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.
- YJL, MY, and ZZ conducted portions of the experiments and contributed to data analysis.
- DRA designed and supervised all experiments, secured funding, prepared final figures, and contributed to data analysis and to the writing of the main text.

32 Abstract

33 Sexual dimorphism is a common feature of animals. Sex determination mechanisms vary widely
 34 and evolve rapidly. In sexually dimorphic cells throughout the body of *Drosophila*, the relative
 35 dosage of autosomes and X chromosomes leads indirectly to alternatively spliced transcripts
 36 from the gene *doublesex*. The female Dsx isoform interacts with the mediator complex protein
 37 encoded by *intersex* to activate female development in flies. In males the transcription factor
 38 encoded by *fruitless* promotes male-specific behavior. In the milkweed bug *Oncopeltus fasciatus*,
 39 we find a requirement for different combinations of these genes during development of distinct
 40 dimorphic structures, within the same sex, suggesting a previously unappreciated level of
 41 diversity in sex determination. While *intersex* and *fruitless* are structurally conserved, *doublesex*
 42 has a history of duplication and divergence among Paraneoptera. Three *doublesex* paralogs in *O.*
 43 *fasciatus* produce multiple transcripts with sex-specific and regional expression. *intersex* and
 44 *fruitless* are expressed across the body, in females and males. RNA interference reveals only one
 45 *doublesex* paralog functions in somatic sex determination. Knockdown of *doublesex* inhibits
 46 adult development of the genitalia and produces intersex phenotypes in two other sexually
 47 dimorphic structures: curvature of the fourth abdominal sternite and abdominal pigmentation.
 48 RNAi targeting *fruitless* and *intersex* also affect the genitalia of both sexes, but have affects
 49 limited to different sexually dimorphic structures in different sexes. These results reveal sex
 50 determination roles for *intersex* and *fruitless* distinct from their orthologs in other insects, and
 51 illuminate a novel form of developmental diversity in insect sex determination.

52 Introduction

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

61 Somatic sex determination requires an initial genetic signal, which varies greatly from species to
62 species. Proteins downstream of this signal then regulate transcription sex-specifically. The
63 best-studied model of insect sex determination is *Drosophila melanogaster*, where a system of
64 female and male chromosomal determinants reside on the X chromosome and autosomes,
65 respectively (Baker and Ridge 1980). Thus, the proportion of X chromosomes to autosomes
66 indirectly determines sex. Sex-specific expression and splicing of downstream genes control
67 female and male sexual differentiation, dosage compensation, and courtship behaviors (Burtis
68 and Baker 1989; McKeown 1992; Kelley et al. 1995; Demir and Dickson 2005; Butler et al.
69 2018).

70 Developmental regulatory genes tend to have a high degree of epistasis and pleiotropy, which
71 limit their evolutionary divergence over time due to stabilizing selection (Stern and Orgogozo,
72 2009). Sex determination pathways provide an intriguing exception to this pattern, with
73 substantial variation, even among Holometabola (Schütt and Nöthiger, 2000; Salz et al. 2011;
74 Herpin and Schartl, 2015). *doublesex* (*dsx*) acts relatively downstream of a gene network,
75 including *Sex-lethal* and *transformer* in *D. melanogaster* sex determination, where alternative
76 splicing produces sex-specific transcription factor isoforms resulting indirectly from X
77 chromosome dosage (Erickson and Quintero 2007). *Dsx* is notable for being conserved in its
78 structure and function in other insects (Schütt and Nöthiger, 2000; Shukla and Nagaraju 2010;
79 Geuverink & Beukeboom 2014; Herpin and Schartl, 2015). Alternative splicing of *dsx* is a
80 consistent mechanism, which produces protein isoforms that orchestrate a cascade of sex-specific
81 gene expression (Burtis and Baker 1989; Kopp 2012). The *Dsx*-ortholog encoded by *Dmrt1* is
82 one of the few genes identified from insects that also has a conserved function in mammalian
83 sex determination. In mice *Dmrt1* is required for testes development and male fertility (Raymond
84 et al. 2000; Matson et al. 2011).

85 Here, we report the study of somatic sex determination in the milkweed bug *Oncopeltus*
86 *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). 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*. Milkweed bug females and males differ in the structure of the genitalia, in the curvature of the fourth abdominal sternite, and in the distribution of abdominal melanization (Fig 1). Previous research has shown that while *ix* is only required for somatic sexual dimorphism in female *D. melanogaster* (Garrett-engele et al., 2002), it is required during genitalia development in females and males of *O. fasciatus* (Aspiras et al. 2011). Surprisingly, we also find a major role for *fru* in development of both female and male sexually dimorphic structures. While there is a common requirement for *dsx* activity in all sexually dimorphic structures in *O. fasciatus*, different dimorphic traits require distinct combinations of these genes. 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* (Aspiras et al. 2011) but only in females of *D. melanogaster* (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 high level 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 high 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 report, 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.

Structure- 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 candidate 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 from 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

158 sampled tissues. In *D. melanogaster* *ix* is required for female somatic development and for
159 sex-specific behavior in females and males (Nagoshi et al. 1988). The gene is transcribed in most
160 tissues of both females and males from prepupal to adult stages (Gelbart and Emmert 2013).
161 High levels of *ix* transcripts were detected in all sampled tissues in both sexes of *O. fasciatus*
162 (Fig 2C). Expression was highest in the female ovipositor.

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

169 These patterns of expression for *ix* and *fru* in the milkweed bug are notably less sexually
170 dimorphic than their orthologs in the fruit fly. Excluding the ovipositor, *fru* and *ix* show a strong
171 correlation in expression (Spearman's rank correlation $\rho = 0.39$, $p = 0.00819$). We tested for
172 gene interactions by measuring gene expression in an RNA interference (RNAi) background.
173 Expression of *fru* was significantly reduced in the bodies of *ix* RNAi males, compared to control
174 males (Fig S5; Wilcoxon rank sum test, $p = 0.019$). This implies that *ix* promotes *fru* expression,
175 although this effect may be indirect. The absence of *fru* expression in the ovipositor (Fig 2C),
176 where *ix* is highly expressed, suggests the action of an unidentified suppressor in that context.

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

178 To investigate the developmental functions of *ix*, *fru*, and the *doublesex* paralogs in *O. fasciatus*,
179 we performed juvenile-stage RNAi targeting each gene, as well as a simultaneous knockdown of
180 all *doublesex* paralogs (Tables S2, S3). We then examined sexually dimorphic traits in the
181 resulting adults to evaluate loss-of-function phenotypes (Fig 3-4).

182 Among *doublesex* paralogs, knockdown of *dxxa* and *dxxb* failed to produce any discernible
183 phenotype. There was no significant difference in genitalia length, in curvature of the A4
184 sternite, or in the distribution of abdominal melanism among putative females and males in
185 RNAi treatments targeting these genes (Fig 4). However, *dsx* knockdown significantly affected
186 development of all sexually dimorphic structures in presumptive females and males. Only
187 dsRNAs targeting the exon containing the DNA-binding domain resulted in phenotypic changes
188 (Table S3). This exon is present in all transcript isoforms produced from the *dsx* locus (Fig 2B).
189 Presumptive female *dsx* RNAi specimens developed ovipositors that were significantly shorter
190 than unmanipulated specimens (Fig 4A) and appearing reduced overall, but particularly in the
191 distal valvulae (Fig 3R-S). The claspers of presumptive males were also dramatically shorter or

absent entirely in some 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 tandem sequences from the DNA-binding domain of each gene. The phenotypes of these treatments were similar to that of *dsx* RNAi alone (Fig 4). 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-Engle et al. 2002). Therefore, we tested *ix* function during *O. fasciatus* development. Knockdown of *ix* by RNAi produced intersex phenotypes (Fig 3F-J; 4A-B,H), 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 4A-B). 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 significantly reduced in size (Fig 3J; 4B).

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 4A-B). While the ovipositors of *ix* RNAi specimens decreased in length, they also increased in thickness and pigmentation, suggestive of partial transformation to 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 presumptive female *fru* RNAi specimens also showed necrosis of the distal valvulae (Fig 3N). In *fru* RNAi males, claspers were reduced in size (Fig 4B) and often misshapen, lacking their characteristic arch (Fig 3O).

***dsx* inhibits intersex sternite shapes in females and in males**

One of the most obvious sexual dimorphisms in milkweed bugs is the posterior projection of the fourth abdominal sternite (Fig 1A). We examined the effects of RNAi gene knockdown on sternite curvature, quantified using nine landmarks placed on the sternite's posterior 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 4D-F). Nevertheless, among dsRNA control specimens, the sternites of females and males showed significantly different median curvatures (Wilcoxon rank sum test, $p < 2.2 \times 10^{-16}$).

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 dimorphism might cause presumptive females to also retain the juvenile sternite shape. Surprisingly, *dsx* RNAi produced sternite shape changes in female and in male bugs. As expected for females, sternite curvature in *dsx* RNAi presumptive females was significantly reduced compared to that of dsRNA control females (Fig 4D; Wilcoxon rank sum test, $p = 4.73 \times 10^{-3}$). However, among presumptive male *dsx* RNAi specimens, sternite curvature increased ($p = 1.12 \times 10^{-7}$), adopting a more typically female phenotype. A similar effect of less magnitude was also produced by the simultaneous knockdown of all three *doublesex* paralogs (females: $p = 0.0458$; males: $p = 4.15 \times 10^{-3}$). Thus, for both sexes, *dsx* RNAi caused sternite shapes to become more intermediate.

Development of female sternite shape requires *fru*

Since knock-down of *ix* and *fru* produced dramatic phenotypes in the genitalia of female and male milkweed bugs, we also examined other sexually dimorphic traits in these specimens. RNAi targeting *ix* did not significantly affect the sternite curvature (Fig 4D-E). Even specimens with dramatic intersex genitalia still displayed sternite curvatures typical of their presumptive sex (Fig S6). In contrast, knockdown of *fru* reduced the curvature of sternites in females ($p = 0.0107$). While some presumptive female *fru* RNAi specimens had sternites with a typically male shape, most developed with an intermediate curvature (Fig 4D). However, presumptive male *fru* RNAi specimens were not different from dsRNA control specimens (Fig 4E; $p = 0.831$). This suggests that *fru* activity is required for the increased curvature of the A4 sternite in females, but not in males, which retain a more juvenile sternite shape.

Sex-specific distribution of abdominal melanism requires *dsx*

The ventral abdomen of *O. fasciatus* typically has several areas of melanic pigmentation. While considerable individual variation exists, females and males have distinct (non-overlapping) ratios of melanism on the fifth and fourth abdominal segments (Fig 4G-I). Females have less melanism on A5, compared to males where melanic pigmentation is more similar on A4 and A5. Within each sex, this trait is independent of the curvature of the A4 sternite (Fig S7; Spearman's rank correlation for females $\rho = -0.079$, $p = 0.72$; for males $\rho = -0.279$, $p = 0.220$).

RNA interference targeting *dsx* affected the distribution of abdominal melanism in both sexes (Fig 3P-Q, 4G-I). Females normally have much less melanic pigmentation on the fifth abdominal sternite, compared to the fourth. However, presumptive female *dsx* RNAi specimens had significantly more even distribution of melanism than control females (Wilcoxon rank sum test,

263 $p = 2.19 \times 10^{-4}$). Conversely, presumptive male *dsx* RNAi specimens had a reduction in the ratio
 264 of A5 to A4 melanic area, compared to male *AmpR* RNAi specimens ($p = 7.62 \times 10^{-7}$). In contrast
 265 to control specimens, where the ratio of abdominal pigmentation is completely non-overlapping,
 266 there is no longer any sexual dimorphism in this trait among *dsx* specimens ($p = 0.25$). Similar
 267 intersex phenotypes are produced by RNAi targeting all three *doublesex* paralogs (for
 268 presumptive females $p = 7.83 \times 10^{-5}$; for males $p = 9.47 \times 10^{-4}$).

269 ***ix* is required in males for sex-specific abdominal melanism**

270 As with sternite shape, we also examined abdominal pigmentation following RNAi targeting *ix*
 271 and *fru*. While *fru* RNAi produced significant changes in the sternite shapes of presumptive
 272 females (Fig 4D), these specimens did not differ significantly from controls in their distribution
 273 of abdominal melanism (Fig 4G; $p = 0.41$). RNAi targeting *ix* did affect abdominal pigmentation,
 274 but only in presumptive males. Often presumptive male *ix* RNAi specimens had reduced
 275 pigmentation on the fifth abdominal segment, leading to a more typically female pigmentation
 276 pattern (Fig 4H) that was significantly less evenly distributed than in control male bugs ($p =$
 277 0.0173).

278 **Discussion**

279 **Sexual dimorphism requires *dsx***

280 Our results suggest a general role for *dsx* in the development of adult sexual dimorphisms in *O.*
 281 *fasciatus*. All three of the sexually dimorphic traits examined in this study require *dsx* function.
 282 The activity of the paralogs, *dxxa* and *dxxb*, seems to be dispensable, at least for these traits, as
 283 RNAi targeting *dxxa* and *dxxb* individually failed to produce significant deviations from the
 284 phenotypes of control specimens.

285 Interestingly, the function of *dsx* was not simply to promote the development of sexually
 286 dimorphic traits. Genitalia are the most obviously dimorphic structures differing in females and
 287 males, and in these appendages *dsx* RNAi reduced their length in all individuals (Fig 4A-B).
 288 However, RNAi knockdown of *dsx* produced more female-like sternite curvature in males and
 289 more male-like curvature in females, suggesting that *dsx* acts in each sex to inhibit intersex
 290 phenotypes. In effect, *dsx* makes the distribution of sternite curvature more bimodal. Each of
 291 these traits develops from a null state in juveniles. Males retain the relatively uncurved juvenile
 292 sternite shape, while neither sex has abdominal melanism as a juvenile. If the role of *dsx* was to
 293 promote sternite curvature, then we would expect male *dsx* RNAi specimens to be comparable to
 294 control males. Similarly, if *dsx* promoted abdominal pigmentation, we might expect reduced
 295 pigmentation overall with no significant effect on its distribution. However, both these traits are

296 more intersex in their value following *dsx* RNAi. It is likely that distinct isoforms of Dsx act on
297 distinct target genes to produce different effects in each sex, and that these intersex phenotypes
298 arise from the absence of this regulation.

299 **Sexual dimorphisms develop by distinct mechanisms in milkweed bugs**

300 Both female and male *O. fasciatus* require *ix* and *fru* for proper development, but in different
301 traits (Fig 4C,F,I). Orthologs of these genes in *D. melanogaster* have much more limited roles,
302 affecting only one sex or the other. Interestingly, while *ix* and *fru* affect genitalia development in
303 both sexes of *O. fasciatus*, these genes affect different sexually dimorphic traits, in different
304 sexes.

305 *fru* RNAi only affected the development of female, but not male, sternite curvature (Fig 4D-F).
306 Because male sternite curvature closely resembles the phenotype of juveniles, it is possible that
307 *fru* is involved in regulating growth and proliferation of these cells, rather than in directly
308 determining sexually dimorphic identity. Knockdown of *ix* did not significantly change sternite
309 curvature, but affected the distribution of abdominal pigmentation only in male milkweed bugs
310 (Fig 4G-I). It is possible that only some Dsx isoforms in *O. fasciatus* interact with Ix, in a
311 structure-specific context, rather than in a sex-specific context. Isoform D of *O. fasciatus dsx* is a
312 strong candidate for this interaction, because of its strong expression in both female and male
313 genitalia, but lower expression in other somatic body regions (Fig 2). Confirmation of this
314 hypothesis must await the development of isoform-specific antibodies from milkweed bug Dsx.
315 Additionally, it is possible that the expression of distinct *dsx* isoforms is regulated by
316 tissue-specific enhancers. A recent study of regulatory sequences in *D. melanogaster* found that
317 expression of *dsx* in distinct sexually dimorphic structures is controlled by separate enhancers
318 (Rice et al. 2019). Similarly, analysis of gene expression in different larval and pupal tissues of
319 the butterfly *Papilio polytes* has suggested distinct tissue-specific targets of *dsx* activity
320 (Deshmukh et al. 2020).

321 These findings support the concept that some aspects of sex determination in insects may be
322 structure-specific. In fact, it is possible that this form of regulation may be an evolutionary
323 intermediate. If a gene has multiple enhancers for expression in different areas, as exemplified by
324 *D. melanogaster dsx*, then a mutation eliminating one enhancer would produce the kind of
325 mosaic genetic requirement seen for *O. fasciatus ix* and *fru*.

326 **Evolution of *doublesex* in Paraneoptera**

327 The presence of multiple *doublesex* paralogs suggests an ancient gene radiation early in the
328 diversification of Paraneoptera. The genomes of early-branching insect lineages appear to
329 contain only one *doublesex* gene, which all clustered with the clade that includes *D.*
330 *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, in the germline, or in other traits not examined by this study. We examined the gonads of RNAi specimens but found no obvious phenotypic differences at the anatomical level (Fig S8). Future studies will be necessary to test whether *dxxa* or *dxxb* have roles in other traits.

Evolution of sex determination mechanisms in insects

Sex determination pathways evolve rapidly and differ substantially among animals (Herpin and Scharf, 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 of 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 understood.

Conclusions

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 merely 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 S8), 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 trait-specific development of sexual dimorphisms, including the development of female and male genitalia and the inhibition of intersex values for sternite curvature and abdominal pigmentation of both sexes. Nevertheless, it is likely that unidentified genes play a more proximate role in milkweed bug sex determination.

More generally, 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 (21-23°C) 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 separated 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 contiguity 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 ix, fru* and *doublesex* transcripts were submitted to GenBank (accession numbers MZ197788 - MZ197800).

All *doublesex* transcripts included 5' exons containing the conserved DNA-binding domain. 5' RACE using gene-specific reverse 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 trait-specific expression, to validate RNAi knockdowns, and to investigate potential 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 template samples. 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 1 µg total RNA using the iScript cDNA kit (BioRad) with a poly-T primer as described in the kit's 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 the consistency of its 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-negative 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 in 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 NaPO₄, 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. (Milkweed bugs have 5 juvenile instars.) Fourth instars were anesthetized using a CO₂ 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 μ l 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) in adults no more than 1 day old. 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 three somatic sexual dimorphisms, genitalia length, sternite curvature, and abdominal pigmentation patterns (Fig 1). Specimens were imaged on a VWR VistaVision dissecting microscope with a Moticam 5 digital camera. A ruler was included in the image for scale. The ImageJ (Schneider 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 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 fourth abdominal sternite (Fig 1A-B). Raw pixel values were converted to metric distances based on measurement of the ruler scale in each image.

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 of *O. fasciatus* adults varies with sex (Fig 1A-B; 4G-I). Rearing temperature can also influence pigmentation (Sharma et al. 2016). Bugs in this study were kept at a consistent temperature (21 - 23°C), limiting this environmental influence. Within 1-2 hours of the imaginal molt, melanic areas are visible on the ventral abdomen. These areas were quantified using the freehand selection tool in ImageJ. Pixel areas were converted to metric area based on measurement of a mm scale in each photograph.

Statistical analyses

Gene expression and phenotypes produced through RNAi 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 examined using Dunn's test, for multiple pairwise comparisons of gene expression (Dinno 2017), or the Wilcoxon rank sum test, for two-sample comparisons of phenotypic values. 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 MZ197788 - MZ197800). 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 morphological measurements and 2D landmark coordinate positions for sternite shapes, gene expression data, and the R scripts used for analysis.

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658 Figure Legends

659 **Figure 1**

660 **Milkweed bugs have several sexually dimorphic features.** (A-B) The fourth abdominal body
661 segment (A4) of females has an exaggerated extension of the sternite at the ventral midline. Nine
662 landmarks were placed along the ventral edge of the sternite to quantify curvature. (C)
663 Appendages from the terminal segments of the female abdomen form an ovipositor. Ovipositor
664 length was measured from the distal tip to the proximal joint. (D) The male abdomen ends in a
665 large genital capsule, which contains the copulatory organ. Externally a pair of small claspers,
666 visible from a posterior perspective, is used for mate guarding. Clasper length was measured
667 from the proximal joint to the tip.

668 **Figure 2**

669 **Structure and expression of candidate sex determination genes in *O. fasciatus*.** (A)
670 Orthologs of *intersex*, *fruitless*, and three *doublesex* paralogs were identified on different

671 genomic scaffolds in the *O. fasciatus* genome. Boxes represent exons; angled lines represent
 672 introns. Introns were very long compared to neighboring gene predictions. Flags indicate the
 673 start of transcription. (B) Transcripts from *ix*, *fru* and *doublesex* paralogs. The first exon of each
 674 transcript is aligned at the left. Omitted exons are left empty. Alternative start or stop sites for
 675 exons are indicated by lowercase letters (e.g. 2a, 2b) Lighter shading indicates UTRs. (C) A
 676 heatmap showing relative transcript abundance from five body regions in each sex. Each panel
 677 represents the mean of 5 to 7 independent measurements. Global differences in means were
 678 tested using the Kruskal-Wallis rank sum test. Where differences were significant, Dunn's test of
 679 multiple comparisons using rank sums was applied post hoc. Lowercase letters indicate
 680 significant differences after correction for multiple tests ($\alpha = 0.05$).

681 **Figure 3**

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

699 **Figure 4**

700 **Quantification of RNAi effects** on genitalia length (A-B), sternite curvature (D-E) and
 701 abdominal melanism (G-H) in females (A,D,G) and males (B,E,H). Dots represent individual
 702 specimens. Gray outlines show the relative distribution of values. The mean of control (*AmpR*
 703 dsRNA) specimens is indicated by the horizontal line in each panel. Stars indicate significant

704 difference from control values, based on one-sided Wilcoxon rank sum tests with FDR
 705 correction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, and are summarized beside a sketch of
 706 typically female and male phenotypes at the right (C,F,I).

A

B

C