

Evolution of Developmental Control Mechanisms

Sex-specific gene interactions in the patterning of insect genitalia

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ABSTRACT

Genitalia play an important role in the life histories of insects, as in other animals. These sexually dimorphic structures evolve rapidly and derive from multiple body segments. Despite the importance of insect genitalia, descriptions of their genetic patterning have been limited to fruit flies. In this study, we report the functions, interactions and regulation of appendage patterning genes (e.g. *homothorax*, *dachshund*, and *Distal-less*) in two insects: the milkweed bug *Oncopeltus fasciatus*, and the red flour beetle *Tribolium castaneum*. These species differ in the anatomical complexity of their genitalia. Females of *T. castaneum* have a terminal ovipositor ending in short styli, while *O. fasciatus* have a multi-jointed subterminal ovipositor. Male *O. fasciatus* have a genital capsule consisting of large gonocoxopodites and claspers; *T. castaneum* males have relatively simple genitalia. The requirement of appendage-patterning genes in males differed between the two species: No defects were observed in *T. castaneum* male genitalia, and while the male claspers of *O. fasciatus* were affected by depletion of appendage-patterning genes, the proximal gonocoxopodite was not, suggesting a non-appendicular origin for this structure. Only the styli of the *T. castaneum* ovipositor were affected by RNAi depletion of appendage-patterning genes (14 genes in all). The posterior Hox genes (*abdominal-A* and *Abdominal-B*) were required for proper genital development in *O. fasciatus* and regulated *Distal-less* and *homothorax* similarly in both sexes. *Distal-less* and *dachshund* were regulated differently in male and female *O. fasciatus*. Knockdown of the sex determination gene *intersex* produced a partial female-to-male transformation of abdominal and genital anatomy and also resulted in abrogation of female-specific regulation of these genes. These results provide developmental genetic support for specific anatomical hypotheses of serial homology. Importantly, these gene functions and interactions describe the developmental patterning of sexually dimorphic structures that have been critical to the diversification of these species-rich insect groups.

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Introduction

In different tissues or organs, differentiation may share many aspects; however especially at later stages distinct cues direct cells to adopt different fates. Serial homology and sexual dimorphism are two phenomena that highlight this issue. In both cases, organs in separate sexes or at different axial body locations share many aspects of development, but diverge in key ways toward distinct phenotypes. Insects exhibit serial homology of body segments and appendages, as well as sometimes dramatic sexual dimorphism. Little is known about the nature of the gene interactions and patterning processes that lead to these distinct developmental end points.

Genitalia play an important role in the life history of most animals. They evolve rapidly and in some cases are the sole divergent morphological characters between closely related species (reviewed by Eberhard, 2011). Due to the dramatic differences between some male genital structures, the so-called “lock and key” hypothesis proposed that genitalia

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

Insect genitalia are complex structures, with elements derived from the internal reproductive organs, posterior abdominal segments, and appendages, which may be elaborated or reduced in different groups. Male genitalia consist of the copulatory organ, and in some groups males possess external claspers (Fig. 1A–C, E). The anatomy of female ovipositors varies greatly among insect groups (Chapman, 1998; Scudder, 1961), but can be divided into two main types, which we will refer to as terminal and subterminal. Terminal ovipositors are modified from the posterior-most region of the abdomen, which telescopes out to deposit eggs on substrate and may be retracted when at rest (Fig. 1G). Coleoptera, Diptera, and some Lepidoptera have terminal ovipositors. Subterminal ovipositors are derived from the appendages of abdominal segments 8 and 9 (A8–A9) and are typically used to deposit eggs on or in specific plant or animal hosts (Fig. 1D–E). Thysanura, some Odonata, Orthoptera, some

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Thysanoptera, Hemiptera and Hymenoptera possess subterminal ovipositors. These orders include many dramatic examples of ovipositor specialization. For example, ichneumonid wasps use an elongated ovipositor to parasitize caterpillars and other insect larvae (Abbott, 1934; Boring et al., 2009), and the ovipositors of cicadas (Hemiptera) are capable of boring through wood.

Despite the biological importance of insect genitalia, their formative genetic patterning has only been examined in *Drosophila melanogaster* (Estrada and Sanchez-Herrero, 2001; Foronda et al., 2006; Gorfinkiel et al., 1999, 2003; Sánchez and Guerrero, 2001; Sánchez et al., 2001), which have relatively simple external genitalia. Genital patterning poses several unique developmental questions. Genitalia are one of only two appendages to appear at adulthood in hemimetabolous insects (wings being the second). Additionally, the genitalia are formed by contributions from several posterior body segments. In *D. melanogaster* A8–A10 contribute to the genital imaginal disc, with A8 and A9 developing into the genitalia and A10 developing into the analia. Furthermore, genitalia are a critical system in which to understand how patterning differs in sexually dimorphic structures.

In many insect groups, the genitalia of both sexes include putatively appendage-derived structures, such as male claspers and lance-like female ovipositors, which have been considered serially homologous to the ventral appendages: the antennae, mouthparts and legs (Boxshall, 2004; Minelli, 2002; Rosa-Molinari and Burke, 2002; Snodgrass, 1935). In *O. fasciatus*, male and female genitalia differ greatly (Bonhag and Wick, 1953). The male genital capsule resembles two coxae fused medially and projecting to the posterior (Fig. 1A–B). Assuming this homology, anatomists have termed these the gonocoxopodites. Heavily sclerotized claspers articulate from the posterior of the genital capsule. Between the claspers, behind a sclerotized ridge, is an opening where

the copulatory organ emerges during mating. In *O. fasciatus* this organ has a sclerotized basophallus and a flexible distal aedeagus that coils into the basophallus when not inflated for copulation (Fig. 1C). Serial homologies between specific segments of the genitalia and other appendages have been uncertain. Since the male external genitalia have a single proximal-distal (PD) PDaxis, they are considered to consist of a single pair of appendage primordia. In comparison, the female ovipositor of *O. fasciatus* is composed of two subterminal pairs of articulated structures, presumably two appendage pairs. Each appendage has a proximal valvifer and distal valvula (Fig. 1D–E). The first and second valvulae remain tightly associated and fused medially in the functional ovipositor.

Genitalia in *T. castaneum* are far less robust than those of *O. fasciatus*. The male genitalia of *Tribolium* consist of a sclerotized aedeagus, which articulates from A8 and is retracted internally when not in copulation. The aedeagus consists of three tightly associated plates (Fig. 1F; Arnaud et al., 2001; Stanley and Grundmann, 1965). The terminal ovipositor of *T. castaneum* consists of membranous telescoping body segments, which remain retracted beneath the A7 sternal and tergal plates. The more anterior of these are regarded as derivatives of abdominal segments 8–10. The coxite is the most posterior segment of the terminal ovipositor and possesses several thinly sclerotized coxite plates. Two cylindrical styli flank the vulva dorsolaterally, and are tipped with several prominent setae (Fig. 1G; Sokoloff, 1972).

In other appendage types, such as legs, development first begins with the specification of appendage tissue during embryogenesis. In the *D. melanogaster* embryo, appendage primordia are specified at segment boundaries by *wingless* activation and inhibited from dorsal and ventral directions by *decapentaplegic* and epidermal growth factor (EGF), respectively. These interactions localize expression of *Distal-less*

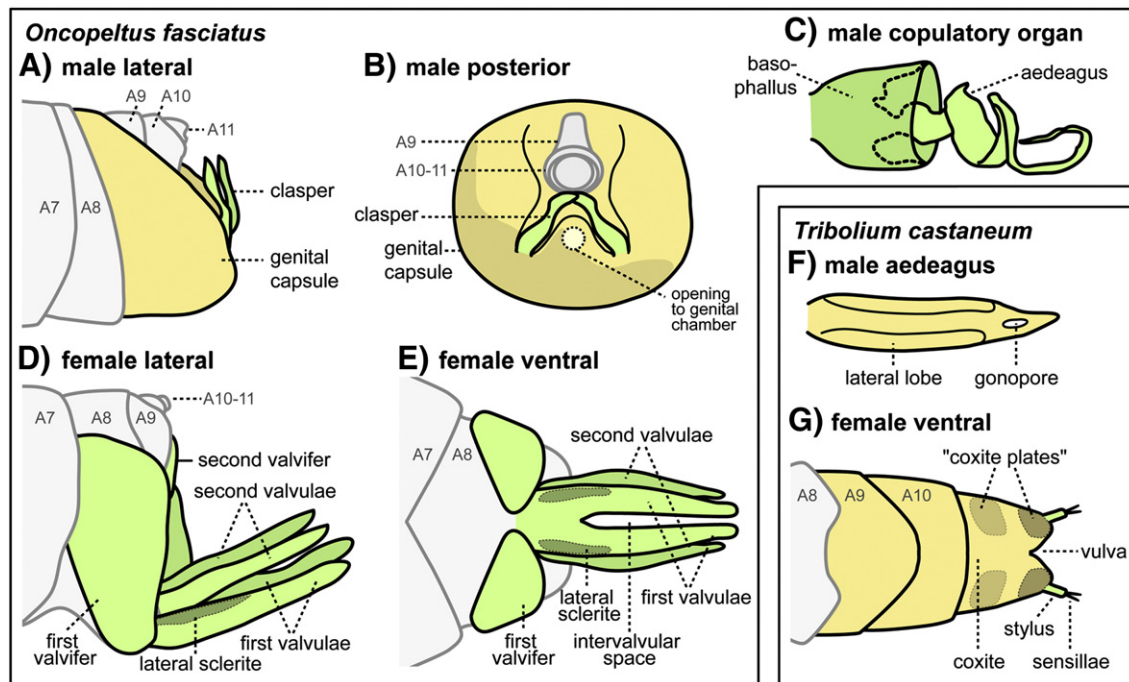


Fig. 1. Diagram of genitalia in *O. fasciatus* (A–E) and *T. castaneum* (F–G). Lateral (A) and posterior (B) sketches of the genitalia of male *O. fasciatus* show the prominent genital capsule, with articulating claspers. The copulatory organ emerges from an opening (dotted circle) obscured by a posterior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

(*Dll*) to the cells of the appendage primordia, which will give rise to the imaginal discs. (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Second, Hox gene expression within the appendage primordia confers appendage identity and regulates appendage patterning to direct segment-specific appendage anatomies. In *D. melanogaster*, *Antennapedia* is required for leg identity (Struhl, 1982). The posterior Hox genes *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) specify identity in the abdomen and terminalia. *Abd-B* expression in the genital imaginal disc directs development of this tissue into the genitalia and analia in both sexes, while *abd-A* represses *Dll* expression in the abdomen and in the genital disc. *abd-A* appears to only be required in the female genitalia of flies, which is mostly derived from the more anterior A8 segment where *abd-A* is expressed. Third, appendage-patterning genes establish regional domains along the limb (Wu and Cohen, 1999). The canonical leg-patterning genes, *homothorax* (*hth*), *dachshund* (*dac*) and *Distal-less* (*Dll*), have conserved roles in arthropod leg development. These genes promote growth and/or regional identity along the PD axis of the legs in *O. fasciatus* (Angelini and Kaufman, 2004; Angelini and Kaufman, 2005), *T. castaneum* (Angelini et al., submitted for publication; Beermann et al., 2001; Suzuki et al., 2009) and other arthropods (Ronco et al., 2008; Schoppmeier and Damen, 2001). Studies exploring the function and expression of these genes in other appendages, such as the antennae and mouthparts, have provided insights into insect appendage development and serial homology (Angelini and Kaufman, 2004; Casares and Mann, 1998; Dong et al., 2000; Morata, 2001; Ronco et al., 2008). For sexually dimorphic characters like genitalia, additional input from the somatic sex determination pathway is also required. In *D. melanogaster* two critical factors in this pathway are *doublesex* (*dsx*), which has sex-specific splicing variants, and *intersex* (*ix*), which encodes a cofactor for the female isoform of Dsx that interacts with the Mediator complex. Both genes are structurally conserved among insects, compared to those acting earlier in the sex determination pathway. Functional conservation of *ix* has been demonstrated through rescue of the *D. melanogaster ix* null mutant with orthologous *ix* sequences from other Diptera and Lepidoptera (Arunkumar and Nagaraju, 2011; Cavaliere et al., 2009; Siegal and Baker, 2005).

Here we present a developmental genetic study of genital patterning in representatives of two species-rich insect orders. The milkweed bug *Oncopeltus fasciatus* has robust male and subterminal female genitalia; females lay eggs (oviposit) in the fluff of milkweed (*Asclepias* sp.) seed pods. In contrast, the genitalia of the red flour

beetle, *Tribolium castaneum*, are less robust, and females oviposit in flour with a terminal ovipositor. We examine the functions, regulation, and interactions of genes involved in appendage development in these species in order to test the extent to which these genital structures share homology with the appendages and explore how sexually dimorphic features are patterned.

Materials and methods

Insect culture

Wildtype cultures of *T. castaneum* and *O. fasciatus* were obtained from Carolina Biological Supply Company. Red flour beetles were maintained at 32 °C under conditions recommended by the supplier. Milkweed bugs were maintained according to Hughes and Kaufman (2000).

Selection and cloning of candidate genes

Candidate genes from *T. castaneum* and *O. fasciatus* were identified for study based on the literature of leg and genital disc development in *D. melanogaster* and comparative data from other arthropods (Table 1). *Dll*, *dac*, and *hth* were the focus of study in both species because these genes have been characterized in great detail in *D. melanogaster* genital development, as well as the development of other appendages types in all three species. The completed genome sequence of *T. castaneum* (Tribolium Genome Sequencing Consortium, 2008) allowed us to also examine several other genes with known functions in appendage development (see references in Table 1). The large size of *O. fasciatus* juveniles facilitates gene expression measurements on single individuals, therefore we include in our study several putative regulators of *Dll*, *dac* and *hth*, such as the posterior Hox genes and *ix*. Most candidate genes used in this study have been cloned previously (Table 1). *Oncopeltus fasciatus ix* was cloned using exact primers (forward: GTAGGTTATTGTGAGTGTTGAGGTTG; reverse: GGTCTGTAGAAAG-GAGGAACCTTTGA) designed from *ix* transcript sequence obtained by Ewen-Campen et al. (2011). The amplified fragment was cloned using standard methods and sequenced to confirm its identity. This *O. fasciatus ix* sequence was deposited in GenBank (Accession JN368475).

Preparation of double-stranded RNA and RNA interference

Phenotypes were generated in adult insects using juvenile RNA interference. Knockdown of gene activity was verified using quantitative

Table 1

Candidate genes used in this study were identified from the literature and clones were obtained based on the referenced studies.

Gene	Symbol	Protein class	GenBank	Reference
<i>Oncopeltus fasciatus</i>				
<i>abdominal-A</i>	<i>abd-A</i>	homeobox TXF	AY627361	Liu and Kaufman, 2004
<i>Abdominal-B</i>	<i>Abd-B</i>	homeobox TXF	AY627362	"
<i>Distal-less</i>	<i>Dll</i>	homeobox TXF	AY584472	Angelini and Kaufman, 2004
<i>dachshund</i>	<i>Dac</i>	Ski/Sno-related TXF	AY584473	"
<i>homothorax</i>	<i>Hth</i>	homeobox TXF	AY584474	"
<i>intersex</i>	<i>Ix</i>	Mediator subunit	JN368475	Ewen-Campen et al., 2011; this study
<i>Tribolium castaneum</i>				
<i>Distal-less</i>	<i>Dll</i>	homeobox TXF	NM_001039439	Jockusch et al., 2004
<i>dachshund</i>	<i>Dac</i>	Ski/Sno-related TXF	XM_964678	Prpic et al., 2001
<i>homothorax</i>	<i>Hth</i>	homeobox TXF	NM_001039400	Angelini and Kaufman, 2004
<i>aristalless</i>	<i>Al</i>	homeobox TXF	NM_001114366	Angelini et al., 2009
<i>abrupt</i>	<i>Ab</i>	BTB/Zn-finger TXF	XM_969854	"
<i>spineless</i>	<i>Ss</i>	bHLH/PAS TXF	XM_962783	"
<i>pdm/nubbin</i>	<i>Pdm</i>	homeobox TXF	XM_963346	"
<i>Notch</i>	<i>N</i>	Notch receptor	NM_001114381	"
<i>Serrate</i>	<i>Ser</i>	Delta/Serrate-type EGF	XM_964393	"
<i>Delta</i>	<i>DI</i>	Delta/Serrate-type EGF	XM_964994	Angelini et al., submitted for publication
<i>odd-skipped</i>	<i>Odd</i>	Zn-finger TXF	XM_966993	Angelini et al., 2009
<i>brother of odd with entrails limited</i>	<i>Bowl</i>	Zn-finger TXF	XM_967045	"
<i>sister of odd & bowl</i>	<i>Sob</i>	Zn-finger TXF	XM_966942	"
<i>drumstick</i>	<i>Drm</i>	Zn-finger TXF	XM_966887	"

Table 2
Summary results for juvenile *O. fasciatus* RNAi.

dsRNA	Number scored		Penetrance	Target gene knockdown
<i>GFP</i>	27	♂	(0)	
	34	♀	(0)	
<i>abd-A</i>	32	♂	84% (27)	37%*
	48	♀	100% (48)	27%*
<i>Abd-B</i>	48	♂	88% (42)	33%*
	51	♀	96% (49)	68%*
<i>Dll</i>	24	♂	92% (22)	55%*
	32	♀	97% (31)	61%*
<i>dac</i>	37	♂	97% (36)	63%*
	35	♀	91% (32)	47%*
<i>Hth</i>	42	♂	74% (31)	74%*
	39	♀	85% (33)	70%*
<i>ix</i>	24	♂	100% (24)	20%
	28	♀	100% (28)	3%
<i>ix, Dll</i>	8	♂	100% (8)	
	12	♀	100% (12)	
<i>ix, dac</i>	6	♂	100% (6)	
	13	♀	100% (13)	
<i>ix, hth</i>	13	♂	100% (13)	
	11	♀	100% (11)	

*Significant difference from gene expression in *GFP* control specimens (Welch's *t*-test, $p < 0.05$).

realtime RT-PCR. To synthesize double-stranded RNA, a template DNA was amplified from a cloned gene fragment, using exact primers with the T7 promoter sequence added to the 5' end. Double-stranded RNA (dsRNA) was transcribed using the MegaScript Transcription Kit (Applied Biosystems) with T7 RNA polymerase, then treated with DNase I to remove plasmid DNA. The product was annealed by cooling and purified by precipitation in ammonium acetate and ethanol. After resuspension in nuclease-free water, dsRNA concentrations were determined through triplicate measurements on a nanoscale spectrophotometer (GE Life Sciences NanoVue) and diluted to 4 µg/µl with 0.05% McCormick green food coloring, 0.01 mM NaPO₄, and 5 mM KCl.

Injection of *O. fasciatus* was done in fourth instar nymphs before the appearance of sexually dimorphic characters. Nymphs were anesthetized using CO₂ or with a 4-minute exposure to diethyl ether vapor. Using a front-loaded pulled-glass capillary needle, approximately 1 µl of 4 µg/µl dsRNA was injected at the base of the right metathoracic coxa. This location facilitated easy delivery into the hemolymph and no defects were observed at the site after ecdysis. Injection of *T. castaneum* pre-pupal larvae was as described by Angelini et al. (2009).

Measurement of gene expression

The extent of gene knockdown was determined using quantitative realtime RT-PCR (qPCR) amplification of target gene sequences. For validation of RNAi, expression was compared between gene-specific and nonspecific control dsRNA treatments (*GFP*). Total RNA was isolated using the PureLink RNA Mini Kit (Life Technologies) from the abdominal tissue (A4-A12) of individual young adult *O. fasciatus* or 10 pooled 3-day-old *T. castaneum* pupae. These stages were chosen because they allowed for consistent selection. Isolated RNA was stored at −80 °C. For all *O. fasciatus* treatments, at least 3 biological replicates were included. Total RNA concentrations were determined by triplicate measures on a nanoscale spectrophotometer and diluted to 100 ng/µl immediately prior to assays. Total RNA was used as template in reverse transcription/ SYBR Green realtime PCR reactions (Quanta BioSciences).

For each gene, exact primers were designed using the Primer3 algorithm (Rozen and Skaletsky, 2000), avoiding conserved functional domains and dsRNA regions. Dissociation curves for each reaction were used to verify that only single products were amplified. To produce quantitative template standards, clones were linearized and

transcribed *in vitro* from T7 promoters to produce single-stranded RNA. This RNA was treated with DNase I to remove template DNA and purified by precipitation in ammonium acetate and ethanol. Immediately before qPCR assays, the RNA concentration was determined in triplicate (as described earlier) and the molar quantity was calculated based on the size of the RNA. Dilution series were then prepared fresh for each plate at concentrations of 10³, 10⁵, and 10⁷ RNA molecules to serve as a standard curve (Pfaffl, 2004). The degree of knockdown in RNAi specimens is given in Table 2 with statistical significance based on significant difference from control *GFP* dsRNA treatment in Tukey's honest significant difference (HSD) test ($p < 0.05$).

Characterization of RNAi effects

Tables 2–3 summarize the phenotypic penetrance and rates of gene knockdown for RNAi. Some treatments did not have a statistically significant knockdown in expression; however phenotypic penetrance was high (Tables 2–3) and phenocopied gene-specific loss-of-function defects in other appendages, similar to those observed previously (Angelini and Kaufman, 2005; Angelini et al., 2009). At the time of injection, juveniles lack obvious sex-specific characters; therefore sex was scored after adult eclosure. Nonspecific *GFP* dsRNA treatment had no effects on genital development.

Specimens of *O. fasciatus* were stored in 70% ethanol within 12 h of adult eclosure. Hox and *ix* dsRNA treated specimens were unable to completely shed the nymphal cuticle, so the loose abdominal exuvia was removed by hand to improve visualization of the genital morphology. Internal anatomy was examined after dissection, and copulatory organs were mounted in Aqua Poly/Mount (Polysciences, Inc.) prior to imaging. Adult *T. castaneum* were cleared overnight in a solution of

Table 3
Summary results for metamorphic *T. castaneum* RNAi.

dsRNA	Number scored		Penetrance		Target gene knockdown
			Genitalia	Other appendages	
Buffer	12	♂	(0)	(0)	
	17	♀	(0)	(0)	
<i>Dll</i>	21	♂	(0)	48% (10)	75% ± 4.9% *
	25	♀	64% (16)	64% (16)	
<i>al</i>	15	♂	(0)	27% (4)	
	27	♀	22% (6)	33% (9)	
<i>ss</i>	5	♂	(0)	100% (5)	
	12	♀	58% (7)	100% (12)	
<i>pdm</i>	20	♂	(0)	95% (19)	61% ± 11% *
	23	♀	78% (18)	78% (18)	
<i>Notch</i>	10	♂	(0)	100% (10)	24% ± 9.0% *
	19	♀	58% (11)	89% (17)	
<i>Ser</i>	27	♂	(0)	96% (26)	
	31	♀	100% (31)	100% (31)	
<i>Delta</i>	35	♂	(0)	49% (17)	71% ± 14% *
	14	♀	36% (5)	57% (8)	
<i>odd</i>	18	♂	(0)	83% (15)	65% ± 5.2% *
	30	♀	53% (16)	87% (26)	
<i>bowl</i>	23	♂	(0)	78% (18)	63% ± 8.0% *
	32	♀	63% (20)	84% (27)	
<i>sob</i>	6	♂	(0)	100% (6)	61% ± 7.3% *
	6	♀	83% (5)	100% (6)	
<i>drm</i>	10	♂	(0)	100% (10)	
	19	♀	95% (18)	95% (18)	
<i>odd, bowl, sob, drm</i>	12	♂	(0)	100% (12)	
	22	♀	86% (19)	100% (22)	
<i>dac</i>	20	♂	(0)	85% (17)	30% ± 16%
	44	♀	0% (0)	77% (34)	
<i>hth</i>	74	♂	(0)	93% (69)	44% ± 7.5% *
	106	♀	0% (0)	91% (96)	
<i>ab</i>	10	♂	(0)	100% (10)	42% ± 3.6% *
	14	♀	0% (0)	86% (12)	

*Significant difference from gene expression in *GFP* control specimens (Welch's *t*-test, $p < 0.05$).

20% glycerol in glacial acetic acid at 50 °C (Van der Meer, 1977), genitalia were dissected in 70% glycerol, and mounted in Aqua Poly/Mount. A representative sample (8 to 10) of each sex for *O. fasciatus* dsRNA treatments were imaged using an Olympus SZX16 dissecting microscope equipped with an Hamamatsu C8484 high-resolution digital camera. The genitalia of *T. castaneum* were imaged with an Olympus digital camera on a Zeiss Axioskop compound microscope.

Genital measurements of *O. fasciatus* were made from digital images using ImageJ (Abramoff et al., 2004). For male specimens, distances were measured from the ventral edge of the genital capsule to the dorsal analia, as well as from base of a clasper to its tip (Fig. 2A,H). For female specimens the length of the first (Fig. 3I) and second valvulae (Fig. 3J) was measured. The distance across the head, between the innermost edges of the eyes (ocular distance), was used to normalize for overall body size. However, no effects of ocular distance were found, therefore figures report absolute measurements. Treatment effects were tested using one-way ANOVA and post-hoc Tukey's HSD tests were used to identify treatments that differed significantly from nonspecific *GFP* dsRNA controls. In all cases, the determination of significance in nonparametric tests (Kruskal–Wallis and Wilcoxon rank sum tests) agreed with ANOVA (treatment effect) and the Tukey's HSD (pairwise difference) tests. All statistical tests were conducted in R (Ihaka and Gentleman, 1996).

Results

RNA interference of appendage-patterning genes produced growth and patterning defects in the genitalia of *O. fasciatus*

In the male genitalia, *Dll* knockdown reduced the length of the claspers (Fig. 2B). This reduction in length was statistically significant (Fig. 2H; Tukey's HSD, $p = 0.0014$). In female *Dll* RNAi specimens, the ovipositor was also reduced (Fig. 3B). Both the first and second valvulae were significantly shorter (Fig. 3J–K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p = 0.039$ for valvula 2). Reduction was more severe in the first valvulae, the more anterior of the two pairs. *Dll* juvenile

RNAi produced allometric reductions of the mouthparts, as well as fusions of the tarsi (not shown).

The clasper length of the *dac* RNAi specimens was significantly shorter than with control dsRNA treatment (Fig. 2C,H; Tukey's HSD, $p = 0.027$). *dac* knockdown in females had a dramatic effect on the genitalia (Fig. 3C). Valvulae were significantly reduced (Fig. 3J–K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p = 1.0 \times 10^{-7}$ for valvula 2). Additionally, the left and right first valvulae did not fuse at the midline, and the membranous tissue of the second valvulae was disorganized and did not fold properly (Fig. 3C). Juvenile *dac* RNAi also caused defects in the stylets, which typically did not extend out of the head to their normal length (not shown). Internal reproductive anatomy was not obviously affected by *dac* depletion, including the single medial spermatheca and its duct.

In male *hth* RNAi, clasper length was not significantly reduced. However, these males developed with reduced and malformed copulatory organs (Fig. 2D–G). This reduction affected both the phallobase and aedeagus (proximal and distal structures; Fig. 2F). The dorsal–ventral depth of the genital capsule was also measured for *Dll*, *dac* and *hth* RNAi treatments, however none of these differed significantly from measurements of *GFP* dsRNA control males (not shown). In females, *hth* RNAi caused the proximal valvifers to become enlarged (Fig. 3E–F), however the valvulae were significantly reduced (Fig. 3D,J–K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p = 8.2 \times 10^{-6}$ for valvula 2). Additionally, the first valvulae failed to fuse medially. *hth* juvenile knockdown also affected the stylets, which failed to lay neatly in the rostrum (not shown).

RNA interference of appendage-patterning genes produced defects in the female *T. castaneum* genital styli

In *T. castaneum* we conducted functional tests for 14 genes (Table 1) involved in appendage patterning, in order to test whether the genitalia require activity of genes involved in the development of appendages. The knockdown of several genes resulted in defects to the female styli. However, there were no obvious defects observed in the coxite

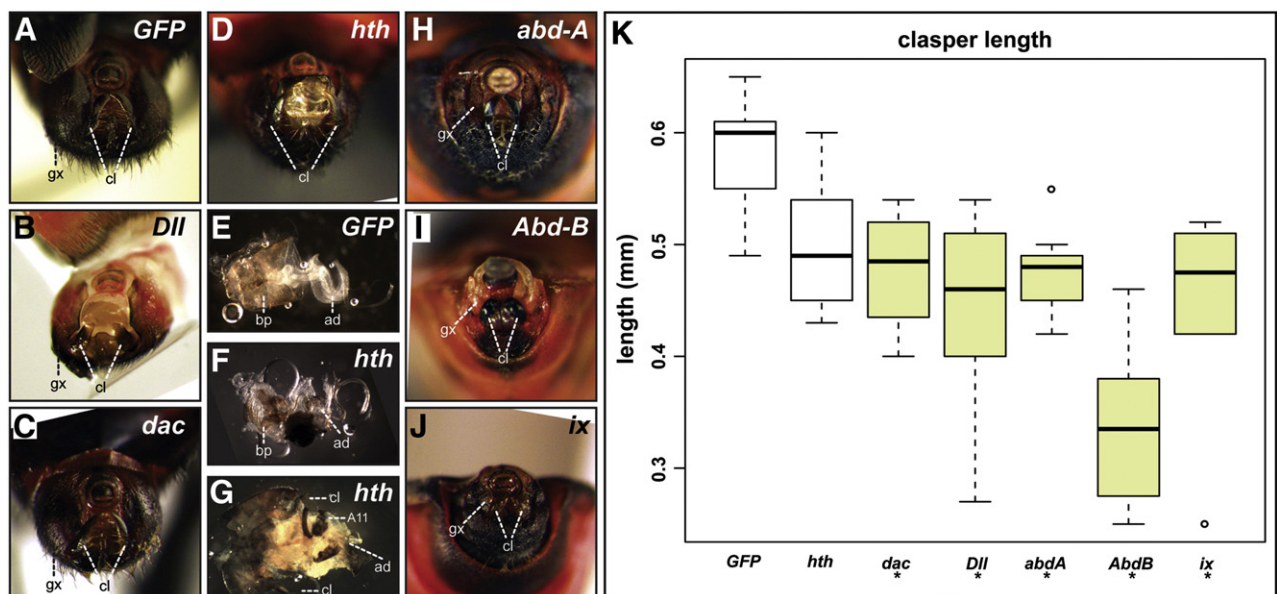


Fig. 2. The male genital capsule of *O. fasciatus*. (A) Nonspecific *GFP* control dsRNA treatments were indistinguishable from unmanipulated males. (B) *Dll* RNAi in males reduced length of the clasper. (C) *dac* RNAi caused reduced claspers. (D) In *hth* RNAi the male copulatory organ (the white tissue) was exposed and malformed. (E) The copulatory organ in control dsRNA males consists of a proximal phallobase and distal aedeagus, which coils up and is stored in the distal part of the phallobase. (F) In mildly affected *hth* RNAi specimens the copulatory organ was reduced overall. (G) In more severe *hth* knockdown males, the copulatory organ lacked an obvious phallobase and a malformed aedeagus protruded externally as in this dissected genital capsule. (H) *abd-A* RNAi caused reduction of the male genitalia. (I) *Abd-B* knockdown caused the most dramatic reduction in male genitalia of all the genes examined. (J) Knockdown of *ix* caused reduction of the male genitalia. (K) Box plots of male clasper length from dsRNA treatments. The dark line represents the median value, the box shows the 25th to the 75th quartiles; dotted lines give outer quartile range; circles show outliers. Treatments differing significantly from nonspecific *GFP* controls are colored and marked by asterisks (Tukey's HSD, $p < 0.05$). Abbreviations: ad, aedeagus; cl, clasper; gx, gonocoxopodite; pb, phallobase.

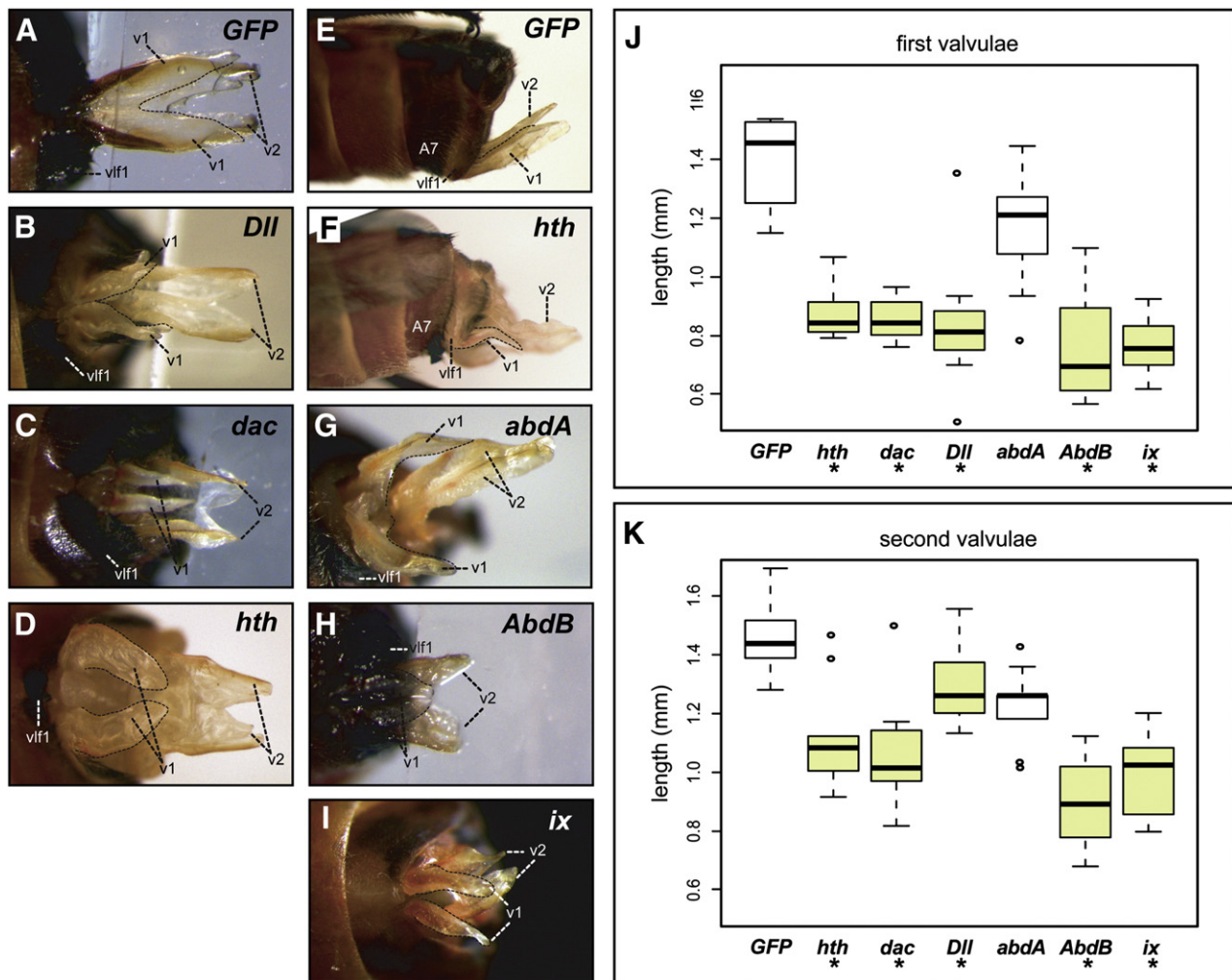


Fig. 3. The ovipositor of *O. fasciatus*. (A) Nonspecific GFP control dsRNA treatments were indistinguishable from unmanipulated females. The view is from a ventral perspective; anterior is to the left. (B) In *Dll* RNAi females both appendage pairs of the ovipositor were significantly shorter. However, first valvulae were dramatically shorter than second valvulae. (C) *dac* knockdown females have reduced valvulae and a failure of medial fusion in the first valvulae. (D) In *hth* RNAi, all valvulae were reduced and first valvulae did not fuse medially. (E) A lateral view of the GFP control ovipositor shows the proximal valvifers. (F) Valvifers were enlarged by *hth* RNAi. (G) *abdA* RNAi produced a transformation of the anterior first valvulae towards the structure of the second valvulae. These structures failed to interlock into a functional ovipositor. (H) The knockdown of *Abd-B* caused dramatic reduction in all components of the female genitalia. (I) *ix* RNAi caused a reduction of female genitalia, a failure of left and right appendages to fuse, and changes in pigmentation and sclerotization that suggest a partial female-to-male transformation. Box plots showing the length of first valvulae (J) and second valvulae (K) from each dsRNA treatment. Range and significance are indicated as in Fig. 2H. Abbreviations: v1, first valvulae; v2, second valvulae; vlf1, first valvifer.

or the more anterior segments of the ovipositor. *Dll* RNAi resulted in a complete deletion of the stylus, including the distal sensillae (Fig. 4B). Knockdown of *pdm* produced a similar effect, in which no stylus was present on the posterior ovipositor (Fig. 4C). Knockdown of *aristaless* (*al*), another gene that functions in distal appendage development (Angelini et al., 2009; Campbell and Tomlinson, 1998), caused a reduction of the stylus and a rounded distal shape, but the distal sensillae were present (Fig. 4D). In *D. melanogaster*, *al* expression in the genital disc is enriched in females (Chatterjee et al., 2011). Depletion of several other genes (*spineless*, *Notch*, and the *odd-skipped* family paralogs) also caused defects in stylus development. In these dsRNA treatments the normal location of the stylus had a small raised bump in the cuticle, topped by the normal sensillae (Fig. 4E–G). The presence of the sensillae in these treatments suggests that cells in this area retain at least partial stylus identity, but are unable to differentiate the cylindrical stylus. Development of the *T. castaneum* ovipositor appeared to be independent of knockdown of a number of other genes that have prominent roles in the development of other appendages, including *hth*, *dac* and *abrupt* (Table 3).

We examined males for defects in the aedeagus (Fig. 4H) but did not observe any among the RNAi treatments included in this study (Table 3) or in knockdown of genes examined in other recent studies (Angelini et al., 2009, submitted for publication). Since the clearing method used on *T. castaneum* specimens does not preserve thin membranous tissue well, we cannot exclude a role for these genes in development of the penis or in internal reproductive structures.

RNA interference of posterior Hox genes

The posterior Hox genes were required for normal development of the genitalia in *O. fasciatus*. *abdominal-A* knockdown significantly reduced male genital characters including the distance between clasper bases (Fig. 2H) and clasper length (Fig. 2K; $p = 0.015$). The genital capsule was also significantly reduced in size (Tukey's HSD, $p < 10^{-16}$) and did not project out from the abdomen as in unmanipulated bugs. Knockdown of *abd-A* in females did not reduce the length of valvulae (Fig. 3J–K). However, the valvulae of *abd-A* depleted females did not nest normally with one another. When at rest, the valvulae of female *O. fasciatus* fold up and are covered by the first valvifers. However, the

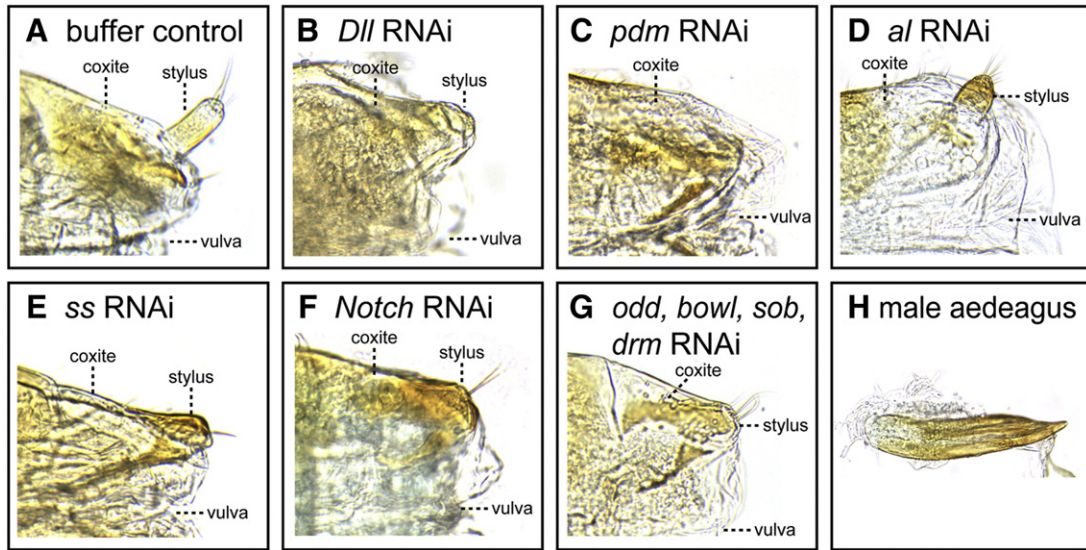


Fig. 4. The genitalia of *T. castaneum*. (A) The stylus of the ovipositor from a buffer-injected control female is joined to the dorsolateral edge of the coxite. Several prominent sensillae tip the stylus. The vulva is at the bottom edge of the panel, and anterior is to the left. All panels are similarly oriented. (B) *Dll* and (C) *pdm* RNAi completely eliminated the stylus. (D) In *al* RNAi, the stylus was reduced in length and rounded at its distal end. Depletion of *ss* (E), *Notch* (F), and *odd*-related paralogs (G) deleted the cylindrical segment of the stylus, but a bulge in the cuticle remained, as did the distal sensillae. (H) The aedeagus of a buffer-injected male. This structure was unaffected by RNAi targeting the candidate genes in this study.

valvulae of *abd-A* knockdown females were positioned abnormally and did not retract. This defect may result from homeotic transformation of the first valvulae towards the structure of the second valvulae (Fig. 3G). Additionally in *abd-A* RNAi, pigmentation of the anterior abdomen (A2–A8) was missing in both sexes. In females the sex-specific A4 sternal process was also absent. These effects resemble embryonic *abd-A* RNAi phenotypes (Angelini et al., 2005) and seem to suggest

the expansion of posterior *Abd-B* activity into more anterior abdominal segments in the absence of normal *abd-A* expression.

Abdominal-B knockdown significantly reduced both male and female genitalia. In males, the gonocoxopodite (Tukey's HSD, $p = 4.2 \times 10^{-5}$) and claspers were significantly reduced (Fig. 2I,K; Tukey's HSD, $p < 10^{-16}$). Abdominal body segments A7–A10 lacked the normal black pigmentation, although A11 remained pigmented. In females,

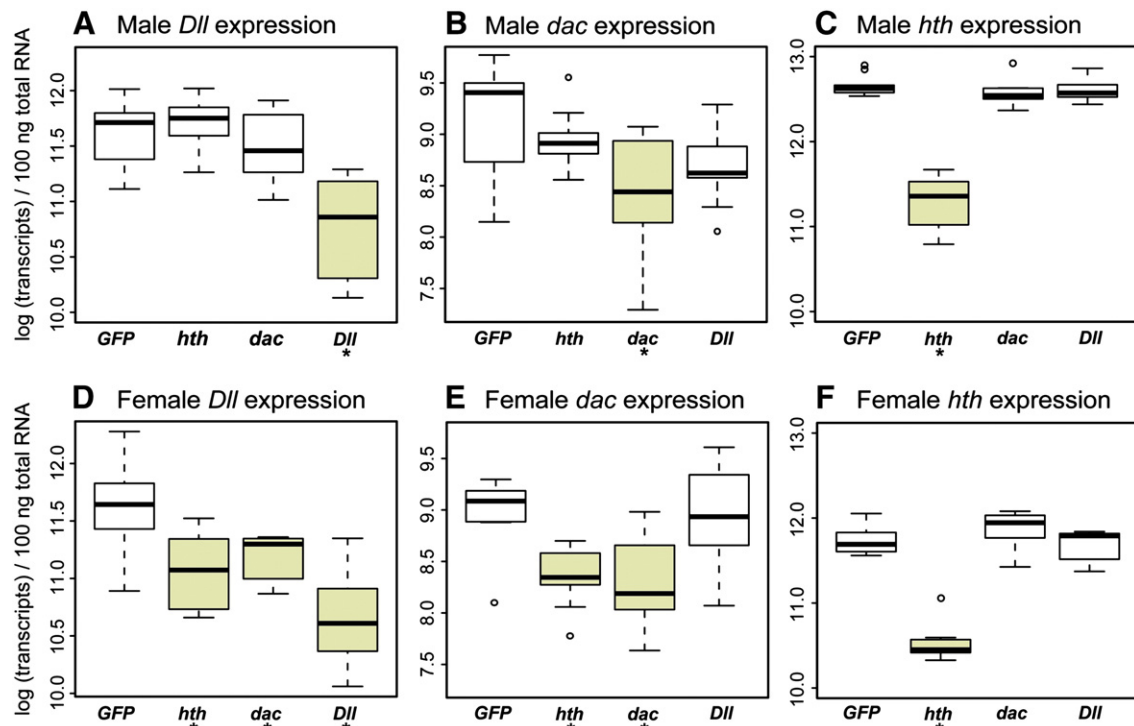


Fig. 5. Interactions among appendage-patterning genes in abdominal tissue of *O. fasciatus* during adult development of each sex. Transcript numbers of *Dll* (A,D), *dac* (B,E) and *hth* (C,F) are shown (log scale) from 100 ng of total RNA isolated from RNAi specimens. Genes targeted for RNAi were significantly reduced in expression. Significant genetic interactions were also detected (D,E) and appear to be sex-specific. Range and significance are indicated as in Fig. 2H.

both first and second valvulae were reduced (Fig. 3HJ–K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p < 10^{-16}$ for valvula 2). Moreover, the proximal valvifers were drastically reduced such that they were typically not visible beneath the overlying A7 sternite (Fig. 3H). In females, A7 lost its normal black pigmentation, although the valvifers remained black. In both sexes, the abdomen was significantly longer in *Abd-B* knockdown, relative to controls (Tukey's HSD, $p = 0.0059$).

Interactions among appendage patterning genes in the pre-adult abdomen

The expression of several genes was determined in the posterior abdomen of newly molted *O. fasciatus* adults after fourth instar RNAi treatment. This allows examination of indirect genetic interactions among genes in this region of the body (summarized in Fig. 8). In the abdomen of females, *Dll* expression was significantly reduced with the knockdown of either *dac* or *hth* (Fig. 5D), implying these genes normally promote activation of *Dll* expression. In males *Dll* expression was independent of *dac* and *hth* (Fig. 5A). A similar sex-specific difference in regulation was found for *dac*. Expression of *dac* was significantly reduced in *hth* RNAi females (Fig. 5E). In males, *hth* RNAi appeared to increase *dac* transcript levels, but the effect was not significant (Fig. 5B; $p = 0.49$). Regulation of *hth* in the posterior abdomen was independent of *dac* and *Dll*, in both sexes (Fig. 5C,F).

Because of the important role of the Hox genes in patterning the posterior abdomen, we also examined regulation of the appendage-patterning genes in this region by the Hox genes (Fig. 6). *Dll* expression was significantly reduced in posterior abdominal tissue in *abd-A* RNAi males (Fig. 6A; Tukey's HSD, $p = 5.1 \times 10^{-4}$). This suggests that *abd-A* normally activates *Dll* expression in *O. fasciatus* males. Reduction of *Dll* expression in *abd-A* RNAi females was suggested, but not significant (Tukey's HSD, $p = 0.38$). These results were surprising, since in *O. fasciatus* embryos *Dll* is repressed by *abd-A* in the abdomen (Angelini et al., 2005), and in *D. melanogaster*, *Dll* is repressed by *abd-A* in the female genital disc (Foronda et al., 2006). In pre-adult *O. fasciatus* abdominal tissue, *Dll* expression was significantly

reduced by *Abd-B* knockdown in both sexes (Fig. 6A,C), suggesting positive regulation. *dac* expression was significantly elevated by *abd-A* RNAi in females (Fig. 6D), indicating negative regulation. *hth* expression was not regulated in a sex-specific manner and increased as a result of depletion of either *abd-A* or *Abd-B* (Fig. 6C,G), suggesting that the Hox genes repress *hth* expression.

No significant transcriptional interactions were detected between the two posterior Hox genes (not shown). However, in *Abd-B* knockdown specimens the most posterior body segments adopt a more anterior fate, implying that *Abd-B* normally acts to inhibit *abd-A* activity in this region. Phenotypic posterior prevalence of Hox genes in the absence of transcription-level regulation has also been reported for *abd-A* and *Abd-B* (as well *Antennapedia* and *Ultrabithorax*) in the embryos of *O. fasciatus* (Angelini et al., 2005).

intersex RNA interference

intersex was selected for functional analysis in *O. fasciatus* as a candidate component of the somatic sex determination pathway. In *D. melanogaster*, *ix* is expressed in both male and female genital imaginal discs, but its activity is only required in females where it acts as a co-factor for *Dsx^F* (Garrett-Engle et al., 2002). Depletion of *ix* in juvenile *O. fasciatus* produced defects in both sexes, reducing the size of both male and female genitalia. In male *ix* specimens, clasper length was significantly reduced (Fig. 2J–K; Tukey's HSD, $p = 7.5 \times 10^{-4}$) and the size of the genital capsule was somewhat reduced ($p = 0.07$). In females, both genital appendage pairs were significantly reduced (Fig. 3I–K; Tukey's HSD, $p < 10^{-16}$ for valvula 1; $p = 4.0 \times 10^{-7}$ for valvula 2). The medial membranous tissue of the intervalvular space was missing, and the valvulae were more rigidly sclerotized and heavily pigmented than in control females, resembling the morphology of the male claspers. In addition to this partial sex-reversal phenotype in the genitalia, *ix* knockdown females lack the sexually dimorphic A4 sternal process. These defects suggest that females have undergone a partial transformation towards

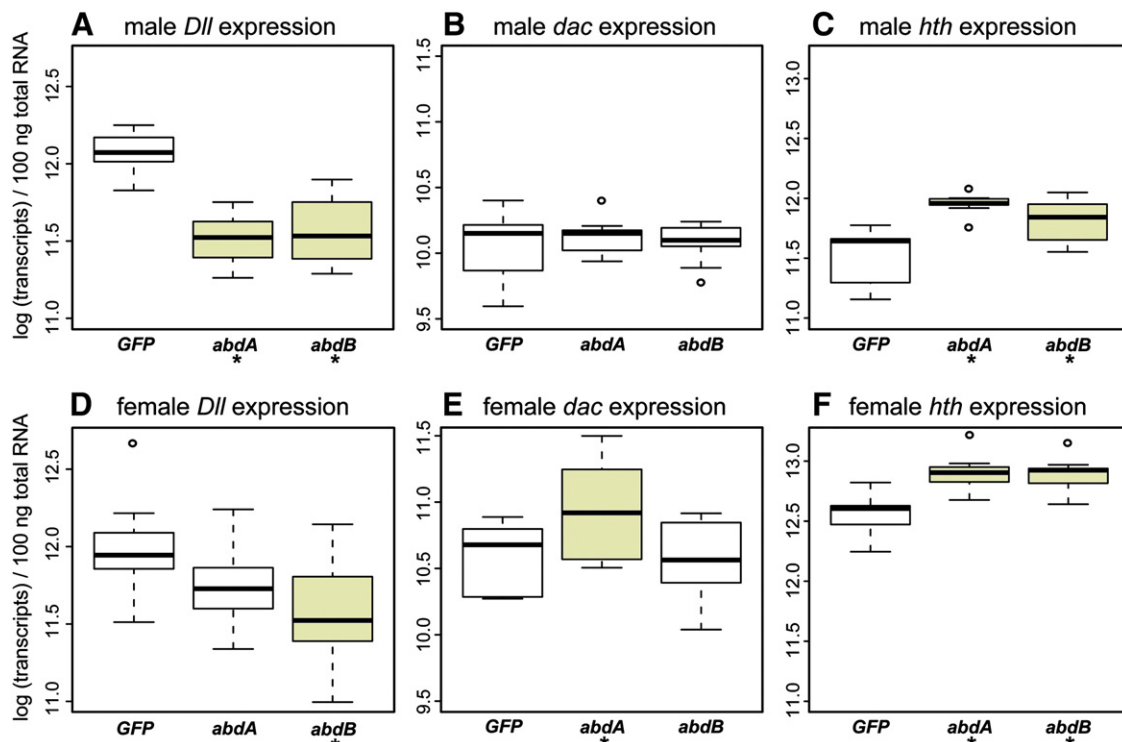


Fig. 6. Regulation of appendage-patterning genes in *O. fasciatus* by posterior Hox genes. Transcript numbers of *Dll* (A,D), *dac* (B,E) and *hth* (C,F) are shown (log scale) from GFP, *abd-A* and *Abd-B* RNAi specimens. *Dll* is activated by both Hox genes in both sexes (A,D), although the interaction is not significant in females. *abd-A* displays negative regulation of *dac* in females (E). Both Hox genes were negative regulators of *hth* in both sexes (C,F). Range and significance are indicated as in Fig. 2H.

male identity. Female-to-male sex reversal is also the main effect of *ix* mutations in *D. melanogaster* (Garrett-Engle et al., 2002).

Sex-dependence of gene interactions

To verify that the observed sex-specific gene regulation is a result of the sex of an individual, we used RNAi to manipulate the somatic sexual differentiation of milkweed bugs. As mentioned previously, *ix* RNAi partially transformed females towards male-like secondary sexual characteristics in the abdomen and genitalia (Fig. 3I). This supports the hypothesis that *O. fasciatus ix* is required for the development of female-specific phenotypes as a member of the somatic sex determination pathway, similar to the role of *ix* in *D. melanogaster*. Thus *ix*-depleted females should display male-like genetic interactions. Therefore, the regulation of *Dll* and *dac* by other appendage patterning genes was examined in an *ix*-depleted environment. In females with unmanipulated *ix* activity, but depleted for *dac* and *hth*, *Dll* transcripts were reduced, suggesting positive regulation (Fig. 7B). However these interactions were absent in male abdominal tissue (Fig. 7A). In an *ix* RNAi background, females displayed a male-like pattern of *Dll* regulation (Fig. 7C–D), with insignificant differences in expression under *dac* and *hth* RNAi. In females *dac* was also regulated by *hth*, with reduced *dac* expression under *hth* RNAi (Fig. 7F). This interaction was absent in males (Fig. 7E). With concomitant knockdown of *ix* and *hth*, *dac* transcript levels were not significantly different from *ix* depletion alone (Fig. 7H), similar to the result from males (Fig. 7G). These results implicate the somatic sex determination pathway in the regulation of *Dll* and *dac* in the developing posterior adult abdomen and confirm that regulation of these genes is sex-specific in the posterior abdomen of developing adults.

Discussion

Genitalia are abdominal appendages—at least in part

Developmental genetics may be examined as one biological level that informs considerations of homology (Wagner, 2007), and we use this data here to evaluate classical hypotheses for serial homology

of genitalia and other appendages (color-coded in Fig. 1). Appendage specification and patterning has been characterized in many insect lineages, through studies of “leg patterning” genes and Hox genes. However, outside of *D. melanogaster*, no functional studies have yet examined the development of anatomically diverse genitalia. *Dll* is a marker of appendage identity and it is known to function in distal outgrowths from the body in many animals (Panganiban et al., 1997). *Dll* expression has been described in the embryonic appendages of diverse insects (Panganiban et al., 1994). The requirement for *Dll* in development of the *O. fasciatus* claspers (Fig. 2B,K) and ovipositors of *O. fasciatus* (Fig. 3C,J–K) and *T. castaneum* (Fig. 4B) is evidence in support of the longstanding hypothesis of homology between these genitalia and the other serially homologous appendages (i.e. antenna, mouthparts and legs). The involvement of the leg patterning genes in genital development, together with previous anatomical studies (Bonhag and Wick, 1953; Marks, 1951; Sokoloff, 1972; Snodgrass, 1935; Truxal, 1952; Tschinkel and Doyen, 1980), provides compelling evidence of the appendicular origin of the genitalia. Additionally, *Dll* RNAi defects in both the first and second valvulae of *O. fasciatus* suggest that the subterminal ovipositor is composed of two pairs of appendage primordia. The failure of left and right valvulae to fuse under most RNAi treatments (Fig. 3B–E,H) also implies that the primordia grow independently and later fuse to produce the adult ovipositor.

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

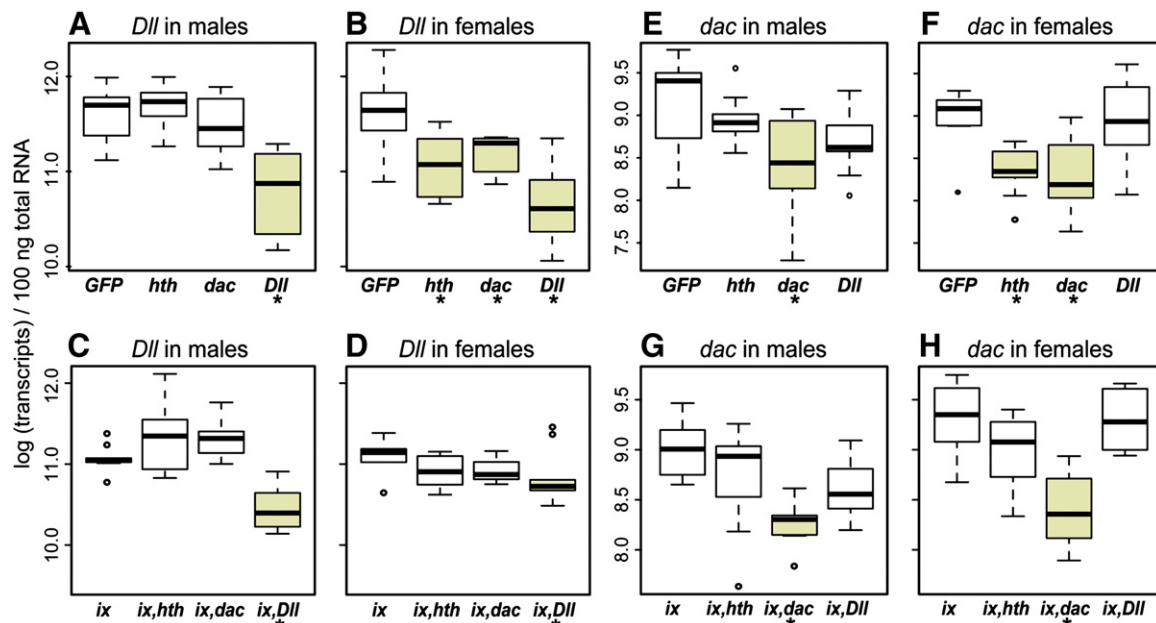


Fig. 7. *intersex* is required for female-specific gene regulation. (A) Male *Dll* expression was independent of *hth* and *dac*. (B) Female *Dll* expression was reduced in *hth* and *dac* RNAi. (C) *ix* RNAi had no effect on *Dll* regulation in males, but (D) abrogated female-specific *Dll* regulation by *hth* and *dac*. (E) Expression of *dac* in males was unaffected by RNAi targeting *hth* and *Dll*. (F) In females, *dac* expression was reduced with *hth* knockdown. (G) In an *ix*-depleted background, male regulation of *dac* remains unchanged. (H) However, in females *dac* expression returned to control levels in *ix*, *hth* double RNAi. Therefore, reduction of *ix* activity altered regulation of *Dll* and *dac* from female to male patterns. Range and significance are indicated as in Fig. 2H.

derive from the abdominal sternum (Marks, 1951; Snodgrass, 1935; Truxal, 1952).

No phenotypes were generated by knockdown of 14 appendage-patterning genes in the male *T. castaneum* aedeagus (Table 3) or the coxite of ovipositor. Therefore these structures are not likely to be derived from appendage primordia. Most authors have assumed that the coxite of terminal ovipositors is appendicular in origin (e.g. Sokoloff, 1972; Tschinkel and Doyen, 1980), but that homology is not supported by our results from *T. castaneum*. Given that *T. castaneum* and most other insects have 11 abdominal segments, it is likely that the 11th (terminal) abdominal segment forms the coxite of the ovipositor, and that the styli are the appendages of A11.

Differences in male and female genital patterning in *O. fasciatus*

Development of the *O. fasciatus* ovipositor requires *Dll*, *dac* and *hth*. Similar reductions of the valvulae were caused by the depletion of each gene (Fig. 3J–K), although *hth* was also required for proximal valvifer development. In the valvulae it is likely that the similarity in phenotypes (the overlapping level of PD defect) is due in part to the positive regulatory interactions among these genes in the female pre-adult abdomen (Fig. 8B).

Interestingly, the functions of both the appendage patterning genes and Hox genes vary between the sexes. Knockdown of *dac* and *hth* had a much greater affect on female genitalia than on males. Valvulae were consistently reduced and failed to fuse medially under *dac* and *hth* RNAi (Fig. 3C–FJ–K). In contrast, the male claspers were not significantly reduced in *hth* treatments, and although the length was significantly reduced by *dac* RNAi (Fig. 2K), the overall morphology of the claspers remained unaffected (Fig. 2C). We also found evidence for regulation of *Dll* by *dac* and *hth* in females (Figs. 5A,C). Part of the sensitivity of females to knockdown of *dac* and *hth* may stem from activation of *Dll* in this sex by these other appendage patterning genes. This difference in *Dll* regulation implies that the somatic sex determination pathway also has regulatory input on *Dll*. Transcriptional profiling of *D. melanogaster* genital discs also identified downstream targets of *dsx*, which were expressed in a sex-specific manner (Chatterjee et al., 2011). *ix* knockdown confirmed that female-specific activity of the somatic sex determination cascade has a role in *Dll* regulation, given that sex-specific *Dll* regulation was abrogated with *ix* RNAi (Fig. 7B,D).

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

Comparisons between the development of the genitalia and other appendages

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

One interesting similarity in gene function among appendages comes from the function of *hth*. In *O. fasciatus* males, *hth* RNAi caused the development of an everted and malformed copulatory organ (Fig. 2D,G). The requirement of *hth* may suggest that the proximal appendicular primordia do contribute to this normally internalized structure. The heteropteran mandibular and maxillary appendages also develop as internalized structures, the retortiform organs, before everting at hatching (and at each molt) to produce functional feeding stylets (Newcomer, 1948). These structures also require *hth* activity, and its depletion by RNAi causes an everted and malformed phenotype (Angelini and Kaufman, 2004) similar to that seen for the copulatory organ. Both the stylets and the copulatory organ have also been proposed as appendage derivatives (Minelli, 2002; Snodgrass, 1921), and *hth* is necessary to direct internalization during the development of both structures.

Comparison of genital development and patterning among insects

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

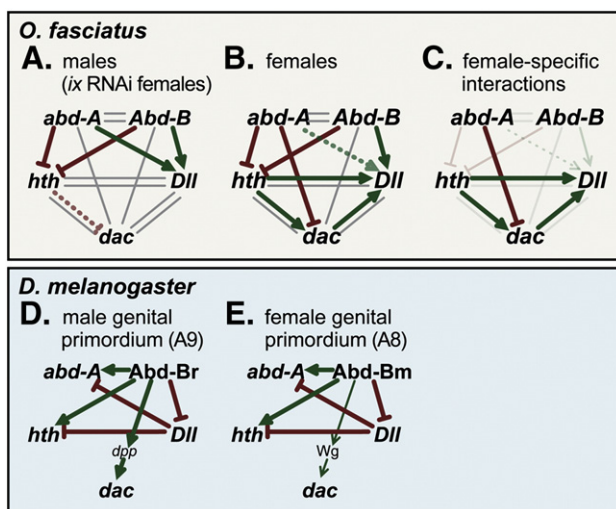


Fig. 8. Summary of regulatory interactions detected in the pre-adult posterior abdomen of *O. fasciatus* males (A) and females (B). (C) Sex-specific interactions are highlighted. Interactions of these genes in the male (D) and female (E) genital imaginal disc of *D. melanogaster*. Green arrows indicate activation; red blunted lines indicate repression. Dotted lines represent relationships that are suggestive but not statistically significant (0.05 < p < 0.1). Light gray lines indicated tested relationships for which no interaction was detected (p > 0.1).

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

In contrast to *D. melanogaster* genital development, *Abd-B* knock-down during *O. fasciatus* genital development did not produce genitalia-to-leg transformations. One possible explanation for this difference may be the inability of RNAi to provide a null phenotype. However it is also possible that while the genital primordia in fifth instar bugs require *Abd-B* for growth, they may no longer be able to adopt another appendage's developmental program. Regarding gene interactions in the developing genitalia, *O. fasciatus* and *D. melanogaster* differ substantially (Fig. 8). To highlight the obvious evolutionary divergence, it is worth noting that *abd-A* and *ix* have novel roles in male genital development not found in *D. melanogaster*. Knockdown of *abd-A* in *O. fasciatus* had a strong effect on male genital development (Fig. 2H), implying that male genitalia derive from a body segment expressing this Hox gene. In the embryo *abd-A* expression includes A2–A9, although embryonic RNAi phenotypes have not been reported for A9 (Angelini et al., 2005). Differences were not limited to external structures. *Drosophila* females have two spermathecae connected by ducts to the uterus, and these ducts are fused in *dac* mutants (Keisman and Baker, 2001). A single medial spermatheca is present in *O. fasciatus* (Bonhag and Wick, 1953), and this structure was not affected in *dac* RNAi (not shown). These differences and others in gene function and interactions likely reflect the dramatic anatomical differences and deep evolutionary divergence between these insects. Similarly, in regard to serial homology, appendage patterning genes play more prominent roles in genital structures that are more anatomically similar to other appendages, such as legs.

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

Conclusions

Genitalia are crucial for copulation and oviposition in insects. Their rapid phenotypic change and potential influence on reproductive isolation makes the underlying development and patterning of genitalia important for understanding insect evolution. We have described the patterning of genitalia in two insects from species-rich orders. Our results indicate that genital structures vary in the extent to which they derive from appendage primordia. For example, while all of the subterminal ovipositor of *O. fasciatus* was affected by knockdown of candidate appendage patterning genes, only the distal styli of the terminal *T. castaneum* ovipositor displayed a requirement for appendage-patterning genes, even with a much wider sampling of candidate genes. In the genitalia of *O. fasciatus*, we identified several sex-specific interactions among appendage-patterning genes (Fig. 7C). This regulation is mediated by the activity of *ix*, a gene involved in somatic sex determination with depletion phenotypes in both sexes of *O. fasciatus*. Therefore, variation in the

regulation and function of conserved developmental genes can play an integral role in appendage development and in the diversification of insects. A theme is emerging from the examination of the developmental genetics of diverse arthropod appendages: divergence in the degree of conservation in anatomy is correlated with divergence of an otherwise highly conserved genetic patterning system.

Database linking and accession numbers

GenBank: Genetic sequence database at the National Center for Biotechnical Information (NCBI).

(GenBank ID: AY627361, AY627362, AY584472, AY584473, AY584474, JN368475, NM_001039439, XM_964678, NM_001039400, NM_001114366, XM_969854, XM_962783, XM_963346, NM_001114381, XM_964393, XM_964994, XM_966993, XM_967045, XM_966942, XM_966887).

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