd-B* during embryogenesis. However, this interaction shifts in the larval genital disc, where *Abd-B* activates *abd-A*, which is in turn inhibited by *Dll* (Foronda et al., 2006). The development of male genitalia depends on repression of the A8 female genital primordium and proliferation of the A9 male genital primordium, which is mediated by the somatic sex determination pathway (Keisman and Baker, 2001).

In contrast to *D. melanogaster* genital development, *Abd-B* knockdown during *O. fasciatus* genital development did not produce genitalia-to-leg transformations. One possible explanation for this difference may be the inability of RNAi to provide a null phenotype. However it is also possible that while the genital primordia in fifth instar bugs require *Abd-B* for growth, they may no longer be able to adopt another appendage's developmental program. Regarding gene interactions in the developing genitalia, *O. fasciatus* and *D. melanogaster* differ substantially (Fig. 8). To highlight the obvious evolutionary divergence, it is worth noting that *abd-A* and *ix* have novel roles in male genital development not found in *D. melanogaster* . Knockdown of *abd-A* in *O. fasciatus* had a strong effect on male genital development (Fig. 5H), implying that male genitalia derive from a body segment expressing this Hox gene. In the embryo *abd-A* expression includes A2-A9, although embryonic RNAi phenotypes have not been reported for A9 (Angelini et al., 2005). Differences were not limited to external structures. *Drosophila* females have two spermathecae connected by ducts to the uterus, and these ducts are fused in *dac* mutants (Keisman and Baker, 2001). A single medial spermatheca is present

in *O. fasciatus* (Bonhag & Wick, 1953), and this structure was not affected in *dac* RNAi (not shown). These differences and others in gene function and interactions likely reflect the dramatic anatomical differences and deep evolutionary divergence between these insects. Similarly, in regard to serial homology, appendage patterning genes play more prominent roles in genital structures that are more anatomically similar to other appendages, such as legs.

In species, such as *T. castaneum*, where male and female genital development is genetically very different, the genitalia of each sex may be regarded as separate developmental modules (Aspiras et.al., *in press*) As such they should be capable of evolving rapidly due to reduced pleiotropic constraints (Schlichting and Pigliucci, 1998; Snell-Rood et al., 2009). In contrast, *O. fasciatus* male and female genitalia, while anatomically distinct, share a requirement for many genes. Thus with greater integration, these genital modules are expected to evolve more slowly. Supporting this conjecture is the fact that lygaeid bugs (e.g. *Oncopeltus*) have relatively conserved genital morphology (Scudder, 1959), while tenebrionid beetles (e.g. *Tribolium*) have diverse male and female forms (Hinton, 1948; Tschinkel and Doyen, 1980).

CHAPTER 6

CONCLUSIONS

Genitalia are crucial for copulation and oviposition in insects. Their rapid phenotypic change and potential influence on reproductive isolation makes the underlying development and patterning of genitalia important for understanding insect evolution. We have described the patterning of genitalia in an insect from a species-rich order. Our results indicate that genital structures vary in the extent to which they derive from appendage primordia. For example, while all of the subterminal ovipositor of *O. fasciatus* was affected by knockdown of candidate appendage patterning genes, Aspiras et.al. (*in press)* found only the distal styli of the terminal *T. castaneum* ovipositor displayed a requirement for appendage-patterning genes, even with a much wider sampling of candidate genes. In the genitalia of *O. fasciatus*, we identified several sex-specific interactions among appendage-patterning genes (Fig. 10C). This regulation is mediated by the activity of *ix*, a gene involved in somatic sex determination with depletion phenotypes in both sexes of *O. fasciatus*. Therefore, variation in the regulation and function of conserved developmental genes can play an integral role in appendage development and in the diversification of insects. A theme is emerging from the examination of the developmental genetics of diverse arthropod appendages: divergence in the degree of conservation in anatomy is correlated with divergence of an otherwise highly conserved genetic patterning system.

CHAPTER 7

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