FUNCTION AND INTERACTION OF SEX DETERMINATION GENES IN THE HEMIMETABOLOUS INSECT *ONCOPELTUS FASCIATUS*

By

Mara Laslo

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Chair:

David Carlini, Ph.D.

David R. Angelini, Ph.D.

Colin Saldanha, Ph.D.

Dean of the College of Arts and Sciences

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Date

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American University

Washington, D.C. 20016

For my Dad.

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ABSTRACT

The regulation and evolution of sex is a fundamental question in developmental biology. Sex determination mechanisms have only been studied in holometabolous insects, or insects with complete metamorphosis. In addition to being closely related to several human and agricultural pests, *O. fasciatus* is a member of the most closely related clade to these extensively studied holometabolous insects. Determining the genetic basis of sex in an outgroup such as *O. fasciatus* will shed light on how sex evolves. Candidate genes were chosen from the sex determination pathway of *D. melanogaster.* Transcript levels of *transformer-2A*, *transformer-2B, intersex,* and *fruitless* were examined at each developmental stage using quantitative PCR. Functional analyses of candidate genes *virilizer, female-2-d, transformer-2, male-specific lethal-2, male-specific lethal-3, intersex,* and *fruitless* were done using RNA interference methods. Results indicate that these sex determination genes play different roles in *O. fasciatus* compared to the established *D. melanogaster* model—most genes do not appear to have a role in sex determination. Additionally, although the genes *fruitless* and *intersex* are conserved, the phenotypes produced by the knockdown are non sex-specific. Interaction data suggests that *fruitless* promotes *intersex* expression—a novel interaction at the most downstream point of the sex determination pathway. These data support the theory that although upstream sex-determining genes vary widely across insect groups, downstream genes remain conserved.

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CHAPTER 1

INTRODUCTION

Overview of Insect Sex Determination

The regulation and development of sex are central issues in the study of developmental biology. The biological importance of sex suggests that sex determination mechanisms might be conserved, to an extent, in sexually reproducing species; however, developmental and genetic studies have revealed incredible diversity in the ways animals determine sex (Bull, 1980; Hodgkin, 2002). Many environmental and genetic factors play different roles in different species, and two main classes of sex determination mechanisms have emerged from studies of gonochoristic species, or species with two sexes, in the past few decades—genetic and environmental sex determination.

Insects are the most numerous, species-rich and anatomically diverse group of animals on the planet, and most use a genetic system of sex determination. Although insects are widely studied, their systems of sex determination have only been examined in a single clade of insects, the Holometabola, those with complete metamorphosis (Gempe & Beye, 2011). Sex determination *in Drosophila melanogaster* occurs autonomously in most, if not all, somatic cells of the individual (Robinett, Vaughan, Knapp, & Baker, 2010; Schutt & Nothiger, 2000). This is hypothesized to be true of all insects; each individual is a mosaic of cells that have an intrinsic sexual identity. This contrasts with mammalian cells where sexually dimorphic characteristics (in tissue other than the gonad) are due to hormonal effects.

This somatic sex determination requires an initial genetic signal, which varies greatly from species to species. Proteins downstream of this signal then regulate transcription sex-specifically. Among hymenopterans (wasps, bees, and ants) the initial sex determination signal is the allelic composition at a single locus. Heterozygotes at the *complementary sex determiner*

(*csd*) locus develop as females while hemi- or homozygotes develop as males (Beye, Hasselmann, Fondrk, Page, & Omholt, 2003). Thus, in the haplodiploid system, all haploid individuals are male and diploid heterozygotes are female (Whiting, 1943). Most lepidopterans such as moths and butterflies have female-determining factors, or a single gene, on the W chromosome, such that heterozygotic insects (ZW) develop as female while homozygous insects (ZZ) lack this gene and develop as male (Fujii & Shimada, 2007). Among different dipteran groups, sex may be chromosomally, environmentally, or maternally determined (Gempe & Beye, 2011). The best-studied model of insect sex determination is the fruit fly *Drosophila melanogaster*. Flies of this species have a system of chromosomal balance where female and male determinants reside on the X chromosome and autosomes, respectively (Christiansen, Keisman, Ahmad, & Baker, 2002). The proportion of X chromosomes to autosomes determines sex. Sex specific expression and splicing of downstream genes control male and female sexual differentiation, dosage compensation, and behavior (Serna et al., 2004).

The Milkweed Bug as a Model Organism

True bugs (Heteroptera), including the milkweed bug, *Oncopeltus fasciatus*, are a diverse and species-rich order that diverged from holometabolous insects, such as Hymenoptera, Lepidoptera and Diptera, more than 300 million years ago (Grimaldi & Engel, 2005). Only holometabolous insect sex determination mechanisms have been studied so far, and despite their importance as disease vectors and agricultural pests (Daly, Doyen, & Purcell, 1998), little is known about the process in Heteroptera. Morphological and molecular phylogenies strongly support the Heteroptera (suborder of Hemiptera) as the sister-clade to all Holometabola (Beutel et al., 2011; Regier et al., 2010). While sex determination mechanisms in invertebrates are known to evolve rapidly (Hill et al., 2006), many developmental genes are highly conserved in

their sequence and function, partly due to their reuse throughout development (pleiotropy) (Stern & Orgogozo, 2009). A study of somatic sex determination in a hemimetabolous out-group, such as *O. fasciatus,* will expand our understanding of the insect developmental diversity and may help illustrate the ancestral function of sex determination genes in Holometabola.

Heteropterans, such as *O. fasciatus*, develop in five juvenile stages (instars or nymphs). Sexually dimorphic characters do not appear until the fifth instar, when females develop a small process on their fourth abdominal (A4) sternite (P. Liu & Kaufman, 2009). *Oncopeltus* has heteromorphic sex chromosomes (LaChance & Richard, 1973), but the mechanisms behind the initial genetic signal and the differentiation of somatic cells remain unclear. A recently published transcriptome of *O. fasciatus* obtained from embryos and ovaries (Ewen-Campen et al., 2011) contains orthologs of many genes involved in the well-studied sex determination pathway of *D. melanogaster,* which can serve as candidate genes for the study of sexual differentiation in *O. fasciatus*. The milkweed bug is a convenient system in which to study sex determination since it has clear sexual dimorphisms, a relatively short life span (2 months), and molecular methods such as RNA interference are highly effective (P. Liu & Kaufman, 2009). This study aims to provide a new evolutionary perspective on insect sex determination mechanisms through examination of the divergence of this process in one member of the Heteroptera, an important and previously unexplored lineage.

Candidate Genes for Milkweed Bug Sex Determination

The published transcriptome of *O. fasciatus* contains several genes involved in the sex differentiation pathway of *D. melanogaster* (Ewen-Campen et al., 2011). Although developmental genes are often highly conserved, even closely related species use different primary signals and gene regulatory networks to determine sex (Sanchez, 2008). Previous studies

comparing *O. fasciatus* to *D. melanogaster* have shown that conserved developmental genes can vary in function and regulation (Angelini & Kaufman, 2005; Aspiras, Smith, & Angelini, 2011). Several genes have emerged from studies of flies, mosquitoes, and wasps that repeatedly play similar roles in sex determination across holometabolous taxa (Figure 1). *transformer* (*tra*) is an early regulator of sex in all these insects. In males, a functional Tra protein is not produced and two important downstream targets (*doublesex* and *fruitless*) are spliced in a default manner. *doublesex* (*dsx*) and *fruitless* (*fru*) directly cause sexually dimorphic development and behavior, respectively. In females, *tra* regulates expression of itself, and it also directs splicing of these two downstream targets. The best-characterized model of insect sex determination is that of *Drosophila melanogaster* (Figure 2). A conservative null model would predict genes involved in sex determination in *Drosophila* would have some role in *O. fasciatus* sex determination and differentiation.

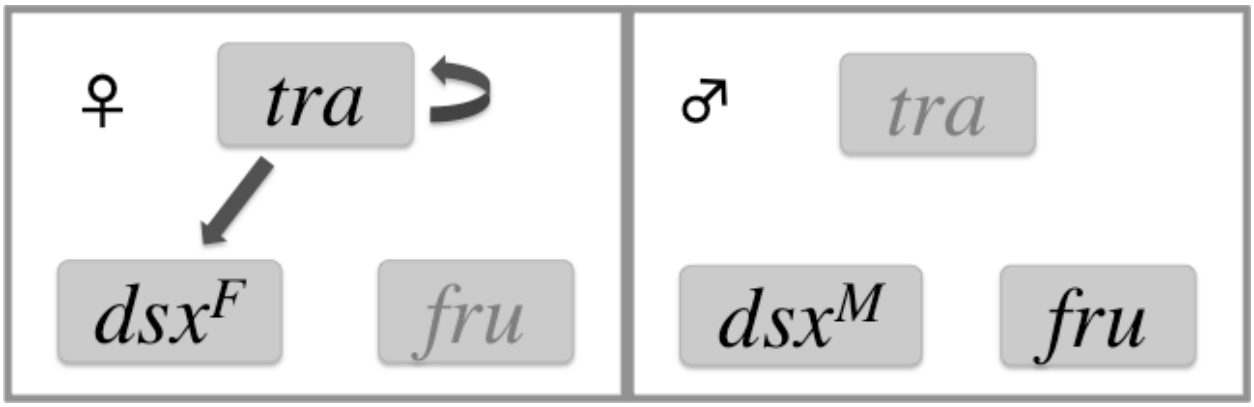

Figure 1. Conserved Aspects of Holometabolous Insect Sex Determination. Gray font indicates a sex specific protein is not produced. In females (left) *transformer* regulates expression of itself as well as splicing of *doublesex* to a female specific isoform. In males (right), functional *transformer* is not produced and *doublesex* and *fruitless* are spliced in a default and male-specific manner.

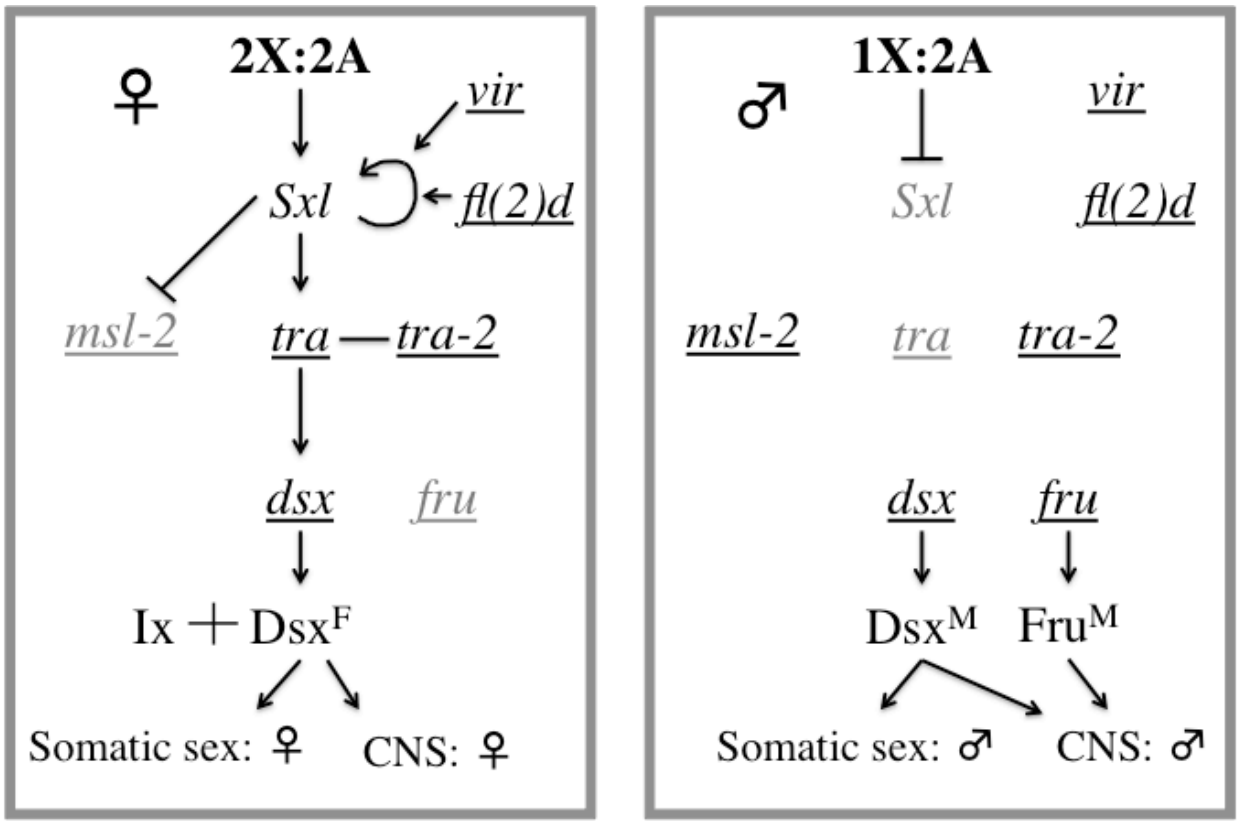

Figure 2. The Sex Determination Pathway in *Drosophila melanogaster*.

virilizer (*vir*) and *female-specific-2-d* (*fl(2)d*)

vir and *fl(2)d* encode nuclear proteins necessary for the auto-regulatory splicing of *Sex-lethal* (*Sxl*) (Niessen, Schneiter, & Nothiger, 2001), which plays a key role in establishing sex-specific genetic pathways in *Drosophila*. Both *vir* and *fl(2)d* transcripts are expressed throughout *D. melanogaster* development. Niessen and coworkers (2001) showed that some *vir* mutations (vir^{22}, vir^{23}) were lethal in the third instar in both sexes, while another allele (vir^{1ts}) is involved in sex regulation. The non-sex specific lethality of vir^{22} and vir^{23} imply that *vir* is involved in a developmental process critical for viability. Based on its sequence, Niessen et al. (2001) predict that *vir* is a nuclear envelope protein involved in mRNA transport. *vir* and *fl(2)d* proteins are also required for female-specific splicing of *transformer* in *Drosophila*; repression of dosage compensation in females also requires *vir* (Hilfiker, Amrein, Dubendorfer, Schneiter, &

Nothiger, 1995). These proteins are likely general splicing factors, since the primary transcript of the homeotic gene *Ultrabithorax* is another known target of *vir* and *fl(2)d*; other essential genes likely require the *vir* and *fl(2)d* products for correct splicing (Burnette, Hatton, & Lopez, 1999).

Homologs of *vir* have been found in the hemipteran *Acyrthosiphon pisum* (pea aphid), the dipteran *Culex quinquefascuiatus* (mosquito), and several species of bees and wasps (Benson, Karsch-Mizrachi, Lipman, Ostell, & Sayers, 2011), although functional studies have not been done in these species. *fl(2)d* is thought to be a key splicing regulator, and is the homolog of a human protein interacting with Wilms' tumor suppressor1 (Ortega et al., 2003). Despite the importance of *Sxl* as a target for sex determination in *Drosophila*, this gene appears to have evolved this function within the dipteran suborder Brachycera (horse flies, bee flies, and robber flies), since homologs in other flies are not involved in sex determination (Serna et al., 2004). It remains to be seen whether *vir* has a role in insect sex determination outside of *Sxl* splicing.

male-specific-lethal (*msl*) Genes

Because *Drosophila* males only have a single X chromosome, expression of X linked loci on this X chromosome must be doubled to compensate for the two X chromosomes in females. The male-specific-lethal (MSL) complex is responsible for hypertranscription of this single X chromosome in males. Three *male-specific-lethal* protein coding genes in *D. melanogaster* form the dosage compensation complex which also includes the proteins MOF (encoded by *males-absent-on-the-first*), and MLE (encoded by *maleless*) (Penalva & Sanchez, 2003). The *msl* genes are transcribed in both sexes, however, only *msl-2* is necessary and sufficient to assemble this complex (Kelley et al., 1995). In female *Drosophila*, *Sxl* represses translation of *msl-2* (Bashaw & Baker, 1996). In the silk moth *Bombyx mori*, where the mechanism of dosage compensation is disputed, *msl-1, msl-2,* and *msl-3* have been identified and exhibit sexually dimorphic expression

patterns (W. Liu, Zhang, Miao, & Huang, 2008). Homologs of *msl-2* have been found in *Danaus plexippus* and *Tribolium casteneum* (Benson et al., 2011), however no functional studies have been done in these species.

transformer (*tra*) and *transformer-2* (*tra-2*)

Whatever the initial sex-determining signal, the downstream cascade involving the splicing proteins *tra* and *tra-2* remain the same in most holometabolous insects examined. *tra* is alternatively spliced only in females to produce a functional Tra protein. In males, Tra is either not produced or has an unknown function unrelated to *dsx* splicing. The Tra protein also establishes an auto-regulatory loop in females, insuring its continued female-specific splicing.

In *Drosophila*, Sxl regulates splicing of the *tra* mRNA at a sex-specific 3' splice site in the first intron, such that functional Tra is only expressed in females (Inoue, Hoshijima, Sakamoto, & Shimura, 1990). In males, *tra* is spliced from an upstream splice site and produces a short (110 nucleotides) non-functional mRNA (Boggs, Gregor, Idriss, Belote, & McKeown, 1987), which is probably rapidly degraded. In female somatic tissue, Tra forms a heterodimer with the product of *transformer2* (*tra-2*), which is constitutively expressed in both sexes. This complex controls the sex-specific splicing of *dsx* and *fru* mRNAs (Mullon, Pomiankowski, & Reuter, 2012). *tra-2* transcripts contain a ribonucleoprotein domain and an arginine-serine rich region, suggesting that *tra-2* is involved in RNA splicing (Mattox, 2012). Its function has been studied extensively in dipterans, where it is necessary for female-specific splicing of *dsx* (Martin, Ruiz, & Sanchez, 2011) and spermatogenesis (Mattox, Palmer et al 1990; Belote and Baker 1983). The RNA-binding domain is conserved in *B. mori tra-2* (Niu et al., 2005). In this lepidopteran, Tra ortholog has not yet been identified. *B. mori dsx* lacks a binding domain for Tra-2 (Suzuki, Ohbayashi, Mita, & Shimada, 2001), indicating an alternative function for Tra-2.

B. mori represents a special case, however, in that default mode of *dsx* splicing is thought to be female; the as yet unknown feminizing factor in *B. mori* is probably a *tra* ortholog.

Orthologs of the *tra* genes have also been identified in Hymenoptera, Coleoptera, and Diptera. Despite large sequence divergence, sex-specific splicing produces a conserved, premature termination codon in males of all these insect groups (Verhulst, van de Zande, & Beukeboom, 2010). Additionally, the sex-specific regulation of *dsx* by *tra* is conserved in these holometabolous orders, suggesting *tra* has an ancestral role as the regulator of somatic sex.

In the haplodiploid system of the honeybee *Apis mellifera*, the *tra* ortholog is known as *feminizer* (*fem*). Having two copies of *csd* directs female-specific splicing of *fem* and results in translation of female-specific DSX^F (Hasselmann et al., 2008). *csd* is hypothesized to have arisen via a recent duplication of the *fem* gene (Schmieder, Colinet, & Poirie, 2012). In the wasp *Nasonia vitripennis*, maternal deposition of *tra* mRNA and the auto-regulatory splicing of *tra* ensures female development, while maternal imprinting prevents zygotic transcription of *tra* mRNA to cause male development (Verhulst, Beukeboom, & van de Zande, 2010).

doublesex (*dsx*) and *intersex* (*ix*)

dsx is the final regulatory gene in the sex determination cascade of *D. melanogaster*, and the Dsx protein acts to directly control differentiation of somatic cells. The male- and female-specific *dsx* mRNAs encode transcription factor isoforms. In both sexes, *dsx* promotes male or female differentiation by repressing and activating target genes. The female isoform Dsx^F, together with the products of *intersex* (*ix*) and *hermaphrodite* (*her*) directly regulate of transcription of terminal sex differentiation proteins (Burtis, Coschigano, Baker, & Wensink, 1991) and represses male development in the dimorphic tissue of females. *ix* is expressed in both

males and females, but does not interact with the male isoform Dsx^M. Dsx^M represses female development in male dimorphic tissue (Kopp, Duncan, Godt, & Carroll, 2000).

dsx also has a role in neuronal differentiation. Rideout et al. (2010) found that Dsx^M is co-expressed with *fru* in brain regions that send projections directly to genitalia. The disruption of *dsx* in these neurons causes abnormal male and female mating behavior, suggesting that *dsx* is required, along with *fru*, for neuronal differentiation underlying mating behavior in both sexes.

dsx orthologs have been characterized in birds, mammals, reptiles, and worms (Hodgkin, 2002; Raymond, Murphy, O'Sullivan, Bardwell, & Zarkower, 2000), as well as in multiple hymenopteran and dipteran species (Cho, Huang, & Zhang, 2007). Overall amino acid identity between *D. melanogaster dsx* and the orthologous *mab-3* in *C. elegans* share only 18% amino acid identity; however, the cysteine rich DNA binding motif is perfectly conserved across vertebrates and invertebrates (Zhu et al., 2000). Outside of this domain, sequences diverge with evolutionary time. Sex-specific alternative splicing and functions for *dsx* have been shown to be conserved in dipterans, *N. vitripennis* (Oliveira et al., 2009), *A. mellifera* (Cho et al., 2007), and *B. mori* (Saccone, Pane, & Polito, 2002). Unlike *tra*, where vertebrate homologs play no role in sex determination, *dsx* has a role in sex determination or development across a wide range of vertebrate species, suggesting an ancient role for *dsx* in animal sex determination. In all species examined thus far, *dsx* has some role in promoting male development (Kopp, 2012).

fruitless (*fru*)

The default splicing of *fru* produces a male-specific protein, while in females splicing of *fru* mRNA by the Tra/Tra-2 heterodimer results in a non-functional protein (Verhulst, van de Zande, et al., 2010). The male-specific form of Fru controls differentiation of the nervous system, and Fru is a conserved regulator of male courtship behavior (Clynen, Ciudad, Belles, &

Piulachs, 2011). In terms of its structure and function, Fru seems to be conserved in most insects examined. Evidence for conserved sex-specific splicing has been found in *Anopheles gambiae*, *N. vitripennis*, and *A. mellifera* (Bertossa, van de Zande, & Beukeboom, 2009; Gailey et al., 2006).

The *fru* gene has a complex molecular structure with four promoters and encodes several zinc-finger protein isoforms that likely act as transcription factors. It is not surprising that *fru* also has an essential (sexually monomorphic) role in adult neuronal development (Anand et al., 2001). Song et al. (2002) have also shown that *fru* functions in embryonic, and probably adult, axonal network formation in *Drosophila* in a manner unrelated to sex. While functional roles of *fru* have not been addressed outside insects, the conservation of this downstream gene coincides with the theory put forward by Wilkins (1995) that sex determination hierarchies evolve from the bottom up, by recruiting new upstream control elements.

Nothing is known about the function of these conserved sex determination genes in *O. fasciatus*. An examination of a representative of a sister taxon of the Holometabola, such as *O. fasciatus*, should enable meaningful evolutionary comparisons to be made between the two, and infer the ancestral state of sex determination mechanisms in insects.

CHAPTER 2

MATERIALS AND METHODS

Insect Culture

Wildtype cultures *O. fasciatus* were obtained from Carolina Biological Supply Company and maintained according to Hughes and Kaufman (2000). Milkweed bug cultures were kept in small aquaria at room temperature. In the wild, *O. fasciatus* feed on milkweed seeds. However, in the lab, they are fed on sunflower seeds. Cotton provides an egg-laying substrate similar to the milkweed pod silk that would be used in the wild. Water is provided in small flasks with wicks made from paper towels.

Selection and Cloning of Candidate Genes

Candidate genes were identified from the literature described above, and sequences were found within the published *O. fasciatus* transcriptome (Ewen-Campen et al., 2011). Exact primers were designed using Primer3 (Rozen & Skaletsky, 2000) in MacVector. After amplification by PCR, DNA fragments were cloned using the Topo4-TA vector (Life Technologies) with One Shot TOP10 chemically competent cells. Plasmids were then isolated using the Purelink Quick Plasmid Miniprep kit (Life Technologies) and sequences were confirmed by Sanger sequencing (Beckman Coulter Genomics, Danvers, MA). A full list of candidate genes, their protein class, and the reciprocal BLAST hit to *Drosophila melanogaster* is listed in Table 1. Transcriptome study data can be found under Genbank study accession number SRP002610. *intersex* was cloned in a prior study by Aspiras et al. (2011).

Table 1. Candidate Genes used in this Study were Identified from the Literature (Ewen-Campen et al., 2004) and Partial Clones were Obtained from the Sequenced *O. fasciatus* Transcriptome.

Gene	Symbol	Cloned?	Protein Class	Genbank Accession	Reciprocal BLASTx to *Drosophila*
virilizer1	*vir1*	X	nuclear transmembrane	-	*virilizer* NP_524900.1
virilizer2	*vir2*	X	nuclear transmembrane	-	*virilizer* NP_524900.1
virilizer3	*vir3*	X	nuclear transmembrane	-	*virilizer* NP_524900.1
female-lethal-2-d	*fl(2)d*	X	nuclear splicing factor	-	*female lethal d* NP_523732.2
male-specific lethal-2	*msl-2*	X	dosage compensation complex	-	*male-specific lethal-3* AFI26241.1
male-specific lethal-3	*msl-3*	X	dosage compensation complex	-	*male-specific lethal-2* ABU96718.1
transformer-2A	*tra-2A*	X	RNA binding protein	-	*transformer-2*, isoform C NP_476765.1
transformer-2B	*tra-2B*	X	RNA binding protein	-	*transformer-2*, isoform E NP_476766.1
intersex	*ix*	X	mediator subunit	JN368475	*intersex* NP_610677.1
fruitless	*fru*	X	BTB-Zn finger TF	-	*fruitless*, isoform A NP_732349.1
doublesex	*dsx*	-	DNA binding protein	-	-
transformer	*tra*	-	RNA binding protein	-	-

In order to better characterize each paralog, similarity of the paralogs to each other (*vir* and *tra-2*) at the nucleotide level was determined with EMBOSS Water local alignment tool and ClustalW (EBI 2013) (Table 2).

Table 2. Percent Identity of Paralogs to Each Other Determined with EMBOSS Water Local Alignment Tool and ClustalW.

Comparison	% Identity w/ Water (local)	% Identity w/ ClustalW
vir1 vs *vir2*	38.2%	66.0%
vir1 vs *vir3*	42.5%	51.0%
vir2 vs *vir3*	40.6%	65.0%
tra-2A vs *tra-2B*	60.8%	63.0%

To provide further evidence that the gene identified in the transcriptome search was comparable to the actual target gene, sequenced clones were compared to the transcriptome sequence at both the nucleotide level using ClustalW and the amino acid level using the tblastx search function of NCBI (Table 3). Clone sequences were between 97% and 99% identical to transcriptome sequences at the nucleotide level, with the exception of *msl-2* at 78%, *vir1* at 63%, and *fl(2)d* at 64% identical to their transcriptome template (Table 3). At the amino acid level, only *msl-2* and *vir1* had poor identity with their transcriptome sequences at 40% and 14%, respectively (Table 3).

Table 3. Nucleotide (ClustalW) and Amino Acid (tblastx) Comparisons of the Target Gene Partial Clones Compared to the Respective Transcriptome Sequence.

	nt Identity	*aa Identity*		
Gene	**% Identity**	**tblastx Total Score**	**Query Coverage**	**E-value**
vir1	63.0%	116	14%	1.4E+00
vir2	99.0%	2414	99%	7.0E-122
vir3	99.0%	4336	100%	0.0E+00
fl(2)d	64.0%	3288	96%	8.0E-152
msl-2	78.0%	196	40%	9.1E-01
msl-3	99.0%	2037	100%	3.0E-103
tra-2A	97.0%	2561	100%	3.0E-141
tra-2B	99.0%	5306	99%	0.0E+00
ix	99.0%	2234	100%	6.0E-118
fru	99.0%	5136	99%	0.0E+00

Preparation of Double-stranded RNA and RNA Interference

Gene function was tested during adult development in *O. fasciatus* using RNA interference (Fire et al., 1998; Hughes & Kaufman, 2000). Knockdown of gene activity was confirmed using quantitative RT-PCR. A DNA template was amplified from cloned fragments using primers with a 10-nucleotide T7 RNA promoter sequence at the 5' end. This linear DNA was used as a template in bidirectional RNA synthesis using the Megascript T7 transcription kit (Life Technologies #AM1334). The product was treated with DNase I to remove template DNA, then annealed by cooling, and purified by precipitation in cold ammonium acetate and ethanol. After resuspension in nuclease-free water, dsRNA concentrations were determined through duplicate measurements on a nanoscale spectrophotometer (GE Life Sciences NanoVue) and diluted to 4 μg/μl. Injection of *O. fasciatus* was done in fourth instar nymphs before the appearance of sexually dimorphic characters. Adult virgin females were also injected for genes that displayed a phenotype in the fourth instar or were hypothesized to act early in development.

Bugs were anesthetized using CO_2 or with a 4-minute exposure to diethyl ether vapor. Using a front-loaded pulled-glass capillary needle, approximately 1μl of 4μg/μl dsRNA was injected at the base of the right metathoracic coxa. This location facilitated easy delivery into the hemocoel and no defects were observed at the site after ecdysis. Phenotypes were observed under a dissection stereomicroscope, and bugs not used for gene expression analysis were preserved in 70-80% ethanol.

Measurement of Gene Expression

Validations of the target gene knockdown and expression timecourses were determined using quantitative real-time RT-PCR (qPCR). For validation of RNAi, expression was compared between a gene-specific and non-specific *green fluorescent protein* (*GFP*) control dsRNA

treatments. Total RNA was isolated from adult *O. fasciatus* abdomens (A4-A12) collected from each RNAi treatment according to the Purelink RNA Mini Kit protocol (Life Technologies). Isolated RNA was stored at −80 °C. For all *O. fasciatus* treatments, at least 3 biological replicates were included for each sex. RNA concentrations from each biological replicate were determined by duplicate measures on a nanoscale spectrophotometer and diluted to 50 ng/µl (knockdown validation) or 30 ng/µl (timecourse) immediately prior to assays. Total RNA was used as template in reverse transcription / SYBR Green real-time PCR reactions (Superscript III). For each gene, exact primers were designed using the Primer3 algorithm (Rozen & Skaletsky, 2000), avoiding conserved functional domains and dsRNA regions. Dissociation curves for each reaction were used to verify that only single products were amplified. Due to its unique sequence, each target gene has a unique dissociation, or denaturing, temperature. When 50% of the target DNA is denatured, a marked reduction in fluorescence is detected by the qPCR machine and indicates amplification of a single, pure product. 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. Before qPCR assays, the RNA concentration was determined in triplicate (as described earlier) 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 10^4, 10^5, 10^7, and 10^9 RNA molecules / µl to serve as a standard curve (Pfaffl, 2004). Welch's t-test was used to determine the significance of gene expression knockdown in comparison to non-gene-specific control treatments using dsRNA with the exogenous sequence of *GFP*.

Characterization of RNAi Effects

Table 4 summarizes the phenotypic penetrance and rates of gene knockdown for RNAi treatments. Percentage knockdown was determined in a representative sample (3 biological replicates) of the total N. Significance and standard errors listed correspond to this smaller sample. The large standard errors seen in some samples are due to single outliers. Juveniles lack obvious sex-specific characters until the fifth instar; therefore sex was scored as fifth instars or after attempted adult eclosure. Non-specific *GFP* dsRNA had no effects on sexual or genital development.

Specimens of *O. fasciatus* were stored in 70-80% ethanol within 12h of adult eclosure. *fru* 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.

A representative sample (4-6) 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, Magalhães, & Ram, 2004). For male specimens, distances were measured from base of a clasper to its tip. For females, the lengths of the first and second valvulae of the ovipositor were measured. The distance across the head, between the innermost edges of the eyes (ocular distance), was used to normalize for overall body size. Because head size differed between treatments, the ratio of genital length to ocular distance is presented in Figures 7 and 8. Differences between dsRNA treatment and non-specific *GFP* dsRNA controls were analyzed using one-way ANOVA and post-hoc Tukey's honest significant difference (HSD) tests. Nonparametric tests (Wilcoxon and Kruskal-Wallis rank sum tests) agreed with the conclusions

of ANOVA (treatment effect) and the Tukey's HSD (pairwise difference) tests. All statistical tests were conducted in R (Ihaka & Gentleman, 1996)

Table 4. Summary Results for *O. fasciatus* RNA Interference. Knockdown was quantified by qPCR in a representative sample (3 biological replicates) of the total N, p-values and standard errors are representative of this smaller sample. Significance was determined using Welch's t-test compared to non-specific *GFP* controls. $p < 0.05$.

Juvenile RNAi	Gene	Number Scored (N)	% Knockdown	SE	p-value	Penetrance
♂	*vir1*	20	88.34%	3.4%	0.03732	
♀		9	68.78%	3.5%	0.02252	
♂	*vir2*	13	27.08%	48.9%	0.69439	
♀		25	15.10%	3.9%	0.01484	
♂	*vir3*	16	72.47%	3.4%	0.00000	
♀		25	62.01%	8.3%	0.00042	
♂	*fl(2)d*	25	49.20%	16.1%	0.26880	
♀		21	71.28%	5.3%	0.00017	
♂	*msl-2*	6	61.77%	2.5%	0.00001	
♀		9	58.54%	1.1%	0.00000	
♂	*msl-3*	17	39.32%	0.9%	0.00605	
♀		35	80.27%	25.5%	0.00000	
♂	*tra-2A*	22	20.56%	3.4%	0.00330	
♀		49	17.71%	2.7%	0.00569	
♂	*tra-2B*	14	85.99%	5.8%	0.00022	
♀		34	60.75%	2.7%	0.00569	
♂	*ix*	6	78.44%	2.6%	0.00039	100.00%
♀		16	90.91%	0.8%	0.00000	100.00%
♂	*fru*	18	65.28%	3.7%	0.00037	38.89%
♀		38	66.75%	6.1%	0.00003	89.47%
Maternal RNAi						
♀	*tra-2A*	4				
♀	*tra-2B*	11				
♀	*ix*	19				100.00%
♀	*fru*	7				

CHAPTER 3

RESULTS

Efforts to Clone *Sex-lethal, doublesex,* and *transformer* were Unsuccessful

Both *Sxl*, *dsx*, and *tra* were absent from the published *O. fasciatus* transcriptome (Ewen-Campen et al 2011). Cloning of these three genes was attempted many times with several sets of degenerate primers, but with no success.

Expression of Somatic Sex Determination Genes Varies during Development

Quantitative PCR was used to chart the expression of several genes of interest in each stage of *O. fasciatus* development. All boxplots in this study represent the interquartile range (box), the median (bold line), and range of the data (whiskers). Significant differences are denoted by bold lines and letters above boxes. A full list of significant differences between developmental stages is available in Appendix A (Table 5 (1st repetition) and Table 6 (2nd repetition)). Each timecourse experiment was repeated twice. The second set of timecourse experiments were done at the same time several months after the first set. *GFP* RNAi adults were used in the first experiment while in the second experiment unmanipulated adults represented the adult life stages. The transcript levels in adults relative to other life stages do not seem to be greatly affected by this; differential RNA amounts in *GFP* RNAi adults were accounted for when making representative graphs.

Two paralogs of *transformer-2* were isolated from the *O. fasciatus* transcriptome, named *transformer-2A (tra-2A)* and *transformer-2B (tra-2B)*. The two paralogs share 63% identity (Table 2). Two independent experiments yielded variable results for expression of *tra-2A* throughout development (Fig. 3a, Fig. 3b). One experiment showed no differences in at any life stage (Fig. 3a), while the second showed a significant decrease in expression in the fifth instar

and adults (Fig. 3b, $p < 0.05$). It is possible that the second experimental replicate is more variable because of RNA degradation over time, although the second experimental set for *tra-2B*, *ix*, and *fru* show a more similar pattern to their corresponding first replicate than *tra-2A*. Some variability could be due to using *GFP* adults rather than unmanipulated adults; however, this is consistent across experiments for different genes and would only affect comparisons involving adults. Expression of *tra-2B* shows a similar pattern, with higher expression in the first and second instars and a decrease in expression beginning in the third instar (Fig. 3c, Fig. 3d).

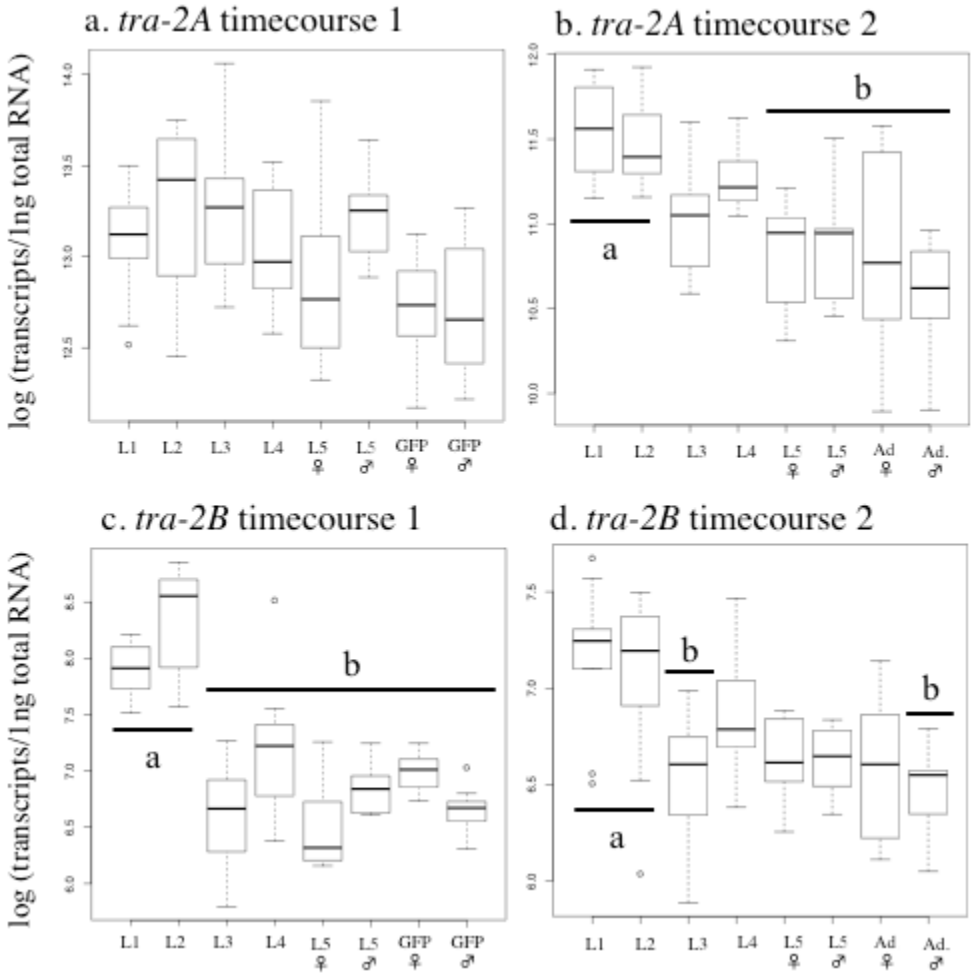

Figure 3. Two Independent Experiments Charting a Timecourse of *tra-2A* (a,b) and *tra-2B* (c,d). L1 indicates first instar, L2, second instar, etc. The first experiments used GFP RNAi adults while unmanipulated adults are used in the second experiments. Significance was determined using a Tukey's HSD test, see Supplementary Tables for all significant comparisons and p-values. Letters denote significantly different groups; $p < 0.05$.

Expression of *ix* and *fru* was also examined over the course of *O. fasciatus* development in two independent experiments each (Fig. 4). These two genes follow a similar general expression pattern to that of the *tra-2* paralogs. *ix* peaks in first and second instars, followed by a significant decrease in expression in the third, fourth, and fifth instars (Fig 4a, Fig. 4b). In both experiments, *ix* expression almost increases towards adulthood; in one experiment, there is a significant increase in expression in the imago molt. (Fig. 4b, $p < 0.05$). *fru* expression again is high in first and second instars, and significantly decreasing in later life stages (Fig. 4c, Fig, 4d). Only *fru* expression exhibited sexually dimorphic expression in one experiment (Fig. 4c, $p < 0.013$) and the second experiment shows the same trend of higher *fru* expression in females, but is not significant. None of other three genes show sexually dimorphic expression patterns in fifth instars or adults (Fig. 3, Fig. 4).

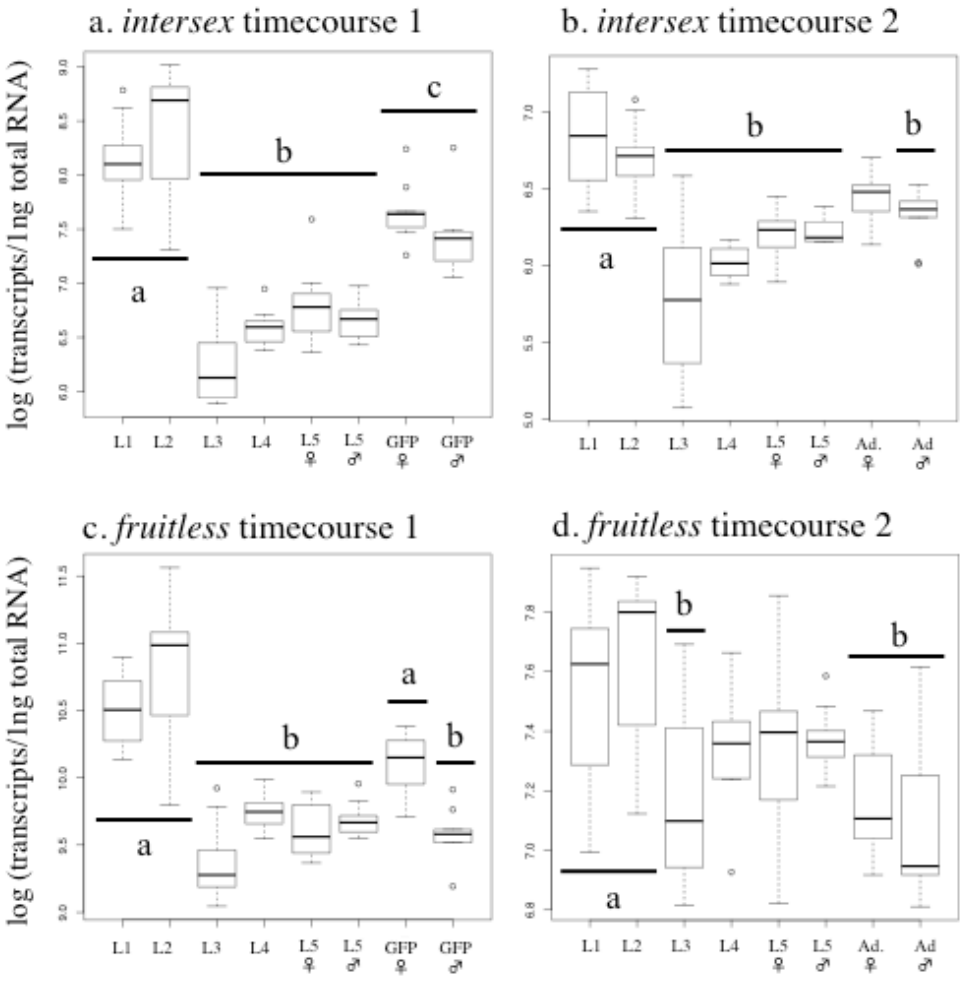

Figure 4. Two Independent Experiments Charting a Timecourse of *intersex* (a,b) and *fruitless* (c,d). The first experiments used GFP RNAi adults while unmanipulated adults are used in the second experiments. Significance was determined using a Tukey's HSD test, see Supplementary Tables for all significant comparisons and p-values. Letters denote significantly different groups; $p < 0.05$.

Juvenile RNA Interference of *virilizer1*, *virilizer2*, *virilizer3*, or *female-lethal-2-d* Caused no Genital Defects

Figure 5 shows the normal structure of male (Fig. 5a) and female (Fig. 5d) internal reproductive structures and external genitalia (Fig. 5b, 5c, 5e, 5f). Three paralogs of *virilizer* were cloned from the *O. fasciatus* transcriptome and share between 51% and 66% identity (Table 2).

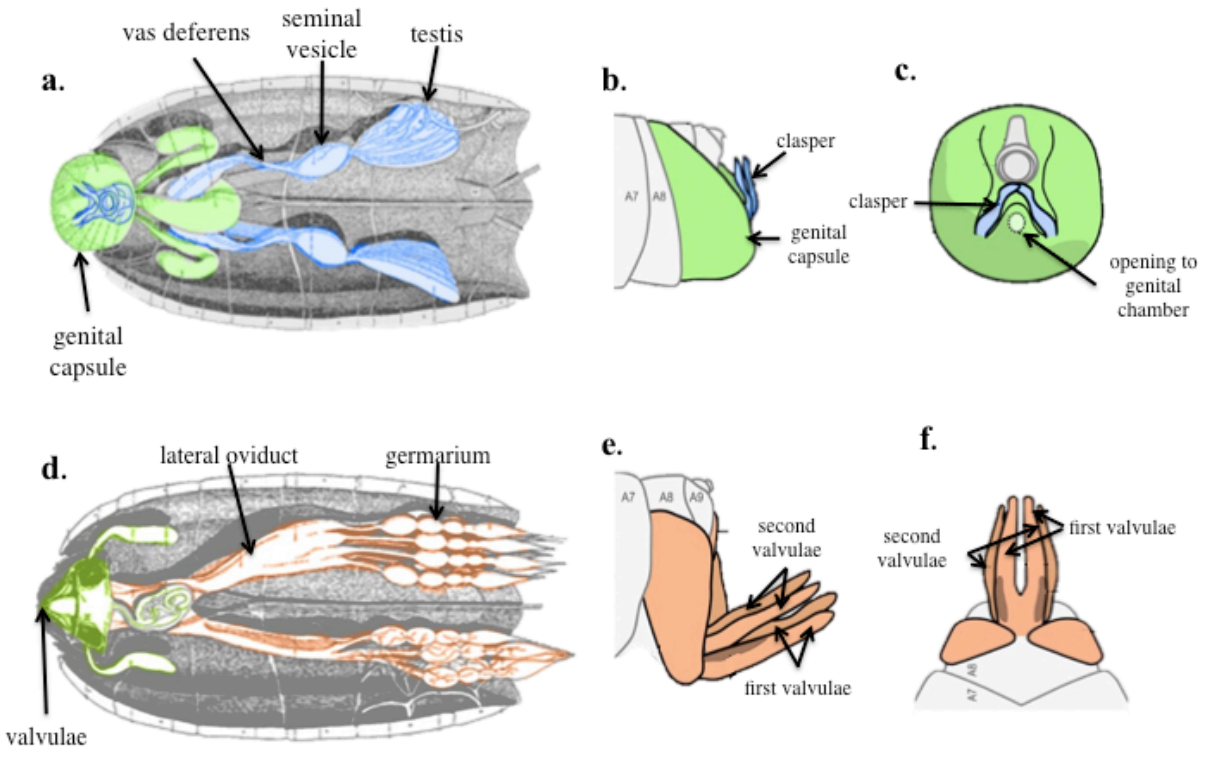

Figure 5. Internal Reproductive Structures and External Genitalia of Male (a,b,c) and Female (d,e,f) *O. fasciatus.* Figures modified from Bonhag, PF and Wick, JR (1953) and Aspiras, AC, Smith, FW and Angelini, DR (2011).

Injection of *vir1, vir2, vir3* and *fl(2)d* dsRNA at the fourth instar did not produce any defects in the genitalia of *O. fasciatus*; however, *fl(2)d* RNAi did cause a wing defect in 37.5% (21/56) of bugs examined (Fig. 6). This defect affected both males (Fig. 6a) and females (Fig.6b). Other than this defect in *fl(2)d* bugs, adult bugs were indistinguishable from the non-specific *GFP* dsRNA controls. Internal reproductive structures of both males and females in these treatments were also indistinguishable from controls. Although reproductive structures are normal in *fl(2)d* specimens, these bugs were never observed mating and produced no viable eggs.

Figure 6. Wing Defect Caused by Juvenile *fl(2)d* RNAi Seen in Males (a) and Females (b).

Juvenile *male-specific lethal-2* and *male-specific lethal-3* RNA Interference Caused no Developmental Defects

Two *male-specific lethal* genes were cloned from the *O. fasciatus* transcriptome, *msl-2* and *msl-3*. The gene called *msl-2* in this study is most similar to *Drosophila msl-3* and vice versa. Attempts to clone *msl-1* were not successful. Internal reproductive structures and genitalia of male and female *msl-2* and *msl-3* RNAi specimens were indistinguishable from *GFP* controls.

Juvenile and Maternal *transformer-2A* and *transformer-2B* RNA Interference Caused no Developmental Defects

Fourth instars and adult virgin females were injected with both paralogs of *tra-2* dsRNA. Internal structures of adult *tra-2A* or *tra-2B* juvenile RNAi specimens were indistinguishable from GFP controls. All injected females were able to mate and each produced several clutches (~4-6) clutches of viable offspring (G_1) that also developed into adults (>100 individuals). The offspring of maternally injected *tra-2A* and *tra-2B* (G_1) displayed normal internal reproductive structures and external genitalia and were able to mate and produce viable offspring of their own (G_2) that also develop into adults (>100 individuals).

Juvenile *intersex* RNA Interference Caused Reduction of the Gonads and Male Genitalia and a Partial Female-to-Male Somatic Transformation

Knockdown of *ix* during juvenile development caused defects in both sexes. The male claspers (Fig. 7, $p = 2.43 \times 10^{-8}$) and both pairs of female valvulae (Fig. 8a, 1st valvulae: $p = 4.62 \times 10^{-11}$; Fig. 8b, 2nd valvulae: $p = 3.079 \times 10^{-9}$) were significantly reduced in *ix* RNAi specimens. The normal ventral fusion of the first valvulae did not occur in *ix* RNAi females. Valvulae were more heavily pigmented, and the abdominal sternal process normally observed in wildtype females was reduced in *ix* RNAi specimens. This partial sex reversal phenotype was also found in an earlier study of *ix* RNAi in *O. fasciatus* (Aspiras et al., 2011).

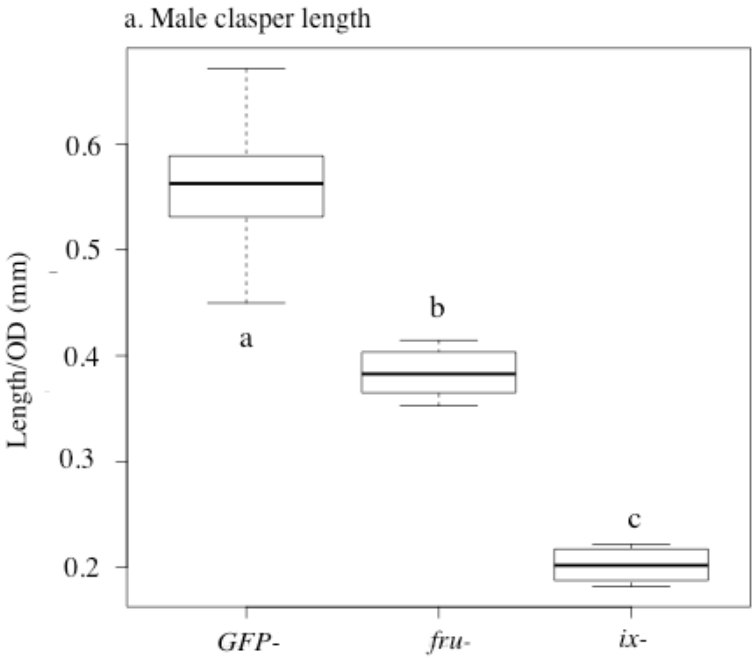

Figure 7. *fruitless* and *intersex* RNAi Shortened the Length of Male Claspers. Letters denote significantly different groups; $p < 0.05$.

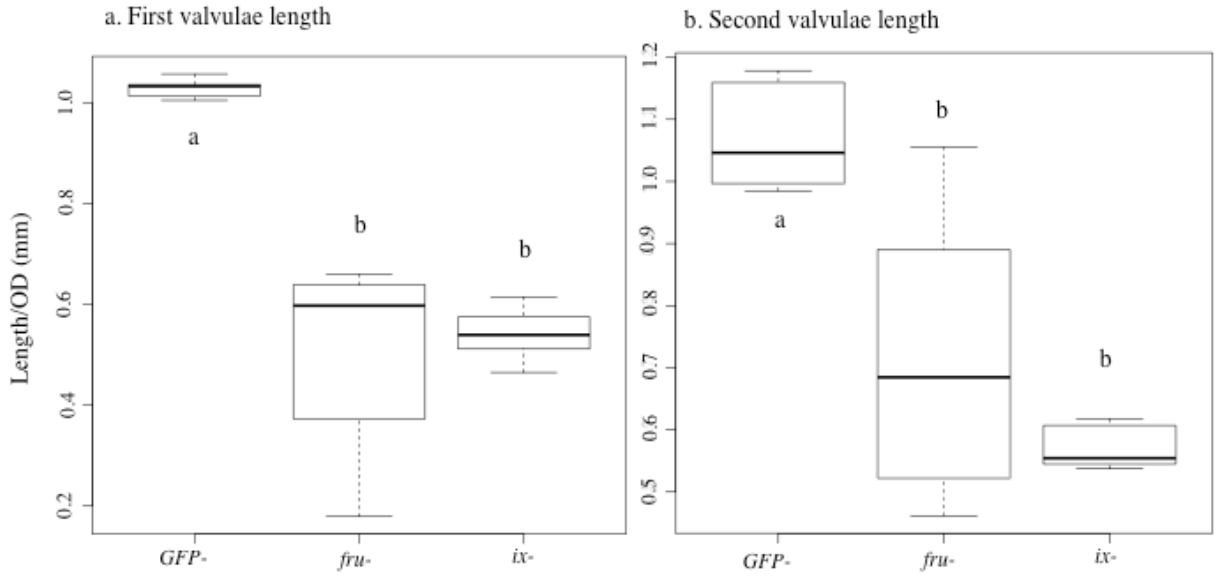

Figure 8. *intersex* & *fruitless* RNAi Shortened Female Valvulae. Letters denote significantly different groups; $p < 0.05$.

Internal reproductive structures were affected by the *ix* knockdown in both sexes. The effect of *ix* RNAi on male internal structures was severe. The testes in *ix* RNAi males lacked their normal color and structure (Fig. 9). Large masses of undifferentiated tissue surrounded both testes and the normal fan-like testes structure was absent. Ovarian follicles are normally separate, but *ix* RNAi female specimens these follicles were fused and surrounded by undifferentiated tissue (Fig. 10). The follicles of *ix* RNAi ovaries were generally reduced in width and length compared to controls.

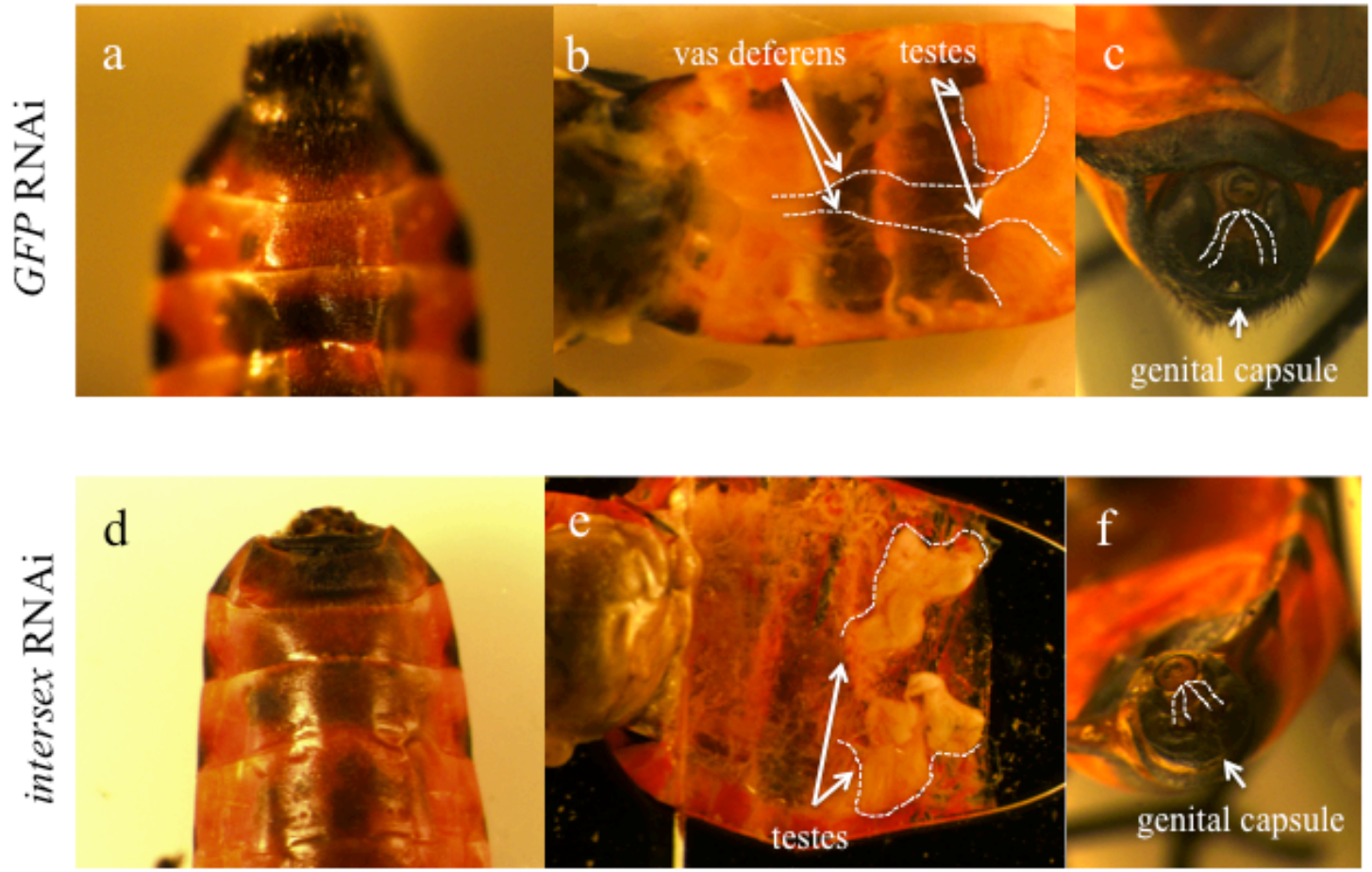

Figure 9. *intersex* RNAi Causes Internal and External Genitalia Defects in Males. Top panel shows a ventral view of a male *GFP* RNAi abdomen (a), wildtype testes and vas deferens (b), and genital capsule (c). Bottom panel shows corresponding views of *intersex* male RNAi abdomen (d), abnormal testes (e), and genital capsule with reduced claspers (f).

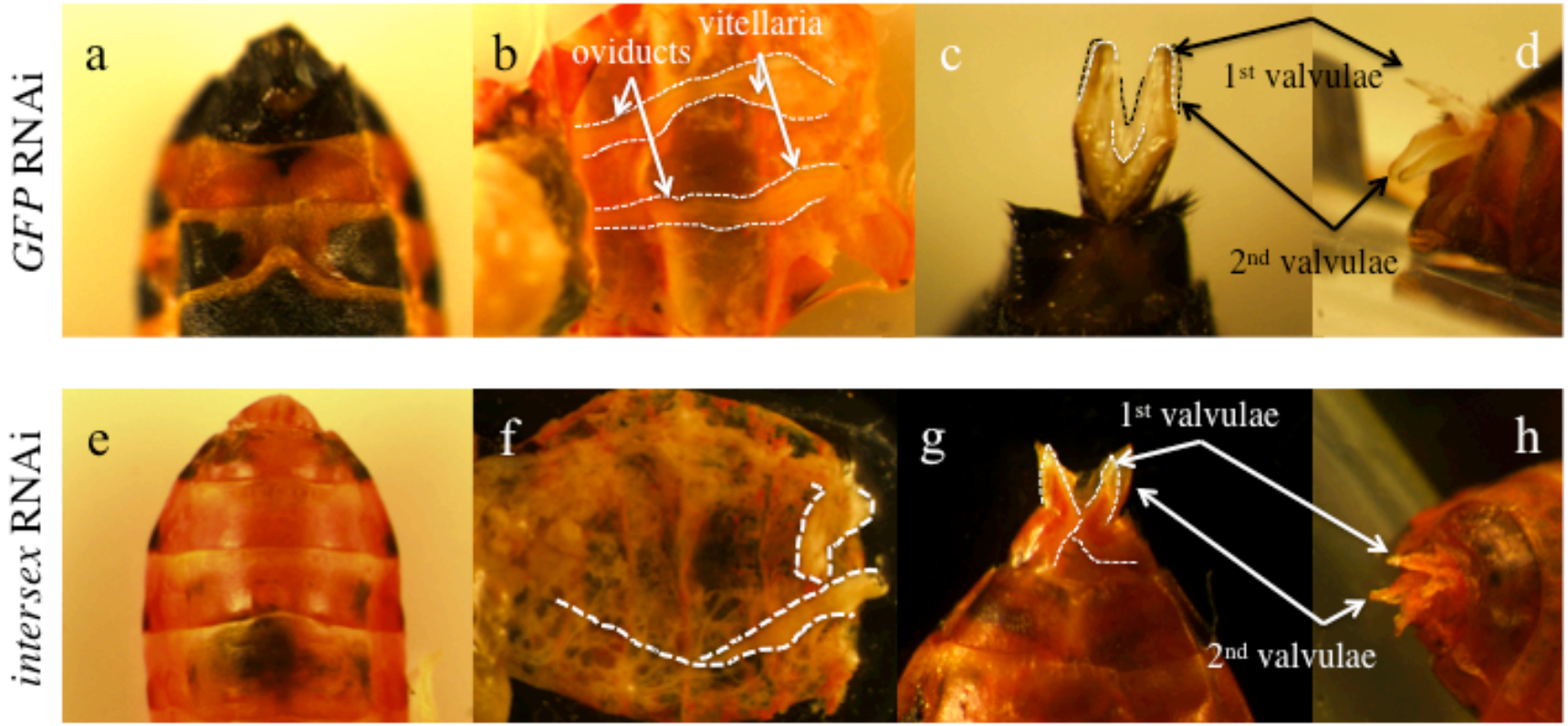

Figure 10. *intersex* RNAi Causes Internal and External Genitalia Defects in Females. Top panel shows a ventral view of a female *GFP* RNAi abdomen (a), wildtype ovaries (b), and a ventral (c) and lateral (d) view of the wildtype ovipositor. Bottom panel shows corresponding views of *intersex* female RNAi abdomen with reduced abdominal sternite (e), abnormal ovaries (f), and ventral (g) and lateral (h) view of the reduced ovipositor.

fruitless RNA Interference caused Defects in the Male and Female Genitalia, but not the Abdomen or Gonads

The claspers of male *fru* RNAi specimens were reduced in pigmentation and cuticle thickness. Claspers were significantly reduced (Fig. 7, $p = 9.755x10^{-6}$) and lightly pigmented compared to controls. Nevertheless, testes in male *fru* RNAi specimens were normal (Fig. 11). In female genitalia, *fru* RNA interference significantly reduced the length of the ovipositor. Both the first and second valvulae were significantly shorter than non-specific controls (Fig. 8a, 1st valvulae: $p = 1.83x10^{-6}$; Fig. 8b, 2nd valvulae: $p = 0.0001581$). Sclerotization and pigmentation were also reduced in *fru* RNAi specimens compared to controls (Fig 12). The second valvulae were so reduced that they no longer had a pointed shape, but appeared as a single sheet of tissue. Necrosis was observed on some parts of the second valvulae and the necrotic tissue was not evenly distributed along the distal edge. However, *fru* RNAi females retained the abdominal sternal process. The ovaries of female *fru* RNAi specimens also appeared to be normal.

Maternal *fru* RNAi did not appear to have any affect on external or internal structures of the G_1 offspring. In a second independent experiment with 3 females, only 2 clutches of eggs were (so far) observed and hatched about 11 individuals. Approximately 38 dead L1s were removed from the cage; observationally, this is more than the normal rate of L1 mortality.

Maternal *ix* RNAi females did not produce any G_1 offspring and eggs were only seen approximately 3 weeks after mating. When the same males used in this experiment were mated with unmanipulated virgin females, they were able to produce >100 healthy offspring, indicating that male fertility was not affected by the *ix* knockdown.

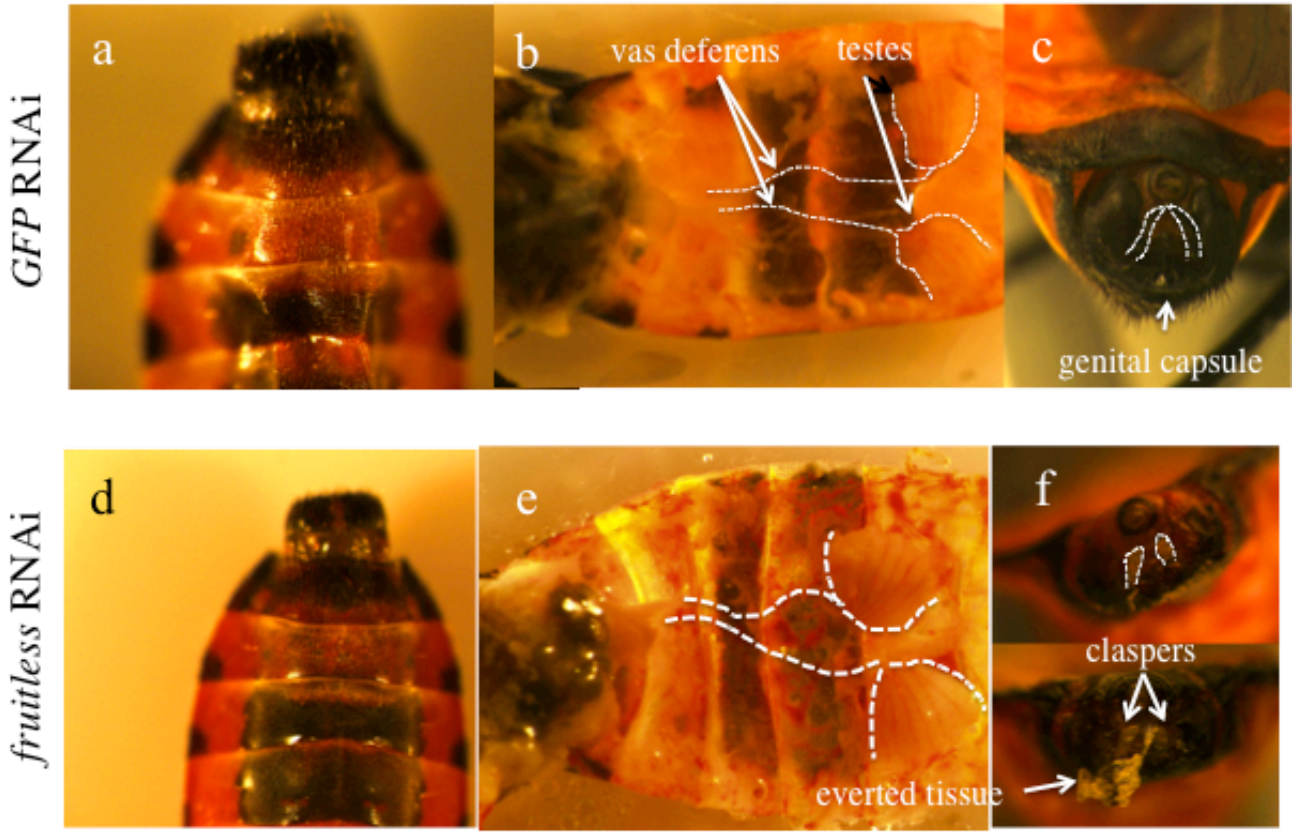

Figure 11. *fruitless* RNAi Causes External Genitalia in Males, but does not Affect Internal Structures. Top panel shows a ventral view of a male *GFP* RNAi abdomen (a), wildtype testes and vas deferens (b), and genital capsule (c). Bottom panel shows corresponding views of *fruitless* male RNAi abdomen (d), wildtype testes (e), and two views of the genital capsule with reduced claspers and necrotic tissue emerging from the genital opening (f).

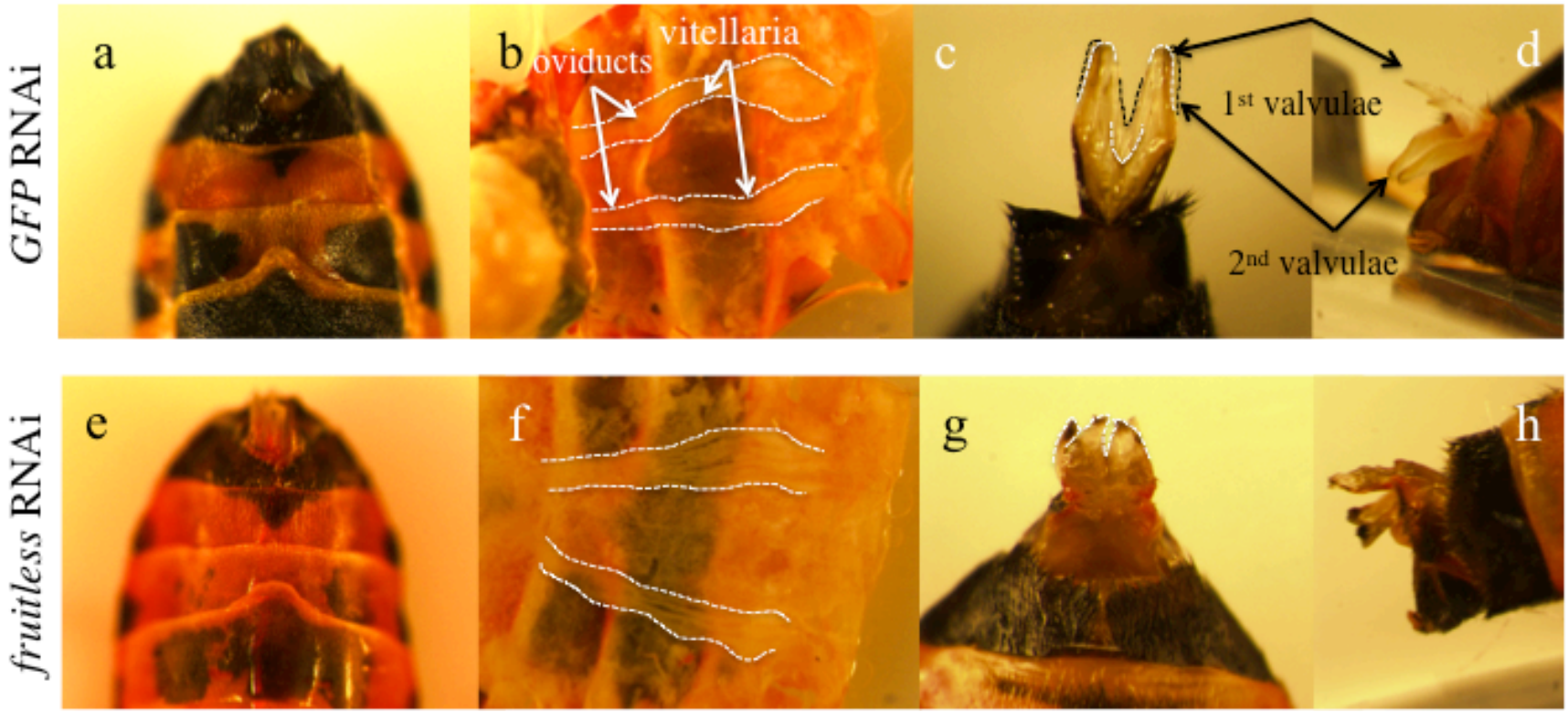

Figure 12. *fruitless* RNAi Causes External Genitalia Defects in Females, but does not Affect Internal Structures. Top panel shows a ventral view of a female *GFP* RNAi abdomen (a), wildtype ovaries (b), and a ventral (c) and lateral (d) view of the wildtype ovipositor. Bottom panel shows corresponding views of *fruitless* female RNAi abdomen (e), wildtype ovaries (f), and ventral (g) and lateral (h) view of the reduced ovipositor.

fruitless Promotes *intersex* Expression in Adult *O. fasciatus*

Because of the importance of *fru* and *ix* in *O. fasciatus* sexual development, the interaction of these genes was examined for comparison to *Drosophila*. To test gene interactions, the expression of one gene is measured in an RNAi background for a second. In RNA isolated from the abdomens of male and female *ix* specimens, *fru* expression was indistinguishable from controls (Fig. 13b, Fig. 13d). Therefore, *fru* expression appears to be unregulated by *ix*. However, in the abdomens of male and female *fru* RNAi specimens, *ix* expression was significantly reduced (Fig. 13a, females: $p = 2.669 \times 10^{-6}$; Fig. 13c, males: $p = 0.001797$,), suggesting that *fru* normally acts to promote *ix* expression. Males and females showed the same pattern of interaction.

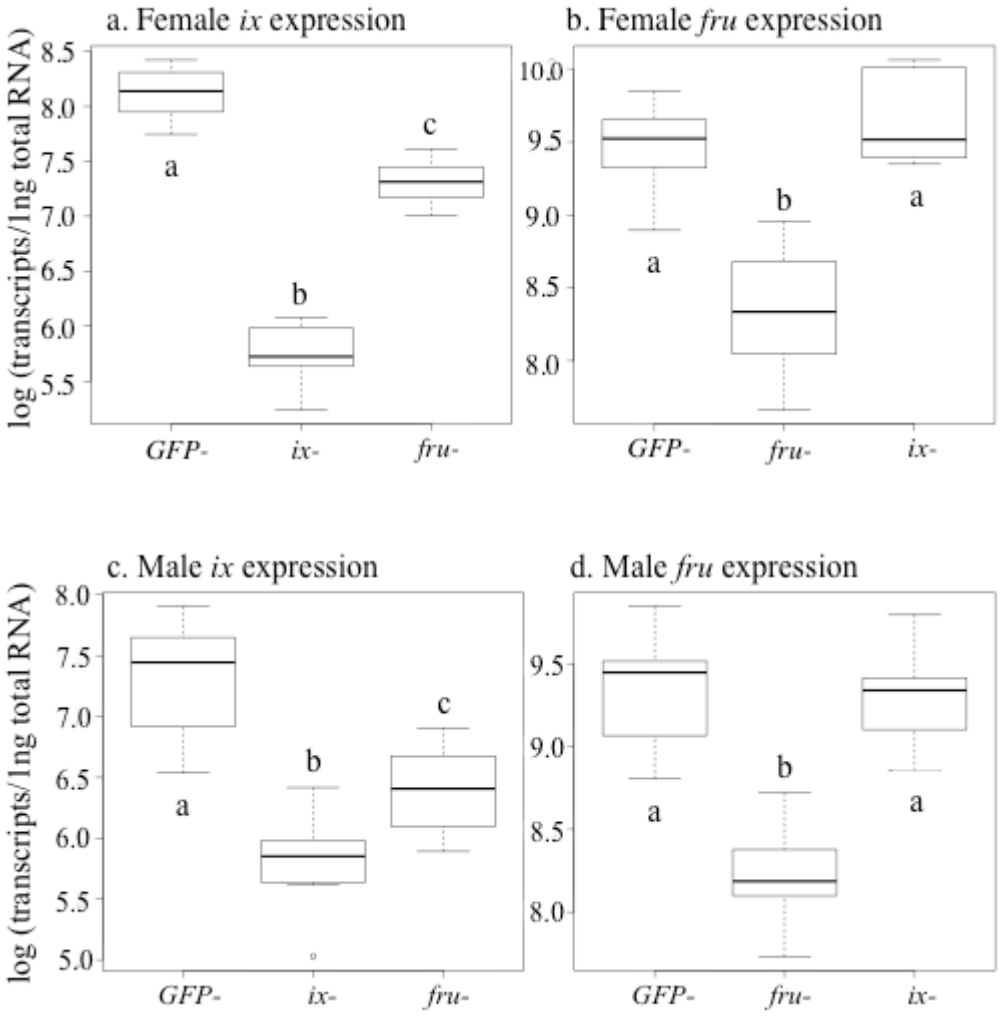

Figure 13. *fruitless* Promotes *intersex* Expression in Females and Males. Each boxplot shows expression of one gene in the RNAi background of another (x-axis). Letters denote significantly different groups; $p < 0.05$.

CHAPTER 4

DISCUSSION

Absence of *Sex-lethal*, *doublesex*, and *transformer* from the Transcriptome

Multiple attempts to clone *Sxl, dsx,* and *tra* using degenerate PCR were unsuccessful. It is possible that these genes were not expressed in the ovarian and embryonic tissue used to assemble the transcriptome or alternatively have been lost in the lineage leading to *O. fasciatus.* Given the derived nature of *Sxl* in sex determination and development, the absence of this gene is not surprising. *Sxl* homologs have been identified in many insect taxa, including the hemipteran *A. pisum*; however, current phylogenetic evidence supports the gain of a sex-determining function for the duplicated *Sxl* only in the *Drosophila* lineage (Traut, Niimi, Ikeo, & Sahara, 2006).

A high expression of *dsx* is observed in dimorphic tissue in adult *Drosophila* (Robinett et al., 2010). If *O. fasciatus* follows the same model as *Drosophila,* it would likely be transcribed in high levels in the ovaries. Alternatively, *O. fasciatus* might not express these downstream genes until they are needed to actively differentiate dimorphic tissue, in which case it seems plausible that they would be absent from already dimorphic adult ovarian and monomorphic embryonic tissue. Other dimorphic differences, however, that require *dsx* in *Drosophila,* such as neural circuitry formation (Song et al., 2002) would have to be formed earlier in development. Other highly conserved developmental genes, such as some Hox genes (Ewen-Campen, personal comm.) are also missing from the transcriptome, suggesting that *dsx* could still be present in the genome. Additionally, genes at the bottom of sex determination networks are known to be highly conserved (Wilkins, 1995). Because *tra* is an upstream regulator, and not found in as broad a range of insect taxa as *dsx* (Suzuki et al., 2001), it is more likely that *tra* is absent from the

genome. Altogether this suggests that *dsx* is probably in the genome and the whole genome sequence will shed more light on its function in *O. fasciatus* sex differentiation.

Most Candidate Genes do not Appear to Have a Role in Sexual Differentiation in *O. fasciatus*

This study describes functional analyses of a series of candidate genes that were predicted to play a role in sex determination in the large milkweed bug, *O. fasciatus*. Knockdown of most of these genes did not cause a developmental defect. There could be several reasons for this. First, post-transcriptional regulation is possible; mRNA stability, translation rates, and protein trafficking and modification could all influence final activity of sex-specific proteins. Protein levels were not examined in any treatment in this study. Additionally, although RNAi experiments were repeated, it is possible that the knockdown was insufficient to cause a developmental disruption. This is true in particular of the partial clones (*msl-2, vir1,* and *fl(2)d*) that do not share high positive identity with the transcriptome sequence used as a template (Table 3). Interestingly, this does not always coincide with low identity at the amino acid level, as only *msl-2* (40%) and *vir1* (14%) are not very similar to proteins translated from the transcriptome sequence (Table 3), although amino acid identity would not have any bearing on the ability of the dsRNA to lower target transcripts. *fl(2)d* shares high identity with the amino acid predicted by the transcriptome sequence at 96%.

Second, although no internal or external developmental defects were observed, there are a myriad of other possible reproductive affects, including defects in gametogenesis, sperm storage, fertilization, or even early embryogenesis. All juvenile RNAi specimens (except *fl(2)d*) were able to mate and produce offspring. Because mating behavior was never observed in these individuals, expression and interactions of *fl(2)d* is being examined in the heads of these animals, although these data are not yet available.

Third, the failure of most RNA interference of genes to produce a developmental phenotype could indicate that these genes (*vir* paralogs, *tra-2* paralogs, *msl-2, msl-3* and *fl(2)d*) have no role in sexual differentiation in *O. fasciatus.* This would suggest that the sex determination function of these genes is derived in certain groups of Holometabola. With regard to the two nuclear proteins *vir* and *fl(2)d*, it is perhaps not surprising that no developmental phenotype is produced, because although they have been implicated in the auto-regulation of *Sxl* and the splicing of *tra* (Hilfiker et al., 1995), *Sxl* has only evolved a sex determination function within the Brachycera (horse flies, robber flies, dance flies, bee flies (Serna et al., 2004) and *tra* was not cloned or examined in this study. The wing defect observed in *fl(2)d* specimens implies a pleiotropic effect of *fl(2)d,* supporting *fl(2)d'*s role as a splicing regulator. Previous studies of the molecular structure of *vir* in *Drosophila* indicate that some alleles have a vital function in mRNA transport (Niessen et al., 2001). Disruption of mRNA transport at any stage of development would be deleterious, and the survival of juvenile RNAi specimens suggests that this is not the function of *vir1* in *O. fasciatus*.

Male-specific-lethal genes (*msl-1*, *msl-2*, *msl-3*) encode proteins that, together with the proteins MOF and MLE, form a protein complex responsible for hypertranscription of the male X chromosome in *Drosophila* (Penalva & Sanchez). The *msl* genes are transcribed in both *Drosophila* sexes, however, only *msl-2* is necessary and sufficient to assemble this complex (Kelley, et al. 1995). Only two *male-specific-lethal* genes were cloned from *O. fasciatus* and they share significant homology to *Drosophila msl-2* and *msl-3* (Table 2). The highly conserved function of *msl* homologs in chromatin modification and transcription regulation in species as diverse as insects and mammals (X. Li & Dou, 2010), is evidence that these genes could be involved in dosage compensation in *O. fasciatus*. Because *O. fasciatus* has heteromorphic sex

chromosomes, some method of X chromosome compensation probably takes place. *msl* genes are merely one part of a complex and varied process and another mechanism may be responsible for this process in *O. fasciatus*. Knocking down *msl* function represents a very focused attempt to learn about dosage compensation when we know very little about how this species may determine sex in the first place. Another gene may be responsible for dosage compensation, or male-specific genes on *O. fasciatus* autosomes may repress female-specific genes when the male ratio of X:A is present. In *B. mori*, *msl-1, msl-2,* and *msl-3* have been identified and exhibit sexually dimorphic expression patterns but the mechanism of dosage compensation is still poorly understood (Liu, et al. 2008). For all of the above genes, it is possible that these genes act at a different stage in development. If a gene plays a key role and it is actively transcribed at a specific developmental time, knocking down expression at this time would have a greater developmental effect.

Two paralogs of *transformer-2 (tra-2)* were isolated from *O. fasciatus* and cluster with other insect isoforms of *tra-2*, not *transformer,* suggesting that these genes did not have recent common evolutionary origins. The expression of each paralog is generally similar throughout development. Each paralog has higher expression in the first and second instar, implying involvement in some process at this developmental stage. The generally similar expression patterns throughout development make it difficult to infer whether the two paralogs have any differing developmental function. There is evidence that *tra-2* is essential and sex specifically regulated in the male germ line in *Drosophila* (Mattox, Palmer et al. 1990); both paralogs here are not sex specifically expressed in the abdomen. The lack of sex differences at measureable stages, ability of individuals to display normal mating behavior and produce viable, fertile offspring is suggestive of activity in a non sex-specific developmental process. Because *tra-2* is

not involved in *dsx* splicing across all holometabolous groups (Niu et al., 2005), this gene could have lost its sex-determining function in certain taxa, including the lineage leading to *O. fasciatus.* There is some extremely preliminary data, however, suggesting that *tra-2* may have a sex-determining function in the crustacean *Fenneropenaeus chinensis* (Chinese shrimp) (S. Li, Li, Wen, & Xiang, 2012), which supports an ancestral sex-determining role for *tra-2*. All told, more data from other basal lineages is required to determine which is the case.

Supporting Evidence for *fruitless*'s Ancestral CNS Development role; Derived Morphological Role in *O. fasciatus*

fru, the master regulator of male mating and courtship behavior, is conserved in most insects examined in structure and function. *Fru* has a complex molecular structure of 4 promoters and encodes several zinc-finger proteins; however, only transcripts from the P1 promoter are sex specifically expressed in a small set of neurons in the CNS that contribute to male behavior. *fru* also has a non sex-specific vital role in embryonic and adult neuronal development (Anand et al., 2001; Song et al., 2002).

The main effect of juvenile *fru* RNAi in *O. fasciatus* sex differentiation is morphological, and affects both males and females, although the effect in females seems more severe. In order to determine if the transcript knocked down in this study is sex specific, the *O. fasciatus* clone was compared to splice variants in a variety of insect taxa. The *fru* clone is most closely related to *D. melanogaster* splice variant L, as determined by maximum likelihood analysis in MEGA 5. Splice variant L is a female specific variant, encoding a female specific isoform. As the best functionally characterized variants, pairwise comparisons were made to *D. melanogaster* variants in ClustalW, which show that the *O. fasciatus* clone aligns best with variants C, F, H, and L, in that order. All of these produce female specific protein isoforms. A knockdown of a female-specific variant would cause more severe gross morphological defects in *fru* RNAi females, as

seen in this study. This is a further departure from the *Drosophila* model—in *Drosophila, fru* transcripts are expressed in both male and female CNS, but female specific transcripts are not translated (Lee et al., 2000). This transcript, whether it is identified as sex specific or not in other insects, has a morphological role in the genitalia and is obviously being expressed outside the CNS.

In contrast to the neuronal and behavioral function of *fru* in many other insects, results reported here indicate that *fru* has a role in genitalia development, as well as another developmental (molting) function in *O. fasciatus*. Because maternal *fru* RNAi specimens produce normal offspring, *fru* seems to have a minimal role in embryonic neuronal development, although this experiment should be repeated. While it is possible that *fru* has a behavioral affect in *O. fasciatus*; it is unlikely that *fru*-depleted individuals would be able to mate and produce offspring because of genitalia defects. If the concentration of *fru* dsRNA were lowered, it may be possible to determine the possible role for *fru* in *O. fasciatus* mating behavior, as was done in a study of mating behavior in *Blatella germanica* (German cockroach). *Fru* regulates of male courtship behavior in this basally branching insect, suggesting an ancestral role in courtship (Clynen et al., 2011). The characteristic male-specific 101 amino acid N-terminal extension ahead of the BTB domain absent in *B. germanica fru* (Clynen et al., 2011) is also lacking in *O. fasciatus.* Another study addressed *fru* structure in three species of the orthopteran genus *Chorthippus* (grasshoppers) and found evidence of alternative splicing at the 5' end of two different *fru* transcripts (Ustinova & Mayer, 2006). Perhaps in basally branching insect lineages, different paralogs may perform different functions while in *D. melanogaster*, differing functions of *fru* stem from alternative splicing of the same transcript from four different promoters.

intersex encodes part of the mediator complex in *D. melanogaster,* and suppresses male development in females, together with DSX^F and Her. The data presented here support a similar role, as juvenile females are masculinized by the *ix* knockdown. In *D. melanogaster,* there is a female specific *ix* loss-of-function phenotype because the action of *ix* is dependent on the presence of DSX^F. Several hypotheses could explain the non-sex specific defects seen in *O. fasciatus*. In this species, IX could interact with both sex specific DSX isoforms, so that *ix* knockdown decreases the efficiency of both proteins in differentiating male and female somatic tissue. Alternatively, IX alone could be the direct actor on certain dimorphic target genes. IX would rely on some other sex specific temporal or spatial cue to dimorphically differentiate tissue. In this case, if DSX were present and acting in concert with IX, DSX would not be sexually dimorphic. The former situation is more likely, however, given the conservation of sex specific splicing of DSX as a mechanism of insect sex determination.

The promotion of *ix* by *fru* could be direct or indirect. If *ix* is a target of the transcription factor encoded by *fru,* the question remains—why don't *fru* RNAi bugs display the same intersexual phenotype? Maternal knockdown of *ix* causes female sterility, but knockdown of *fru* (as yet) does not affect female fertility. Observational data of the second maternal *fru* RNAi experiment does suggest there is some decrease in number of offspring produced, but this was not quantified here. A compensatory mechanism could exist to post-transcriptionally up-regulate Ix when low levels of Fru are detected.

intersex knockdown produces a consistently different phenotype than *fru* knockdown. Because fusion of the second valvulae occurs only in *fru* RNAi specimens, but not in *ix* knockdowns, only *fru* must be involved in the regulation of second valvulae fusion/genitalia development. This is an independent role of *fru,* in addition to promoting *ix* expression. The

localized necrosis often observed in the second valvulae is perhaps caused by poor maintenance of an open tracheal system during development or molting. Lack of movement as the nymph attempts to molt could cause the circulation of the hemolymph to slow, restricting flow of nutrients to the developing genital tissue. A potential role for *fru* or *ix* could be as a repressor of apoptosis during the formation of the separate first valvulae. Apoptosis continues in the *ix* knockdowns and thus causes the unfused first valvulae in females.

Evolution of Sex Determination Networks

The genetic and molecular techniques of the past decades have provided greater understanding of how a wide variety of organisms determine sex. It is known that sexual selection drives morphological sexual dimorphisms and is a source of rapid evolutionary change (Darwin, 1871). Because sex determination mechanisms can impact organismal fitness (Warner & Shine, 2008), sexual selection probably also drives mechanistic dimorphism in sex determination networks. This makes sense given the huge diversity of sex determination mechanisms in closely related insect groups. There is a large body of theoretical work on how these mechanisms change quickly over evolutionary time.

Male-female genomic conflict (Werren & Beukeboom, 1998) is one of these theories. In diploid insects, the paternal genome is predicted to prevent female-specific splicing of *tra* in a variety of ways. Autosomal repressors in *D. melanogaster* prevent *Sxl* auto-regulation and therefore the downstream expression of *tra.* In diploid organisms with a male-determining Y chromosome, such as *Ceratitis capitata* and *Musca domestica*, the male determinant actively blocks expression of *tra* and thus prevents the establishment of the auto-regulatory *tra* loop (Hediger et al., 2010; Pane, Salvemini, Delli Bovi, Polito, & Saccone, 2002). In Lepidoptera, where females are the heterogametic sex and possess a female-determining factor, the role of the

paternal genome in *tra* activity is not as clear. A theory put forward by Pomiankowski et al. (2004) suggests how a homozygous *tra* genotype which promotes male development could have evolved to eliminate this genomic conflict.

Wilkins (1995) put forward a theory that sex determination hierarchies evolve via sex ratio selection from the bottom up, by recruiting new upstream control elements. The most downstream elements, such as *dsx*, are thus considered the most ancient and under the most purifying selective pressure. This kind of downstream conservation is present in other sex-determining networks: DMRT genes generally promote male development in vertebrates including mammals and reptiles, while the male determining SRY gene is found only within mammalian taxa (Kopp, 2012; Wallis, Waters, & Graves, 2008). Pomiankowski et al. (2004) hypothesize that the *Drosophila* sex determination pathway could have evolved in this fashion where selection favors mutations that reduce expression of sex specific transcripts in the wrong sex. In light of this theory and the extreme functional conservation of *dsx*, it becomes even more unlikely that it is truly absent in *O. fasciatus*. However, *tra* has not yet been shown to have a sex-determining role outside Holometabola. There is large sequence divergence of *tra* across insect species outside of the RNA binding domain and large divergence between the functional domains of *tra* merely within *Drosophila*, indicating that this is a rapidly evolving gene (O'Neil & Belote). This rapid evolution could be one reason for the difficulty in isolating *O. fasciatus tra.* If *tra* is present in the genome, functional tests would reveal another role for this gene in *O. fasciatus* or if *tra* has been recruited as an upstream regulator of *dsx* in Holometabola.

Recent experimental evidence suggests that sex determination networks are flexible and can evolve via compensatory adaptation (Chandler, Chadderdon, Phillips, Dworkin, & Janzen, 2012). When the sex-determining pathway is mutated to be biased towards one sex, standing

genetic variation and pre-existing alleles in the population restore a normal sex ratio via alternate genetic pathways, or via regulatory changes in genes not normally associated with the sex-determination pathway. This an interesting twist to recent evo-devo hypotheses about the predictability of phenotypic evolution (Stern & Orgogozo, 2009); there are multiple pathways to the evolutionary end of a sexually reproducing population. Altogether, the evidence found here supports the existing paradigm that diverse sex determination networks evolve from the bottom up. Genes acting above the final transcription factors vary greatly among species in their functions and interactions while downstream genes are conserved.

APPENDIX A

COMPLETE LIST OF ANOVA COMPARISONS FOR TIMECOURSE EXPERIMENTS

Table 5. All Significant Differences in Means of first *transformer-2A, transformer-2B, intersex,* and *fruitless* Timecourse Experiments.

Gene	Life Stage 1	Mean 1	SE	Life Stage 2	Mean 2	SE	p-value
tra-2A							
tra-2B	L1	7.91	0.08	L3	6.58	0.12	<0.00001
	L1	7.91	0.08	L4	7.18	0.14	<0.01
	L1	7.91	0.08	L5 ♀	6.52	0.13	<0.00001
	L1	7.91	0.08	L5 ♂	6.84	0.07	<0.0001
	L1	7.91	0.08	GFP ♀	7.00	0.06	<0.001
	L1	7.91	0.08	GFP ♂	6.64	0.07	<0.00001
	L2	8.38	0.16	L3	6.58	0.12	<0.00001
	L2	8.38	0.16	L4	7.18	0.14	<0.00001
	L2	8.38	0.16	L5 ♀	6.52	0.13	<0.00001
	L2	8.38	0.16	L5 ♂	6.84	0.07	<0.00001
	L2	8.38	0.16	GFP ♀	7.00	0.06	<0.00001
	L2	8.38	0.16	GFP ♂	6.64	0.07	<0.00001
	L3	6.58	0.12	L4	7.18	0.14	<0.01
	L4	7.18	0.14	L5 ♀	6.52	0.13	<0.05
ix	L1	8.13	0.13	L3	6.25	0.09	<0.00001
	L1	8.13	0.13	L4	6.58	0.04	<0.00001
	L1	8.13	0.13	L5 ♀	6.80	0.12	<0.00001
	L1	8.13	0.13	L5 ♂	6.67	0.06	<0.00001
	L1	8.13	0.13	GFP ♂	7.42	0.12	<0.01
	L2	8.43	0.21	L3	6.25	0.09	<0.00001
	L2	8.43	0.21	L4	6.58	0.04	<0.00001
	L2	8.43	0.21	L5 ♀	6.80	0.12	<0.00001
	L2	8.43	0.21	L5 ♂	6.67	0.06	<0.00001
	L2	8.43	0.21	GFP ♀	7.65	0.09	<0.01
	L2	8.43	0.21	GFP ♂	7.42	0.12	<0.00001
	L3	6.25	0.09	L5 ♀	6.80	0.12	<0.05
	L3	6.25	0.09	GFP ♀	7.65	0.09	<0.00001
	L3	6.25	0.09	GFP ♂	7.42	0.12	<0.00001
	L4	6.58	0.04	GFP ♀	7.65	0.09	<0.00001
	L4	6.58	0.04	GFP ♂	7.42	0.12	<0.00001
	L5 ♀	6.80	0.12	GFP ♀	7.65	0.09	<0.001

	L5 ♀	6.80	0.12	GFP ♂	7.42	0.12	<0.05
	L5 ♂	6.67	0.06	GFP ♀	7.65	0.09	<0.0001
	L5 ♂	6.67	0.06	GFP ♂	7.42	0.12	<0.01
fru	L1	10.49	0.09	L3	9.35	0.07	<0.00001
	L1	10.49	0.09	L4	9.74	0.03	<0.00001
	L1	10.49	0.09	L5 ♀	9.62	0.06	<0.00001
	L1	10.49	0.09	L5 ♂	9.69	0.04	<0.00001
	L1	10.49	0.09	GFP ♂	9.59	0.07	<0.00001
	L2	10.82	0.18	L3	9.35	0.07	<0.00001
	L2	10.82	0.18	L4	9.74	0.03	<0.00001
	L2	10.82	0.18	L5 ♀	9.62	0.06	<0.00001
	L2	10.82	0.18	GFP ♀	10.09	0.08	<0.00001
	L2	10.82	0.18	L5 ♂	9.69	0.04	<0.00001
	L2	10.82	0.18	GFP ♂	9.59	0.07	<0.00001
	L3	9.35	0.07	L4	9.74	0.03	<0.05
	L3	9.35	0.07	GFP ♀	10.09	0.08	<0.00001
	L5 ♀	9.62	0.06	GFP ♀	10.09	0.08	<0.05
	GFP ♀	10.09	0.08	GFP ♂	9.59	0.07	<0.05

Table 6. All Significant Differences in Means of Second *transformer-2A, transformer-2B, intersex,* and *fruitless* Timecourse Experiments.

Gene	Life Stage 1	Mean 1	SE	Life Stage 2	Mean 2	SE	p-value
tra-2A	L1	11.56	0.10	L5 ♀	10.82	0.11	<0.01
	L1	11.56	0.10	L5 ♂	10.86	0.11	<0.01
	L1	11.56	0.10	Ad ♀	10.77	0.21	<0.001
	L1	11.56	0.10	Ad ♂	10.55	0.12	<0.00001
	L2	11.48	0.08	L5 ♀	10.82	0.11	<0.05
	L2	11.48	0.08	L5 ♂	10.86	0.11	<0.05
	L2	11.48	0.08	Ad ♀	10.77	0.21	<0.01
	L2	11.48	0.08	Ad ♂	10.55	0.12	<0.00001
	L4	11.27	0.05	Ad ♂	10.55	0.12	<0.001
tra-2B	L1	7.15	0.13	L3	6.52	0.09	<0.01
	L1	7.15	0.13	Ad ♀	6.55	0.13	<0.05
	L1	7.15	0.13	Ad ♂	6.44	0.08	<0.01
	L2	7.05	0.16	L3	6.52	0.09	<0.05
	L2	7.05	0.16	Ad ♂	6.44	0.08	<0.05
ix	L1	6.85	0.11	L3	5.75	0.12	<0.00001
	L1	6.85	0.11	L4	6.02	0.03	<0.00001
	L1	6.85	0.11	L5 ♀	6.21	0.06	<0.001
	L1	6.85	0.11	L5 ♂	6.24	0.03	<0.01
	L1	6.85	0.11	Ad ♂	6.32	0.06	<0.05
	L2	6.69	0.09	L3	5.75	0.12	<0.00001
	L2	6.69	0.09	L4	6.02	0.03	<0.0001
	L2	6.69	0.09	L5 ♀	6.21	0.06	<0.05
	L3	5.75	0.12	L5 ♂	6.24	0.03	<0.01
	L3	5.75	0.12	L5 ♀	6.21	0.06	<0.05
	L3	5.75	0.12	Ad ♂	6.32	0.06	<0.001
	L3	5.75	0.12	Ad ♀	6.46	0.06	<0.00001
	L4	6.02	0.03	Ad ♀	6.46	0.06	<0.05
fru	L2	7.62	0.10	Ad ♀	7.15	0.06	<0.05
	L2	7.62	0.10	Ad ♂	7.09	0.09	<0.01
	L1	7.55	0.11	L3	7.17	0.07	<0.05
	L2	7.62	0.10	L3	7.17	0.07	<0.01

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