Distinct developmental mechanisms influence sexual dimorphisms in the milkweed bug *Oncopeltus fasciatus*

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Author contributions

- JJ and ML designed and conducted most experiments and contributed equally to data analysis, creation of draft figures, and writing of the main text.
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Abstract

Sexual dimorphism is a common feature of animals. Sex determination mechanisms vary widely among species and evolve rapidly. Until recently studies have found consistent mechanisms across the body of each individual determine female or male dimorphic body structures. In sexually dimorphic cells throughout the body of *Drosophila*, the relative dosage of autosomes and X chromosomes leads indirectly to alternatively spliced transcripts from the gene *doublesex*. The female Dsx isoform interacts with the mediator complex protein encoded by *intersex* to activate female development in flies. In males the transcription factor encoded by *fruitless* promotes male-specific behavior. In the milkweed bug *Oncopeltus fasciatus*, we find a requirement for different combinations of these genes during development of distinct dimorphic structures, within the same sex, suggesting a previously unappreciated level of diversity in sex determination. While *intersex* and *fruitless* are structurally conserved, *doublesex* has a history of duplication and divergence among Paraneoptera. Three *doublesex* paralogs in *O. fasciatus* produce multiple transcripts with sex- and tissue-specific expression. *intersex* and *fruitless* are expressed across the body, in females and males. RNA interference reveals only one *doublesex* paralog functions in somatic sex determination. Knockdown of *doublesex* and *fruitless* produces intersex phenotypic conditions in two sexually dimorphic structures: genitalia and abdominal sternites. In contrast, *intersex* is required for dimorphic development of female and male genitalia, but not for sternite dimorphism. These results reveal sex determination roles for *intersex* and *fruitless* distinct from their orthologs in other insects. Our results illuminate a novel form of developmental diversity in insect sex determination.

Significance Statement

Phenotypic differences between females and males, sexual dimorphisms, are common in animals. Sexual characters are controlled by a rapidly evolving developmental pathway. To explore this diversity we have examined somatic sex determination in the milkweed bug, *Oncopeltus fasciatus*. We show that (1) the key regulatory gene *doublesex* has undergone multiple duplications during insect evolution, (2) *doublesex* isoforms are expressed in sex- and tissue-specific patterns, and (3) *doublesex* and two other conserved genes, *intersex* and *fruitless*, are required for sex determination in females and males. However, different combinations of these genes are required for development of different dimorphic structures within the same sex. Our results illuminate a previously unappreciated form of developmental diversity in insect sex determination.

Introduction

Sexual dimorphisms are wide-spread among animals, but their development has been studied in only a small number of species. These genetic model organisms have revealed a surprising degree of diversity in sex determination mechansisms (Schütt and Nöthiger, 2000; Kopp 2012; Herpin and Schartl, 2015), despite the deep evolutionary conservation of sex itself. However, sexual dimorphisms across the body are typically assumed to share similar regulation. Even in insects, where somatic sex determination is cell autonomous, similar mechanisms are thought to control sex-specific differentiation in multiple tissues and organs (Arbeitman et al. 2004; Rideout et al. 2010; Robinett et al. 2010; Tanaka et al. 2011).

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. The best-studied model of insect sex determination is *Drosophila melanogaster*, where a system of female and male chromosomal determinants reside on the X chromosome and autosomes, respectively (Baker and Ridge 1980). Thus, the proportion of X chromosomes to autosomes determines sex. Sex-specific expression and splicing of downstream genes control female and male sexual differentiation, dosage compensation, and behavior (Burtis and Baker 1989; McKeown 1992; Kelley et al. 1995; Demir and Dickson 2005; Butler et al. 2018).

Developmental regulatory genes tend to have a high degree of epistasis and pleiotropy, which limit their evolutionary divergence over time due to stabilizing selection (Stern and Orgogozo, 2009). Sex determination pathways provide an intriguing exception to this pattern, with substantial variation, even among Holometabola (Schütt and Nöthiger, 2000; Salz et al. 2011; Herpin and Schartl, 2015). *doublesex* (*dsx*) acts relatively downstream of a gene network, including *Sex-lethal* and *transformer* in *D. melanogaster* sex determination, where alternative splicing produces sex-specific transcription factor isoforms. This gene is notable for being conserved in its structure and function in other insects (Schütt and Nöthiger, 2000; Shukla and Nagaraju 2010; Geuverink & Beukeboom 2014; Herpin and Schartl, 2015). Alternative splicing of *dsx* is a consistent mechanism, which produces protein isoforms that orchestrate a cascade of sex-specific gene expression (Burtis and Baker 1989; Kopp 2012). The Dsx-ortholog encoded by *Dmrt1* is one of the few genes identified in insect sex determaintion that is also conserved in mammals. *Dmrt1* is required in mice for testes development and male fertility (Raymond et al. 2000; Matson et al. 2011).

Here, we report the study of somatic sex determination in the milkweed bug *Oncopeltus fasciatus* (Heteroptera: Lygaeidae), focusing on *doublesex* paralogs and two sex determination genes with divergent functions: *intersex* (*ix*) and *fruitless* (*fru*). The true bugs are a diverse and species-rich hemimetabolous order that diverged from holometabolous insects more than 370

million years ago (Misof et al. 2014). Milkweed bug females and males differ in the structure of the genitalia and in the curvature of the second abdominal sternite (Fig 1). We find that *doublesex* paralogs diversified at the base of Paraneoptera, but three paralogs are maintained in Heteroptera, with only one required for somatic sexual dimorphisms in *O. fasciatus.* Previous research has shown that while *ix* is only required for somatic sexual dimorphism in female *D. melanogaster*, it is required in females and males of *O. fasciatus* (Garrett-engele et al., 2002; Aspiras et al. 2011). Surprisingly, we also find a major role for *fru* in development of both female and male sexually dimorphic structures. These findings indicate that the sex determination pathway of milkweed bugs has diverged significantly from that of other insects.

Results

doublesex paralogs in Paraneoptera

To examine the determination of somatic sexual dimorphisms in *O. fasciatus*, we cloned several candidate genes based on genetic models from *D. melanogaster* (Arbeitman et al. 2004), including *dsx*, *ix* and *fru*. *dsx* is a key regulator of sex determination in many holometabolous insects (Garrett-engele et al., 2002; Herpin and Schartl, 2015; Schütt and Nöthiger, 2000; Geuverink and Beukeboom 2014). *ix* is required for genitalia development in both sexes in *O. fasciatus* while only in females in *D. melanogaster* (Aspiras et al. 2011, Garrett-engele et al., 2002). In *D. melanogaster,* the transcription factor encoded by *fruitless* (*fru*) is required for sexually dimorphic brain development, necessary for male courtship behavior (Hall 1978; Demir and Dickson 2005; Ryner et al., 1996). Single copies of *ix* and *fru* were isolated from *O. fasciatus* (Fig 2A). Transcriptome and genome sequences also supported single instances of these genes. However, three genomic loci have sequence similarity to *D. melanogaster dsx* (Fig 2A). We used phylogenetic inference to verify orthology and to explore the relationships of these genes (Fig S1-S3). We found single orthologs of *ix* and *fru* in most available insect genomes, and phylogenies based on their aligned amino acid sequences mostly reflect relationships among insect taxa (Fig S1-S2).

In contrast, the Dsx phylogeny does not recapitulate most taxonomic relationships. Single *dsx* orthologs were recovered from holometabolous insect genomes, but multiple sequences with similarity to *D. melanogaster dsx* were found in the genomes or transcriptomes of most Paraneoptera. These sequences clustered by paralog-group, and topologies within groups reproduced some higher-level taxonomic relationships (Fig S3). We refer to the clade containing holometabolous *doublesex* sequences as the *dsx* clade, and the *O. fasciatus* sequence it contains as *dsx*. We dub the sibling clades *doublesex-like-a* (*dxxa*) and *doublesex-like-b* (*dxxb*). The *dsx* and *dxxb* clades are supported in more than 80% of bootstrap replicates. Throughout this article, we will use "*doublesex*" to refer to the broader group of genes with homology to *D.*

melanogaster doublesex, reserving "*dsx*" to refer to genes in the clade that excludes the *dsx*-like paralogs of Paraneoptera.

Tissue- and sex-specific transcript expression

To effect sexually dimorphic development, genes are often alternatively spliced. Using rapid amplification of cDNA ends (RACE) we isolated transcripts for all genes (Fig 2B). We supplemented this survey by searching available transcriptome data, but this approach did not identify any additional transcripts. Five alternative transcripts were found for *O. fasciatus dsx*, and three each for *dxxa* and *dxxb*. All transcript isoforms from each *doublesex* paralog included a DNA-binding domain in the 5' exon. A DMRTA domain was present in the downstream exons of some transcript isoforms of each paralog.

We designed exon-specific primers to examine the expression of these transcripts using qRT-PCR (Table S1). Expression was measured in tissues isolated from teneral adults. Expression of the three *doublesex* paralogs, measured from exons containing the DNA-binding domain, showed significant correlations with one another (Spearman's rank correlation, $rho > 0.6$, $p < 10^{-5}$). Expression of *doublesex* paralog transcripts was generally low compared to other genes, and showed considerable variation by sex, tissue and exon (Fig 2C). While our primer sets do not allow unambiguous identification of transcripts in all instances, some determinations about transcript-specific expression can be made from these data.

Expression of *dsx* was generally higher in females (Fig 2C). Transcript isoform D, which contains exon 6, was the most abundant isoform, with particularly strong expression in the ovaries and ovipositor. We also detected this transcript in the male genital capsule, albeit at lower levels. Isoforms of *dsx* containing the conserved DMRTA domain were not detected in testes, but were expressed at low levels in the ovaries. *dxxa* was not detected in any male tissues, but showed relatively consistent, low expression in female tissues, excluding the ovipositor. We failed to detect *dxxa* exon 2, suggesting that all detectable expression in the sampled tissues reflects expression of transcript isoform B. Expression of *dxxb* was high in the gonads of both sexes, but low or undetectable in other tissues. This expression was greater in testes than in ovaries. We did not detect any expression of *dxxb* exon 4a in females, suggesting male specificity in transcript isoform B.

We also isolated transcripts from *ix* and *fru* using RACE. In contrast to *O. fasciatus doublesex* paralogs, *ix* and *fru* appeared to be expressed strongly and more uniformly across most of the sampled tissues. In *D. melanogaster ix* is required for female somatic development and for sex-specific behavior in females and males (Nagoshi et al. 1988). The gene is transcribed in most tissues of both females and males from prepupal to adult stages (Gelbart and Emmert 2013).

High levels of *ix* transcripts were detected in all sampled tissues in both sexes of *O. fasciatus* (Fig 2C). Expression was highest in the female ovipositor.

fruitless is expressed much more strongly in male than female *D. melanogaster*, particularly in older males (Gelbart and Emmert 2013) where it is required for courtship behavior, fertility, and minor anatomical features specific to males (Hall 1978; Hall 1994; Usui-Aoki et al. 2000). Expression of *fru* in *O. fasciatus* was detected in all tissues, except for the female ovipositor. Expression was strongest in the testes. Interestingly, *fru* expression was comparable and moderate in the heads of females and males (Fig 2C).

These patterns of expression for *ix* and *fru* in the milkweed bug are notably less sexually dimorphic than their orthologs in the fruit fly. Excluding the ovipositor, *fru* and *ix* show a strong correlation in expression ($rho = 0.39$, $p = 0.00819$). We tested for gene interactions by measuring gene expression in an RNA interference (RNAi) background. Expression of *fru* was significantly reduced in the bodies of *ix* RNAi males, compared to control males (Fig S5; Wilcoxon RST, $p = 0.019$). This implies that *ix* promotes *fru* expression, although this effect may be indirect. The absence of *fru* expression in the ovipositor, where *ix* is highly expressed, suggests the action of an unidentified suppressor in that context.

Development of female and male genitalia requires *ix*, *fru* and *dsx*

To investigate the developmental functions of *ix*, *fru*, and the *doublesex* paralogs in *O. fasciatus*, we performed juvenile-stage RNAi targeting each gene, as well as a simultaneous knockdown of all *doublesex* paralogs (Tables S2, S3). We then examined sexually dimorphic structures in the resulting adults to evaluate loss-of-function phenotypes (Figs. 3-5).

Among *doublesex* paralogs, knockdown of *dxxa* and *dxxb* failed to produce any discernible phenotype. Genitalia length and sternite curvature were not significantly altered in any RNAi treatments targeting these genes (Figs. 4-5). However, *dsx* knockdown significantly affected development of sexually dimorphic structures. Only dsRNAs targeting the exon containing the DNA-binding domain resulted in phenotypic changes. This exon is present in all transcript isoforms produced from the *dsx* locus. Female *dsx* RNAi specimens developed ovipositors that looked similar to the wildtype in overall shape, but were significantly shorter (Fig 3R-S; 4A). Male claspers were also dramatically shorter or absent entirely in many specimens (Fig 3T; 4B). Male *dsx* RNAi individuals often died while molting to adulthood, when the genital capsule did not complete molting.

We also tested combined knockdown of all *doublesex* paralogs, by co-injection of three dsRNAs and by injection of a single dsRNA containing sequences from the DNA-binding domain of each gene. The phenotypes of these treatments were similar to that of *dsx* RNAi alone. Lengths of ovipositors and claspers were significantly reduced in simultaneous knockdown of *dsx*, *dxxa* and

dxxb, compared to controls. The magnitude of this effect was comparable to the *dsx* RNAi treatment (Fig 4).

In *D. melanogaster* the female isoform of Dsx interacts with the protein encoded by *ix* to activate female-specific target gene expression (Garrett-Engele et al. 2002). Therefore, we tested *ix* function during *O. fasciatus* development. Knockdown of *ix* by RNAi produced intersex phenotypes (Fig 3F-J), although it was still possible to identify bugs unambiguously as female and male based on the number of terminal appendages and the presence of a genital capsule in presumptive males. Length was significantly reduced in ovipositors and claspers of *ix* RNAi specimens (Fig 4). Distally the ovipositor developed with heavy sclerotization, suggesting partial transformation toward a clasper-like identity (Fig 3H-I). In presumptive males, the genital capsule and claspers were dramatically reduced in size (Fig 3J).

The transcription factor encoded by *fru* is conserved among insects (Fig S2). In *D. melanogaster* *fru* is required for male-specific behaviors, but plays a relatively minor role in anatomical development (Ryner et al. 1996). Surprisingly, knockdown of *fru* in *O. fasciatus* caused dramatic defects in sexually dimorphic adult structures (Fig 3K-O), similar in many ways to the effects of *dsx* and *ix* knockdowns. *fru* RNAi significantly reduced the length of the ovipositor and claspers (Fig 4). While the ovipositors of *ix* RNAi specimens decreased in length, they also increased in thickness and red pigmentation suggestive of clasper identity. In contrast, the ovipositors of *fru* RNAi specimens did not appear transformed, and retained a membranous structure (compare Fig 3H-I to Fig 3M-N). Some specimens also showed necrosis of the distal valvulae (Fig 3N). In *fru* RNAi males, claspers were reduced in size and often misshapen, lacking their characteristic arch (Fig 3O).

Development of female sternite shape requires *fru*

One of the most obvious sexual dimorphisms in milkweed bugs is the posterior projection of the second abdominal sternite (Fig 1A). We examined sternite curvature in the same adult bugs produced through RNAi. Sternite curvature was quantified based on nine landmarks placed on the sternite edge (Fig 1A-B). After Procrustes alignment of the coordinate positions, these landmarks were regressed to a line. The square-root of the mean of squared residuals (residual mean standard deviation) was used as a metric of curvature. (See Materials and Methods for more details.)

Sternite curvature is not a binary trait in milkweed bugs. Females and males display a continuous range of curvature (Fig 5). Nevertheless, among dsRNA control specimens, female and male sternites showed significantly different mean curvatures (Wilcoxon rank sum test, $W = 2132$, $p < 2.2\times10^{-16}$). Surprisingly, RNAi targeting *ix* did not significantly affect the sternite curvature. Even specimens with dramatic intersex genitalia phenotypes still displayed sternite curvatures typical of their presumptive sex. In contrast, knockdown of *fru* reduced the curvature of sternites

in females ($W = 589$, $p = 0.0256$). While some *fru* RNAi females had sternites with a typically male shape, most developed with an intermediate curvature. Male *fru* RNAi specimens were not different from dsRNA control specimens ($W = 473$, $p = 0.595$).

dsx is required in females and males for sex-specific sternite shapes

Male milkweed bugs retain a juvenile sternite shape, while the female sternite projection arises during the last two molts before adulthood. Therefore, we predicted that inhibiting genes required for the development of sexual dimorphisms might cause presumptive females to also retain the juvenile sternite shape. This effect was produced by *fru* RNAi. In contrast, *dsx* RNAi produced sternite shape changes in female and in male bugs (Fig 5). Sternite curvature in *dsx* RNAi specimens was significantly reduced compared to that of dsRNA controls for females ($W = 2689$, $p = 0.0256$) and increased for males ($W = 446$, $p = 6.70\times10^{-7}$). A similar effect of less magnitude was also produced by the simultaneous knockdown of all three *doublesex* paralogs (females: $W = 1596$, $p = 0.0787$; males: $W = 663$, $p = 7.44\times10^{-3}$). In both sexes, *dsx* RNAi caused the mean sternite shape to become more intermediate (Fig 5).

Discussion

Sexual dimorphisms use distinct mechanisms in milkweed bugs

Our results suggest structure-specific functions for *ix*, *fru* and *dsx* in *O. fasciatus*. All three genes are required for the development of genitalia (Fig 3-4). However, sternite curvature was not affected by knockdown of *ix*. Moreover, *fru* RNAi only affected the development of female, but not male, sternite curvature (Fig 5). Because male sternite curvature closely resembles the phenotype of juveniles, it is possible that *fru* is involved in regulating growth and proliferation of these cells, rather than in directly determining sexually dimorphic identity. However, this developmental role contrasts with *dsx*. RNAi knockdown of *dsx* produced more female-like curvature in males and more male-like curvature in females (Fig 5), suggesting that *dsx* acts in each sex to inhibit intersex phenotypes. In effect, *dsx* makes the distribution of sternite curvature more bimodal.

Therefore, while *dsx* activity is required in all sexually dimorphic structures, other components of the sex determination pathway are required in more limited developmental contexts. In *Drosophila* females, the female-specific isoform of Dsx interacts directly with Ix to activate female-specific target genes (Garrett-Engele et al. 2002). It is possible that only some Dsx isoforms in *O. fasciatus* interact with Ix, in a structure-specific context, rather than in a sex-specific context. Isoform D of *O. fasciatus dsx* is a strong candidate for this interaction, because of its strong expression in both female and male genitalia, but lower expression in other

somatic body regions (Fig 2). Confirmation of this hypothesis must await the development of isoform-specific antibodies from milkweed bug Dsx.

Additionally, it is possible that the expression of distinct *dsx* isoforms is regulated by tissue-specific enhancers. A recent study of regulatory sequences in *D. melanogaster* found that expression of *dsx* in distinct sexually dimorphic structures is controlled by separate enhancers (Rice et al. 2019). Similarly, analysis of gene expression in different larval and pupal tissues of the butterfly *Papilio polytes* has suggested distinct tissue-specific targets of *dsx* activity (Deshmukh et al. 2020). These findings support the concept that some aspects of sex determination in insects may be structure-specific. In fact, it is possible that this form of regulation may be an evolutionary intermediate. If a gene has multiple enhancers for expression in different areas, as exemplified by *D. melanogaster dsx*, then a mutation eliminating one enhancer would produce the kind of mosaic genetic requirement seen for *O. fasciatus ix*.

Evolution of *doublesex* in Paraneoptera

The presence of multiple *doublesex* paralogs suggests an ancient gene radiation early in the diversification of Paraneoptera. The genomes of early-branching insect lineages appear to contain only one *doublesex* gene, which all clustered with the clade that includes *D. melanogaster dsx* (Fig S3). This result is consistent with two duplication events in the lineage leading to Heteroptera. Paraneopteran *dsx* sequences had relatively long branches, suggesting that they underwent greater diversification after the duplications that produced the *dxxa* and *dxxb* paralog groups. The absence of obvious external phenotypes for *dxxa* or *dxxb* RNAi suggests that these genes have diverged from *dsx* in their function. Four *doublesex* paralogs have been identified in the brown planthopper *Nilaparvata lugens* (Hemiptera: Delphacidae). However, like *O. fasciatus*, only one *N. lugens* paralog, nested in the *doublesex* paralog group (Fig S3), has a functional requirement during somatic sex determination (Zhuo et al. 2018). Nevertheless, the persistence of the *doublesex*-like paralogs over more than 290 million years (Misof et al. 2014) suggests a critical function. We found *dxxa* expression exclusively in female *O. fasciatus*, while *dxxb* was highly expressed in testes (Fig 2C). These patterns may suggest sex-specific roles for these genes in development of the gonads or germline. We examined the gonads of RNAi specimens but found no obvious phenotypic differences at the anatomical level (Fig S6). Future studies will be necessary to test whether *dxxa* or *dxxb* have roles at the cellular or molecular levels.

Evolution of sex determination mechanisms in insects

Sex determination pathways evolve rapidly and differ substantially among animals (Herpin and Schartl, 2015; Kopp, 2012; Schütt and Nöthiger, 2000). Additionally, changes in alternative splicing and the interactions of genes, as well as their expression patterns, are mechanisms by

which these pathways evolve (Baker, 1998; Salz, 2011; Shukla and Nagaraju 2010). The results we present here further illuminate the rapid evolution of sex determination genes and offer insight into the sex determination mechanisms in hemimetabolous insects.

Compared to other genes in insects involved in sexually dimorphic development, such as *ix* and *fru, doublesex* orthologs evolve rapidly in their protein sequence and have undergone multiple duplications in different lineages (Fig S3). This could suggest that *dsx* occupies a space in the developmental network where mutations are more readily tolerated. Other genes, including *ix* and *fru,* may be more limited in the evolution of their protein sequences by stabilizing selection, perhaps due to greater pleiotropy. However, differences in the functions of these genes among insect species suggest that regulatory changes still occur without comparable constraints.

The functions of *ix* and *fru* in *O. fasciatus* are different from those reported in other insects. In *D. melanogaster, ix* is only required in females for genitalia development while both female and male milkweed bugs require *ix* for genitalia development (Aspiras et al, 2011; Fig 3-4). *ix* RNAi knockdown in *N. lugens* produced genitalia defects in females, but not in males (Zhang et al. 2021). *fru* is a transcription factor required for development of male-specific neurons in *D. melanogaster* (Hall 1994; Ryner et al. 1996; Demir and Dickson 2005). In *O. fasciatus*, we find a more expansive requirement for *fru* in female and male genitalia development (Fig 3-4) and in the development of female sternite curvature (Fig 5). To the best of our knowledge, such a prominent function for *fru* outside the nervous system has not been previously reported. Further studies of *fru* outside drosophilids are needed to better understand its functional diversity across the insects and to determine when during drosophilid evolution it became restricted to the nervous system. Overall, these differences in the functions of *ix* and *fru* suggest that somatic sex determination may have a much greater diversity of mechanisms among insects than is currently is understood.

In insects such as the fruit fly, *dsx* functions as a critical downstream regulator of somatic sex determination (Arbeitman et al. 2004; Kopp 2012). Our results are not consistent with this role for *dsx* in *O. fasciatus*. We did not detect sex-specific transcript isoforms of *dsx*, although expression was female-biased. Similarly, in the sweet potato whitefly, *Bemisia tabaci*, a single *dsx* ortholog also has sex-biased expression (Guo et al. 2018). Although in that member of the Sternorrhyncha, *dsx* is more strongly expressed in males and RNAi knockdown revealed a requirement in male genitalia development, and only a role in vitellogenin regulation in females. The expression of *dxxa* in *O. fasciatus* was limited to females and high in ovaries, while expression of *dxxb* was high in testes. Similarly, *dsx* isoforms containing the DMRTA domain were only detected in ovaries, not testes. While gonads of RNAi specimens did not display anatomical defects (Fig S6), these expression patterns suggest that duplication and sex-specific divergence of these genes and transcripts may play a role in sex-specific development of the germline in *O. fasciatus*. Knockdowns of *dsx* or all three *doublesex* paralogs during juvenile

development do not completely eliminate adult sexual dimorphism. Rather, *dsx*, together with *ix* and *fru*, appears to help facilitate tissue-specific development of sexual dimorphisms, including the development of female and male genitalia and the inhibition of intersex sternite phenotypes in both sexes. Nevertheless, it is likely that unidentified genes play a more proximate role in milkweed bug sex determination.

Conclusions

Based on our results, we propose the following model for the development of adult sexual dimorphisms in *O. fasciatus* (Fig 6). *fru* promotes the acquisition of female sternite curvature from the juvenile sternite shape present through the fourth instar. *dsx* maintains the sternite extension in females while preventing it in males. Thus, it prevents both sexes from entering intersex morphospace. Both sexes require *ix*, *fru* and *dsx* to promote growth of the genitalia. Unidentified factors must also be required to specify female and male sexual traits. Collectively, these results show that genes may act to control the development of somatic sexual dimorphisms by acting in tissue-specific as well as sex-specific contexts. The sex determination cascades of other insects should be investigated in more detail to expand our understanding of the evolution and diversity of sex determination mechanisms. Such studies will broaden our perspective on developmental pathway evolution and help us better understand how genetic networks bias evolutionary outcomes.

Materials and Methods

Insect culture

Oncopeltus fasciatus were obtained from Carolina Biological Supply Company and maintained as described by Liu and Kaufman (2009). Milkweed bug cultures were kept in terraria at room temperature and fed on sunflower seeds. Loose cotton balls provided an egg-laying substrate. Water was provided in flasks with paper towel wicks.

Sequence orthology

An ortholog of *ix* from *O. fasciatus*, GenBank accession AEM16993, was identified as part of a previous study (Aspiras et al. 2011). Sequences with similarity to *fru* and *dsx* were obtained from *O. fasciatus* using degenerate PCR and RACE and by PCR with exact primers based on the available genome and gene set annotation (Ewen-Campen et al. 2011; Panfilio et al. 2019). Initial identification was based on tBLASTn search using sequences from *D. melanogaster*. One genomic locus had high similarity to *fru*, while three genomic loci closely resembled *dsx*.

Orthology was confirmed using phylogenetic inference. Additional orthologs from insects were obtained from GenBank using BLAST. Alignments of amino acid sequences were performed by ClustalW in Geneious Prime (version 2019.2.3) using a gap cost of 5 and an extension cost of 0.05 with free end gaps. Alignment of *ix* and *fru* included all amino acids. Alignment of *doublesex* orthologs was restricted to exons containing the conserved DNA-binding domain. Phylogenies were inferred using RAxML version 8.2.11 on a multiprocessor computing cluster. Mutation rates were modeled using the BLOSUM62 matrix with a gamma distribution. The program was called as "`raxml-mpi -d -f a -x 123 -p 123 -# autoMRE -m PROTCATBLOSUM62 -s X.phy`" where `X` stands in for the alignment name. Trees were rooted with sequences from other arthropod classes for *ix*, collembola for *fru*, and rooted at the midpoint for *doublesex*. Node supports were determined by bootstrapping.

Isolation of mRNA isoforms

Alternative splicing of mRNAs was examined using Rapid Amplification of cDNA Ends (RACE). Initial cloning of candidate genes was done via degenerate PCR with primers designed to conserved protein domains, or with exact primers designed from publicly available transcriptome data. To obtain alternative mRNA sequences, total RNA was extracted from a mix of late fifth instars and teneral adults seperated by sex, using the PureLink RNA Mini Kit (ThermoFisher). First strand cDNA synthesis was done using the GeneRacer Kit with AMV reverse transcriptase (Invitrogen). This procedure ligates a specific DNA adapter sequence to the 5' or 3' ends of cDNAs, which are then used in PCR with gene-specific and adapter-specific primers. Nested PCR was used to enrich the template pool for the target sequences. From multiple independent PCRs, 120 products were isolated using gel excision and cloned using the Topo-TA cloning kit (Invitrogen). Sanger sequencing and alignment confirmed 51 of these clones had continuity with potential *doublesex* paralogs. These were determined to represent 3 genomic loci with a total of 11 splicing isoforms (Fig 2A-B). Sequences from *O. fasciatus fru* and *doublesex* transcripts were submitted to GenBank (accession numbers TBD).

All *doublesex* transcripts included 5' exons containing the conserved DNA-binding domain. 5' RACE using gene-specific primers in more downstream exons failed to isolate other transcript variants. BLAST searches of available *O. fasciatus* transcriptomes (Ewen-Campen et al. 2011; Panfilio et al. 2019) using downstream exons as a query also failed to identify additional transcripts.

Gene expression

Quantitative real-time PCR (qRT-PCR) was used to measure sex- and tissue-specific expression, to validate RNAi knockdowns, and to investigate gene interactions. Specific probe and primer sets were created for multiple exons in each *doublesex* paralog. Dual-labeled probes

(SigmaAldrich) allowed reaction multiplexing to conserve the template. Primers and probes were designed using Primer3 in Geneious Prime and are listed in Supplemental Table 1.

Milkweed bugs were collected without agitation and placed at -80 °C for fifteen minutes. For tissue-specific assays, specimens were dissected on ice and immediately proceeded into RNA isolation. RNA was extracted following the Maxwell 16 LEV Tissue RNA Kit protocol (Promega). For tissue-specific assays, RNAi validation and studies of gene interactions, expression was measured in templates prepared from teneral adults. Between five and seven biological replicates of each sex-by-tissue combination were measured independently. RNA was stored at -80 °C. First-strand cDNA was generated from 1µg total RNA using the iScript cDNA kit (BioRad) with a poly-T primer as per kit instructions. qRT-PCR was carried out on a CFX96 Touch qPCR System (bioRad) using iQ Supermix (BioRad). Expression was calculated from the mean of technical triplicate reactions. Each plate included four DNA standards diluted from known concentrations of synthesized gene fragments (IDT gBlocks) with the addition of nonspecific salmon sperm DNA (ThermoFisher). All plates included control reactions with water in place of the cDNA template (no-template controls) and with total RNA in place of cDNA (no-RT controls). Whenever primer sets were used on multiple plates, a minimum of two templates (in addition to concentration standards) were included on those plates to allow inter-plate calibration. *Elongation Factor 1-α* (*EF1-α*) was used as an endogenous reference gene due to its fairly consistent expression throughout development (Su et al. 2011).

We expected that in some tissues, certain transcripts would not be expressed. Therefore, we developed a method for calculating normalized gene expression that allows zero values to be determined in comparison to negative controls from the same plate. Typically, normalized target gene expression is determined by subtracting the mean C_q for a reference gene from the mean C_q of the gene of interest. In such cases, zero has no special meaning. Instead, we coded all measurements of gene expression as zero if they were within 0.5 cycles of any template controls on the same plate, and maintained that value throughout analyses. Non-zero values were normalized as usual to the expression of *EF1-α*. Next, expression values were scaled, so that the mean of non-zero values was standardized to 1. Finally, variance was scaled to maintain the relative distance from zero of the global non-zero minimum value prior to standardization.

RNA interference

Loss-of-function phenotypes from RNA interference (RNAi) were used to investigate gene function as previously described (Aspiras et al. 2011). Knockdown was verified by qRT-PCR. Double-stranded RNA (dsRNA) encoding *Ampicillin Resistance* (*AmpR*) was used as a control for dsRNA toxicity and injection mortality. This sequence was chosen because it is present on the pCR-Topo4 plasmid vector used in cloning of gene fragments.

A synthetic gene fragment (IDT gBlock) or cloned gene fragment was used to create a PCR template for the synthesis of dsRNA. PCR primers were designed with an approximately 20-bp exact match to the target sequence, avoiding sequences that also occur in paralogs (Table S2). A 5' T7 polymerase promoter sequence was added to both primers. Following PCR, dsRNA was transcribed using the T7 MegaScript Transcription Kit (ThermoFisher). Template DNA was removed by treatment with DNase I. Slow cooling to room temperature allowed dsRNA annealing. Samples were cleaned by precipitation with cold ethanol and ammonium acetate. The product was resuspended in nuclease-free water and stored at -20 °C. Double-stranded RNA structure was confirmed by a coherent band on an agarose electrophoretic gel. dsRNA concentrations were measured using a nanoscale spectrophotometer. dsRNA solutions were diluted in saline buffer (0.01 mM $NaPO_4$, 5 mM KCl, green food coloring) and stored at -20 °C.

RNAi was tested at several dsRNA concentrations (Table S3). Fourth instar *O. fasciatus* are not yet sexually dimorphic. Bugs at this stage were anesthetized using a CO_2 pad and injected with dsRNA solutions using a borosilicate glass microcapillary, pulled on a Sutter Instruments pipette puller into an injection needle. Approximately 0.5 µl of dsRNA solution was injected into the left side of the abdomen of each bug.

The effectiveness of RNAi was validated using qRT-PCR. Gene expression was measured in control (*AmpR* dsRNA) specimens and RNAi treatments targeting the genes of interest (Fig S4). Primers used for validation PCRs did not overlap with the dsRNA sequences. RNAi validation of *dxxa* RNAi was not possible, because expression of *dxxa* could not be detected in the whole bodies of 28 *AmpR* or 16 *dxxa* RNAi specimens examined. While *dxxa* expression was detected in specific body regions of unmanipulated bugs (Fig 2C), the overall expression of *dxxa* appears to be very low.

Morphometry

We examined two somatic sexual dimorphisms, genitalia length and sternite curvature (Fig 1). Specimens were imaged on a VWR VistaVision dissecting microscope with a Moticam 5 digital camera. Whenever possible, a ruler was included in the image. Otherwise, the magnification was calibrated to enable conversion of pixel to metric distances. The ImageJ (Schindelin et al. 2012) line-tool was used to measure the length of genitalia and the femur, which was used to normalize for body size. In females, the absolute length of the first valvulae of the ovipositor was measured from the point of emergence to the distal tip (Fig 1C). For males, the segmented line-tool was used to measure absolute clasper length from the proximal joint to the tip (Fig 1D). Using the multi-point tool, nine points were placed across the posterior edge of the second abdominal sternite. Raw pixel values were converted to metric distances.

To quantify sternite curvature, landmark coordinate positions from each specimen were marked in ImageJ and stored in the TPS format (Rohlf 2015). Coordinates were then aligned using

generalized Procrustes alignment, as implemented in the R package geomorph (Adams et al. 2020). Landmarks at the left and right sides and on the anatomical midline were fixed, but others were treated as semilandmarks. Curvature was defined as the residual mean standard deviation (RMSD) from a linear regression of Procrustes-aligned coordinate values. Larger RMSD values indicated a higher amount of curvature in the sternite, the more characteristically female phenotype.

Ventral abdominal pigmentation in *O. fasciatus* does not reliably correlate with sex. Rearing temperature is a much stronger influence on pigmentation extent and pattern (Sharma et al. 2016). To the extent that pigmentation correlates with sex, the pattern is influenced by the size and shape of the ventral sternite, which is quantified. Due to the high natural variation in abdominal pigmentation within each sex, we chose to exclude it from our phenotypic analysis.

Statistical analyses

Gene expression and phenotypes produced through RNA interference are expected to produce skewed disruptions. Therefore, we used nonparametric statistical methods to analyze these data. We used the Kruskal-Wallis analysis of variance to detect overall effects. Post-hoc contrasts were done using Dunn's test, for pairwise comparisons (Dinno 2017), or the Wilcoxon rank sum test, for two-sample comparisons. We used one-sided tests for the comparison of sex-specific phenotypes. Where appropriate, correction for multiple tests was done using the FDR method. R version 4.0.3 was used for all statistical analyses and the ggplot2 package was used for plotting data. Figures were assembled in Adobe Illustrator.

Data availability

DNA sequences were archived in GenBank (accession numbers JJ999999-JJ999999). All other data were archived at Dryad (https://doi.org/10.5061/dryad.kwh70rz3q), including amino acid sequence alignments of candidate gene orthologs and resulting Newick tree files, tables of linear morphological measurements and 2D landmark coordinate positions for sternite shapes, gene expression data, and the R script used to analyze the sternite shape data.

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Figure Legends

Figure 1

Milkweed bugs have several sexually dimorphic features. (A-B) The second abdominal body segment (A2) of females has an exaggerated extension of the sternite at the ventral midline. Nine landmarks were placed along the ventral edge of the sternite to quantify curvature. (C) Appendages from the terminal segments of the female abdomen form an ovipositor. Ovipositor length was measured from the distal tip to the proximal joint. (D) The male abdomen ends in a large genital capsule, which contains the copulatory organ. Externally a pair of small claspers, visible from a posterior perspective, is used for mate guarding. Clasper length was measured from the proximal joint to the tip.

Figure 2

Structure and expression of candidate sex determination genes in *O. fasciatus*. (A) Orthologs of *intersex*, *fruitless*, and three *doublesex* paralogs were identified on different genomic scaffolds in the *O. fasciatus* genome. Boxes represent exons; angled lines represent introns. Introns were very long compared to neighboring gene predictions. Flags indicate the start of transcription. (B) Transcripts from *ix*, *fru* and *doublesex* paralogs. The first exon of each transcript is aligned at the left. Omitted exons are left empty. Alternative start or stop sites are

indicated by lowercase letters (e.g. 2a, 2b) Lighter shading indicates UTRs. (C) A heatmap showing relative transcript abundance from five tissues in each sex. Each panel represents the mean of 5 to 7 independent measurements. Global differences in means were tested using the Kruskal-Wallis rank sum test. Where differences were significant, Dunn's test of multiple comparisons using rank sums was applied post hoc. Lowercase letters indicate significant differences after correction for multiple tests ($\alpha = 0.05$).

Figure 3

Effects of RNA interference on sexual dimorphism. (**A-E**) Unmanipulated *O. fasciatus.* Ventral views of the female and male abdomen differ in curvature of the second sternite and in the terminalia. Abdomens are shown with anterior towards the top of the page (A,B). The ovipositor is a jointed structure formed by two nested appendages that end in membranous valvulae. Ovipositors are shown from ventral perspective, with anterior up (C), and from left lateral perspective, with dorsal up (D). The male genital capsule (E) is visible from a posterior view; dorsal is up. The posterior of the capsule ends in dorsally pointing claspers (white arrow), used by the male to hold onto females during and after mating. The abdominal panels share the same scale, while the ovipositor and clasper images share a separate scale. One millimeter scale bars are given for each group of panels. Strongly affected RNAi specimens are shown to illustrate phenotypes. (**F-J**) *ix* RNAi produces intersex phenotypes in females and males. The female ovipositor is reduced in length and heavily sclerotized, suggesting partial transformation toward a clasper-like identity. The male genital capsule and claspers (red arrow) are severely reduced. (**K-O**) *fru* RNAi also reduced the size of the ovipositor and claspers. Claspers are also misshapen (red arrow). (**P-T**) RNAi targeting *dsx* results in extreme reduction of terminal appendages in both sexes. Only a small nub of tissue represents the valvulae and claspers (red arrow) in severely affected specimens.

Figure 4

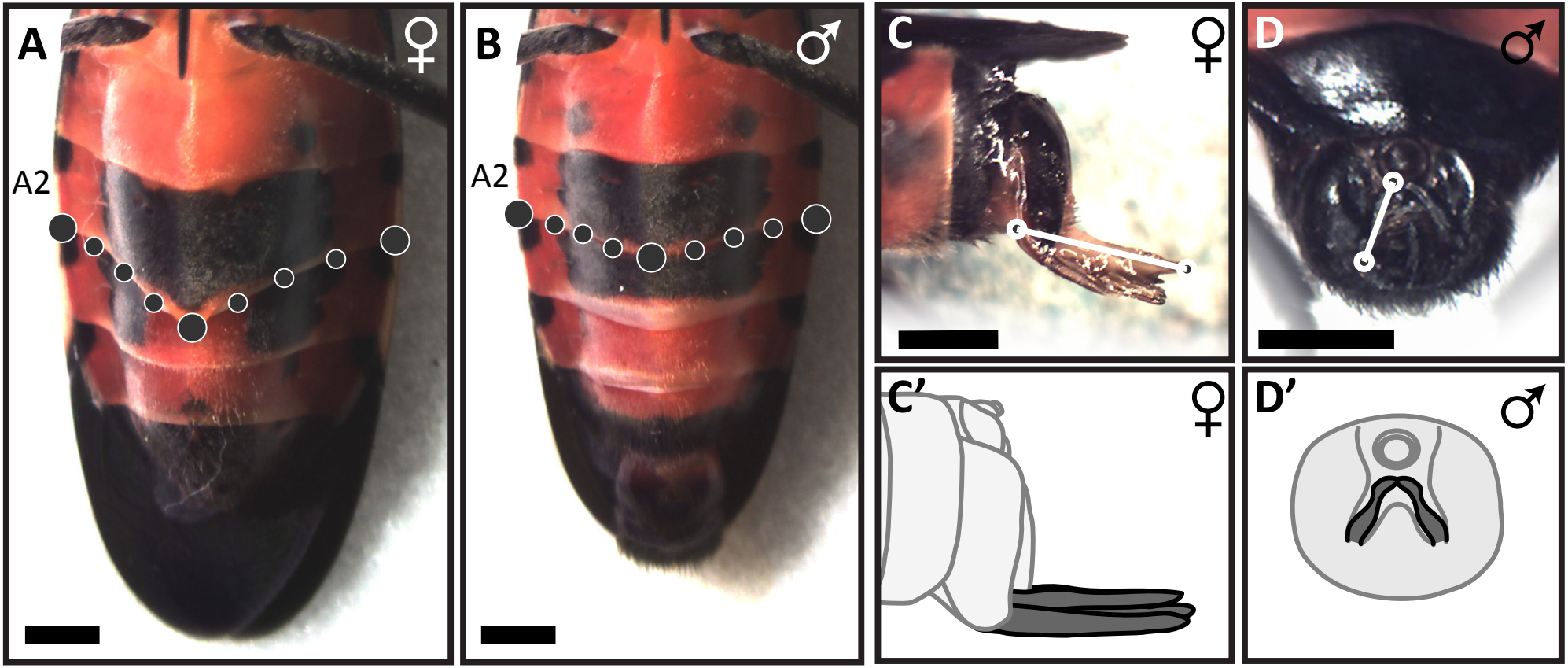
RNAi effects on genitalia length in females (A) and males (B). Dots represent individual normalized structure length. Gray outlines show the relative distribution of values. The mean of control (*AmpR* dsRNA) specimens is indicated by the horizontal line in each panel. Stars indicate significant difference from control values, based on one-sided Wilcoxon rank sum tests with FDR correction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5

RNAi effects on the curvature of the second abdominal sternite in females (A) and males (B). Dots represent individual specimen values. Gray outlines show the relative distribution of values. The means of control (*AmpR* dsRNA) females and males are indicated by the orange and purple horizontal lines, respectively, in each panel. Stars indicate significant difference from control values, based on one-sided Wilcoxon rank sum tests with FDR correction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 6

Proposed morphogenic trajectories of juvenile milkweed bugs. Genitalia development in females and males requires *ix*, *fru* and *dsx*. Development of female abdominal sternite curvature requires *fru*. Activity of *dsx* is also necessary to prevent an intersex sternite curvature in both sexes. Intersex morphospace is represented by space in between the final female and male positions.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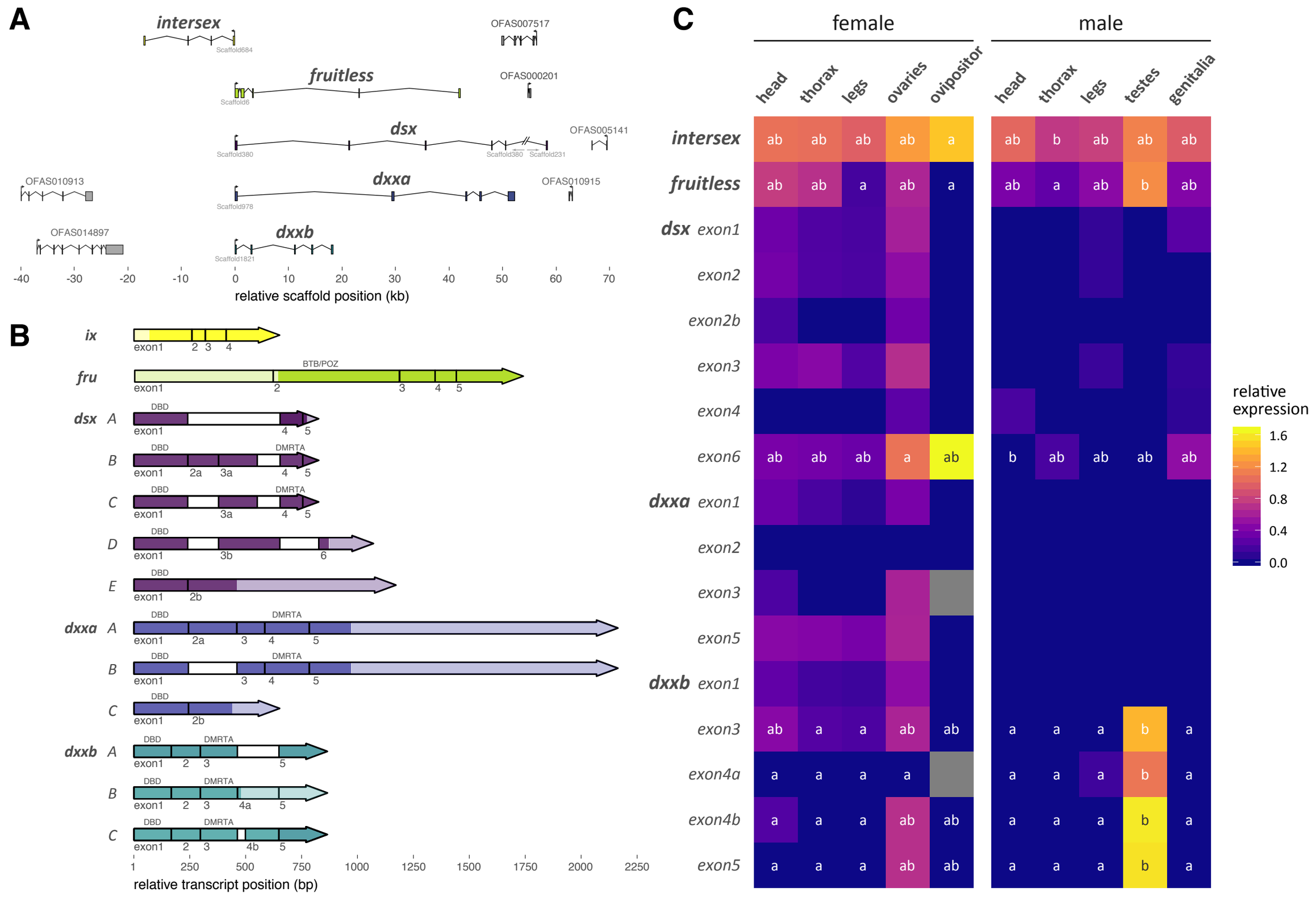
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fruitless ab ab a ab a ab a ab b ab
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exon6 ab ab ab a ab b ab ab ab ab
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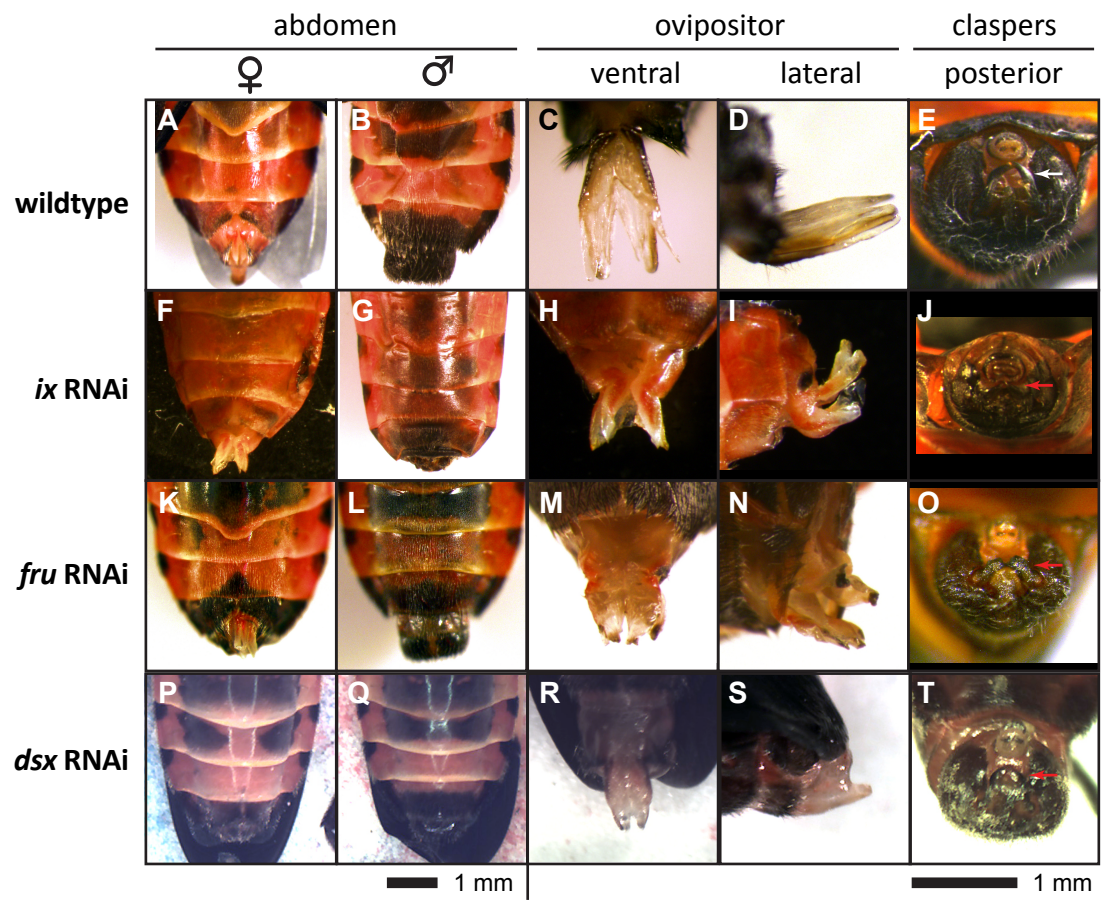
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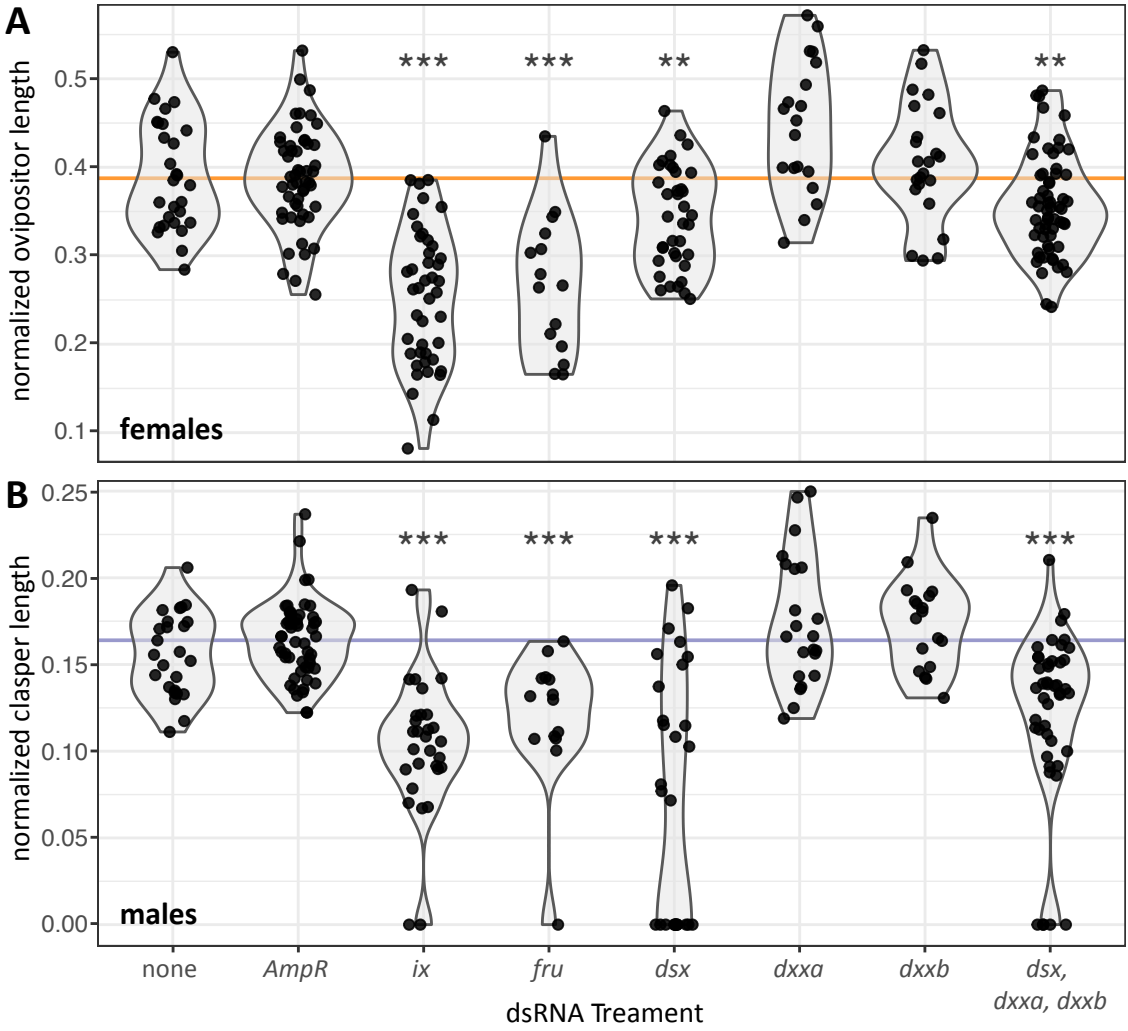
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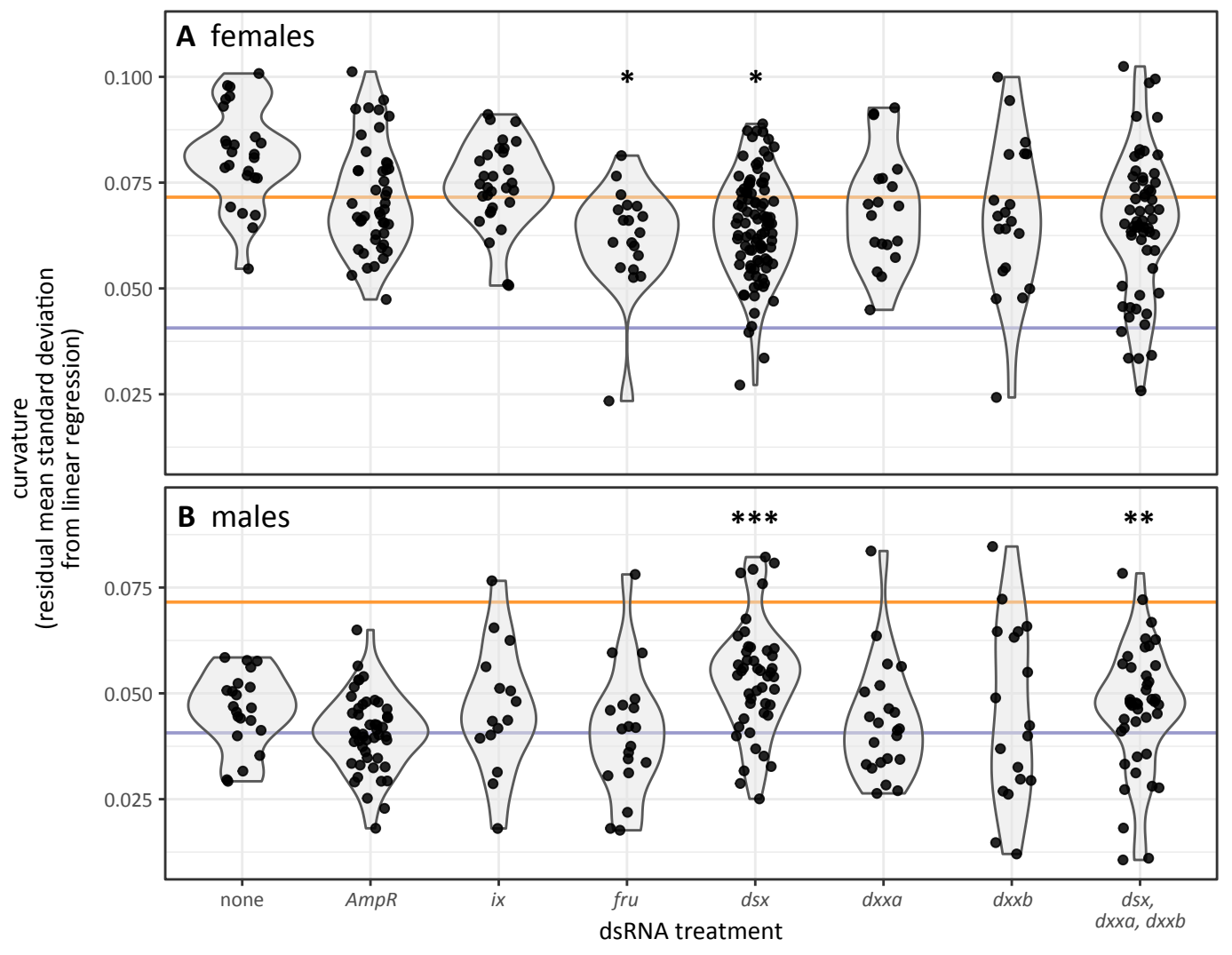
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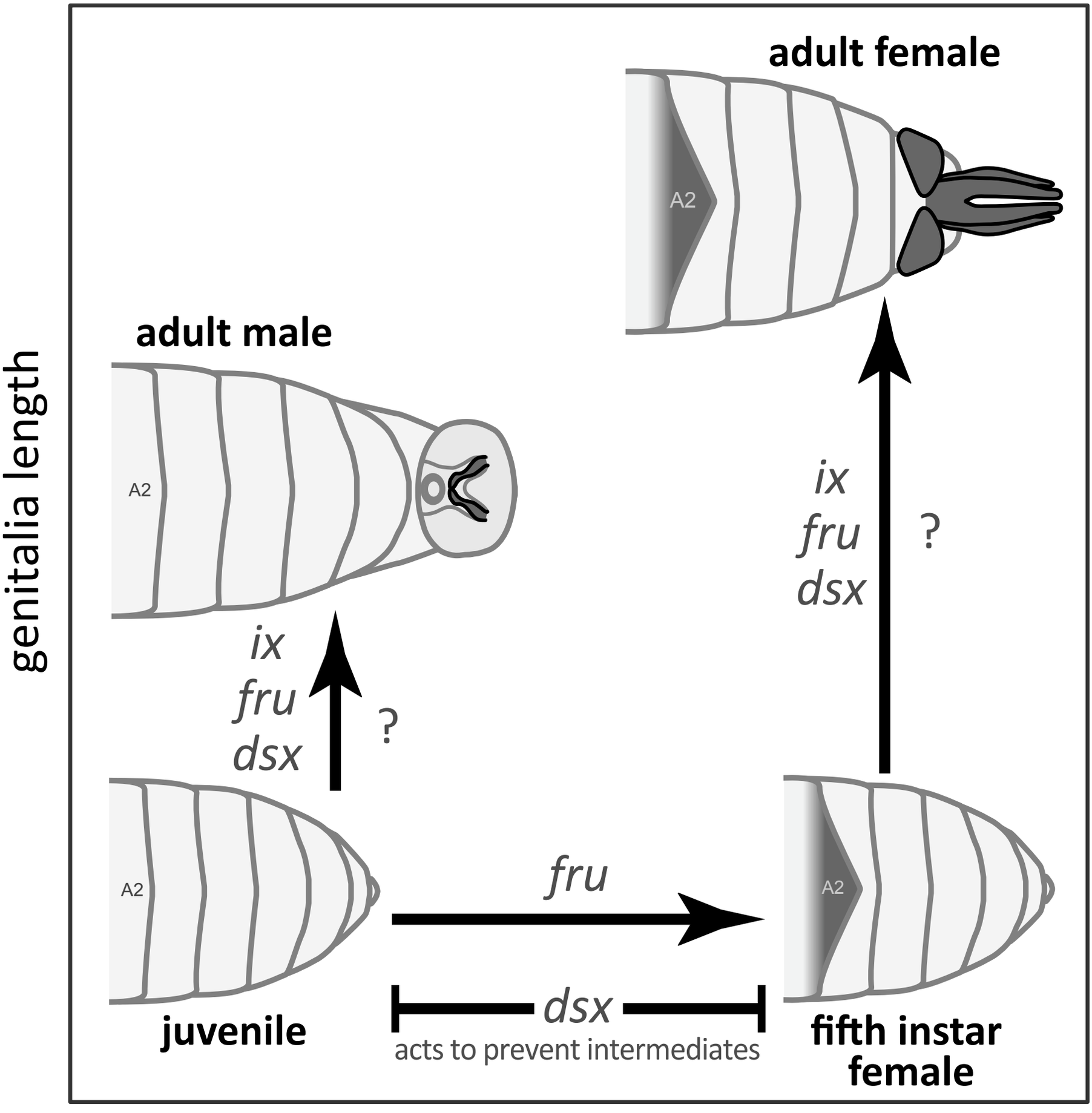
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