Diversification of *doublesex* function underlies morph-, sex-, and species-specific development of beetle horns

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Sex-specific trait expression is frequently associated with highly variable, condition-dependent expression within sexes and rapid divergence among closely related species. Horned beetles are an excellent example for studying the molecular basis of these phenomena because horn morphology varies markedly among species, between sexes, and among alternative, nutritionally-cued morphs within sexes. In addition, horns lack obvious homology to other insect traits and provide a good opportunity to explore the molecular basis of the rapid diversification of a novel trait within and between species. Here we show that the sex-determination gene *doublesex* (*dsx*) underlies important aspects of horn development, including differences between sexes, morphs, and species. In male *Onthophagus taurus*, *dsx* transcripts were preferentially expressed in the horns of the large, horned morph, and RNAi-mediated knockdown of *dsx* dramatically altered male horn allometry by massively reducing horn development in large males, but not in smaller males. Conversely, *dsx* RNAi induced ectopic, nutrition-sensitive horn development in otherwise hornless females. Finally, in a closely related species (*Onthophagus sagittarius*) that has recently evolved a rare reversed sexual dimorphism, *dsx* RNAi revealed reversed as well as novel *dsx* functions despite an overall conservation of *dsx* expression. This suggests that rapid evolution of *dsx* functions has facilitated the transition from a regular sexual dimorphism to a reversed sexual dimorphism in this species. Our findings add beetle horns to existing examples of a close relationship between *dsx* and sexual trait development, and suggest that *dsx* function has been coopted to facilitate both the evolution of environmentally-cued intrasexual dimorphisms and rapid species divergences in a novel trait.

cooption | polyphenism | evolutionary novelty | interchangeability | weak linkage

Exaggerated secondary sexual traits are common products of sexual selection, often diverge rapidly between closely related species, and frequently exhibit condition-dependent expression (1). In extreme cases, conditional expression of secondary sexual traits is discrete and results in the production of alternative, intrasexual morphs (2). For instance, the exaggerated horns of horned beetles are typically present only in males; have diversified dramatically in size, shape, and location across thousands of species; and—among conspecific males—frequently develop in a conditional, nutrition-dependent manner. In such cases, only males with access to optimal feeding conditions during the larval stage grow into large, major males with fully developed horns, which are then used as weapons in aggressive encounters with conspecific males. In contrast, poor larval feeding conditions result in males developing into smaller, nonaggressive sneaker or minor morphs with disproportionately smaller horns (3, 4).

In this study, we investigated whether morph-, sex-, and species-specific development of secondary sexual traits, as seen in horned beetles, may arise through the use of a shared developmental genetic machinery. In particular, we focused on the somatic sex-determination gene *doublesex* (*dsx*), for three reasons. First, recent array-based studies on horned beetles of the genus *Onthophagus* suggested that differential *dsx* expression is associated with morph-specific development of horns. Specifically, relative to the abdominal epidermis of the same individual, *dsx* expression was consistently elevated in the horn tissue, but not the legs, of horned males (5, 6).

Second, previous studies have shown that *dsx* has a highly conserved function as the terminal gene in the sex determination pathway that regulates the sex-limited expression of downstream genes, leading to sexually dimorphic development and behavior across diverse insects (7–9). The structure and function of *dsx* are best understood from studies in *Drosophila*. In *Drosophila melanogaster*, a hierarchy of sex-determination genes acts to regulate the expression of sex-specific isoforms of the Dsx transcription factor, which in turn regulates the sex-limited expression of downstream cytodifferentiation genes responsible for the elaboration of sexually dimorphic traits (10). Comparative analyses have revealed that even though the sex-determination pathway upstream of *dsx* is divergent across insect orders, *dsx* itself is highly conserved. In particular, in all insects examined thus far, the key features of *dsx* structure and function appear to be conserved—specifically, the expression of male- and female-specific Dsx transcription factors generated through alternative splicing of an exon included in *dsx* transcripts in males and excluded in females (8).

Third, recent studies have also revealed that *dsx* is a nexus for the evolution of sexually dimorphic traits; all cases understood at the molecular level involve either changes in *cis*-regulatory sequences of *dsx* target genes (11, 12) or changes in the expression of *dsx* itself (13, 14). In addition, the recent finding that developing *Drosophila* are mosaic for *dsx* expression—and thus are mosaic for the potential for sexual differentiation—has led to increased appreciation for the potential of evolution through tissue-specific changes in *dsx* activity (15). Indeed, it was recently found that the diversification of sex combs in *Drosophila* species is correlated with changes in *dsx* expression patterns (14).

Here we investigated the gene structure, expression, and function of *dsx* in two congeneric species of horned beetles, *Onthophagus taurus* and *Onthophagus sagittarius,* which exhibit highly divergent patterns of phenotype expression. *O. taurus* has a dramatic intrasexual and intersexual dimorphism in horn development typical for the genus and thought to reflect the ancestral character state of horn development in *Onthophagus*. *O. sagittarius* shared a common ancestor with *O. taurus* ~5 Mya, but has since evolved a pattern of horn development radically divergent from the ancestral pattern still seen in *O. taurus*, including the

rare expression of a reversed sexual dimorphism as well as horn development in a novel location not used by *O. taurus* or more basal branches in the *Onthophagus* phylogeny (16). We sought to investigate the degree to which *dsx* might mediate the differential development of horns across different developmental and evolutionary contexts. Our results suggest that *dsx* functions to regulate sex-specific development of horns, that this function has been coopted to enable the nutritionally-cued development of alternative male morphs, and that *dsx* function has further diversified to facilitate rapid and extreme species divergences in the expression of a novel trait.

Results and Discussion

dsx Gene Structure and Expression Are Conserved in *O. taurus* Relative to Other Insects.

We first investigated the gene structure and function of *dsx* in *O. taurus*, a species with a typical intrasexual and intersexual dimorphism in horn development (Figs. 1 and 2). The *O. taurus dsx* gene architecture and inferred protein sequence are similar to those in other insects (Fig. 1 *A* and *B*). Specifically, *O. taurus* expresses sex-specific *dsx* mRNA isoforms (Fig. 1 *B* and *C*), with one isoform found only in males (*Otdsxm*), and at least five isoforms found only in females (*Otdsxf1–5*). In addition, we detected

a single *dsx* isoform that was expressed in both sexes (*Otdsxb*; Fig. 1 *B* and *C*).

Each of the *Otdsxf* transcripts has female-specific sequences, including translation termination codons, that are absent from the *Otdsxm* and *Otdsxb* transcripts (Fig. S1 *A* and *B*). Thus, the organization of female-specific transcripts is most similar to that of the silkmoth *Bombyx mori*, which also expresses multiple *dsxf* transcripts that encode DsxF protein isoforms with different C-terminal sequences (8, 17) (Fig. S1C). Unlike in *Drosophila*, the male *dsx* isoform shares all of its putative coding exons with the female isoforms (Fig. 1*B* and Fig. S1*A*). We found that when translated, all sex-specific isoforms have all of the functional domains commonly present in other insects, including the oligomerization domains (ODs) 1 and 2 (Fig. S1 *B–D*). Specifically, overall identity of translated sequences is 46% for the male-specific isoform, 45–47% for DsxF1, and 46–47% for DsxF2 compared with *B. mori*. The predicted OD1 (also known as DM domain) and OD2 share 93% and 55% sequence identity, respectively, with the corresponding regions in *D. melanogaster*. In contrast, the *dsx* transcript shared by both sexes (*Otdsxb*) includes at least one exon that is absent from any of the other isoforms and lacks obvious similarities to published *dsx* sequences (Fig. S1*B*). Moreover, the *Otdsxb* transcript does not encode OD2 (Fig. S1 *A* and *B*). A sex-shared insect *dsx* isoform, also lacking OD2, has so far been reported only from the honeybee (18), and its functional significance, if any, remains unclear. Combined, these data suggest that male- and female-specific *Otdsx* isoforms may function as sex-specific transcription factors, but also raise the possibility that non–sex-specific functions may be performed by an additional, distinct *dsx* isoform whose expression is shared across sexes.

dsx Controls Sex-Specific Development of Horns in *O. taurus*.

To investigate possible functions of male, female, and nonspecific *dsx* isoforms, we used RNAi to knock down *dsx* expression. We then used morphometric measurements on the resulting pupae and adults to quantify the effects of *dsx* knockdown on male and female horn development compared with individuals receiving mock double-stranded RNA (dsRNA) injections and untreated WT individuals. We first injected dsRNA directed to the transcript region unique to *Otdsxb*, the isoform expressed in both sexes (indicated by the black bar in Fig. 1*B* and Fig. S1*A*). Compared with mock injection, *Otdsxb* dsRNA had no apparent effects on horn development (Fig. S2), genital development, or any other phenotypic trait in males or females. These findings suggest that this isoform may lack a function during sexual differentiation, at least to the limits of detection of our experiment.

We then investigated possible functions of *Otdsxm* and *Otdsxf*. Because these isoforms are spliced strictly in a sex-specific manner, we used dsRNA directed to the common region to knock down the expression of *Otdsxm* in males and *Otdsxf* in females. We refer to this construct as *OtdsxC* dsRNA (indicated by the gray bar in Fig. 1 and Fig. S1*A*). We replicated this approach using dsRNA directed to a gene region specific to female isoforms (*Otdsxf* dsRNA, indicated by the orange bar in Fig. 1*B* and Fig. S1*A*). However, because all coding regions of *Otdsxm* are also contained within the transcripts of female-specific isoforms, it was not possible to design a corresponding dsRNA construct that would affect only *Otdsxm*.

Injecting *OtdsxC* dsRNA into third instar larvae resulted in decreased expression of *dsx* in both sexes (Fig. S3*A*), defects in the development of male genitalia (Fig. S3 *B* and *C*), and obvious alterations to horn development in both sexes (detailed below), validating the effectiveness of our approach. Specifically, knocking down *dsx* dramatically affected the sexually dimorphic development of horns in *O. taurus*. Large adults normally express sexually dimorphic head horns, such that large males have prominent paired horns on the posterior head, whereas females feature a small continuous ridge in the corresponding location. *Otdsx*
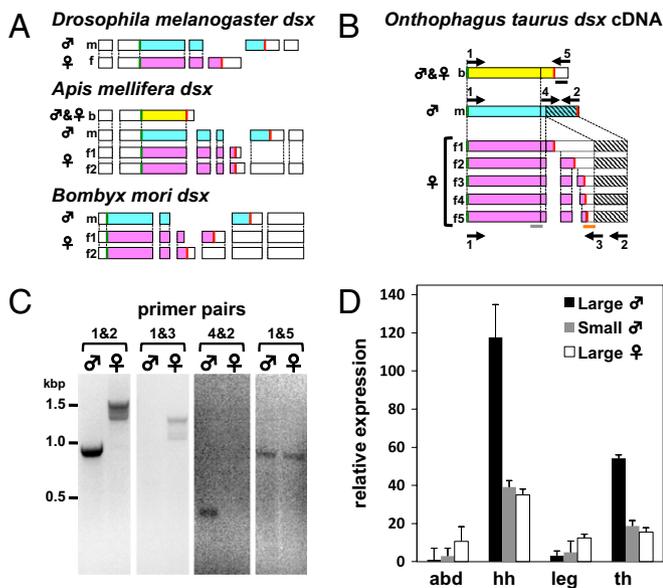
Fig. 1. Structure and expression of *O. taurus dsx*. (*A*) *dsx* transcripts in *D. melanogaster* (*Top*), *A. mellifera* (*Middle*), and *B. mori* (*Bottom*). The gene structures of transcripts are indicated by rectangles, with ORFs shaded either blue (male), pink (female), or yellow (non–sex-specific isoform). Start and stop codons are indicated by green and red lines, respectively. (*B*) Cloned cDNA and inferred ORFs of *O. taurus dsx*, with the same color coding as in *A*. The 3′ region of *Otdsxm* is shared with five *Otdsxf* isoforms (shaded by hatched lines). Note that *D. melanogaster dsx*, *A. mellifera dsx*, and *B. mori dsx* are shown based on genomic structures, whereas *Onthophagus dsx* is reconstructed based on cDNA sequence matches across transcripts. Putative splice donor and acceptor sites in *Otdsxf2–5* are shown in Fig. S1. The horizontal solid gray bar on *O. taurus dsx* indicates the region used to generate *dsxC* dsRNA and used for qRT-PCR. Orange and black solid horizontal bars indicate the regions used to generate *dsxf* dsRNA and *dsxb* dsRNA to knockdown female and sex-shared *dsx* splice variants, respectively. Arrows and numbers indicate primers used to assess gene structures. (*C*) RT-PCR results confirm sex-specific splicing pattern of *O. taurus dsx*. The RT-PCR primer pairs correspond to those shown in *B*. (*D*) Relative expression of *dsx* (qRT-PCR, estimates relative to *actin*; 1 indicates *dsx* expression in the abdominal epithelium of large males) in the indicated tissues [abd, abdominal epithelium; hh, head horn; leg, all six legs combined; th, thoracic (pronotal) horn] of *O. taurus* large males (black bars), small males (gray bars), and large females (white bars).
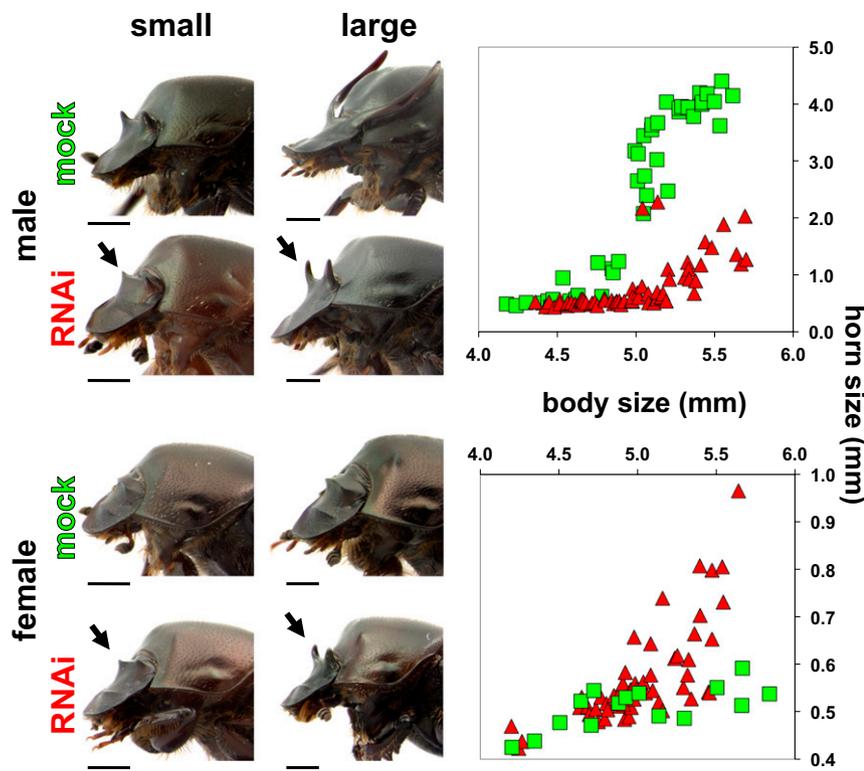
Fig. 2. Effects of *dsx* RNAi on horn development in adult *O. taurus* males and females. (*Left*) Representative animals obtained after mock (control) injections (*Upper*) and *dsx* dsRNA injections (*Lower*). Small individuals are shown on the left; large individuals, on the right. Anterior is to the left, and filled arrows indicate locations of head horn development of RNAi individuals. (Scale bars: 1 mm.) (*Right*) Bivariate plots of body size (*x*-axis) and head horn length (*y*-axis) for male (*Upper*) and female (*Lower*) *O. taurus*. Mock-injected and RNAi individuals are plotted as green squares and red triangles, respectively. The *dsx* RNAi substantially reduced horn expression in large males ($T_{115} = 6.82$, $P < 0.001$) and induced conspicuous ectopic head horns in females (ANOVA, $F_{2,104} = 9.35$, $P < 0.001$).

knockdown dramatically reduced the development of head horns in large males (Fig. 2; *t* test, $T_{115} = 6.82$, $P < 0.001$), whereas knocking down *dsx* in large females induced the development of paired ectopic head horns (Fig. 2; ANOVA, $F_{2,104} = 9.35$, $P < 0.001$). Injecting *Otdsxf* dsRNA, which specifically targets *Otdsxf* isoforms only, similarly induced paired ectopic head horns in females but left male horn development completely unaffected (Fig. S4). Collectively, these results indicate that *Otdsxm* promotes horn development in large males, whereas *Otdsxf* inhibits horn formation in females, and that reduced expression of either *Otdsxm* or *Otdsxf* causes males and females to default to a similar small-horned phenotype.

Similar sex-specific effects on horn growth were also observed in thoracic (pronotal) horns in pupae. Pupal thoracic horns are ubiquitous in *Onthophagus* and function as molting organs, designed to remove the rigid larval head capsule during the larval-to-pupal molt (19). Secondary cooption of pupal thoracic horns into the adult stage is believed to have enabled the evolution of adult thoracic horns seen now in a subset of extant species (19, 20). In *O. taurus*, pupae develop conspicuous thoracic horns that are completely resorbed before the pupal-adult molt through programmed cell death in both sexes (19, 21). Moreover, in this species, pupal thoracic horns are sexually dimorphic, with males having larger horns than females (21). *dsx* RNAi reduced pupal thoracic horn length in males (ANOVA, $F_{2,156} = 12.75$, $P < 0.001$), but increased it in females (ANOVA, $F_{2,118} = 13.02$, $P < 0.001$), again causing both sexes to converge toward an intermediate phenotype when *dsx* function was reduced (Fig. S5). In contrast, mock injections using nonsense dsRNA did not affect horn location and allometries in male and female *O. taurus* pupae or adults compared with untreated WT individuals (Fig. S6).

Nutrition Influences *dsx* Function in Regulating Sex-Specific Horn Development. Normally, the development of head horns in males differs distinctly among morphs. Large, well-nourished males have prominent horns nearly 10-fold longer than those of smaller males that received suboptimal nutrition (Fig. 2). If *dsx* regulates horn

development independent of nutritional conditions, we would predict *dsx* RNAi to result in the same relative reduction in horn length regardless of male body size. Instead, sensitivity to *dsx* knockdown increased dramatically with adult body size, ranging from no change to only modest reductions in horn length in small and medium-sized males to as much as an 80% reduction in horn length in the largest males (Fig. S7). A nutrition-dependent *dsx* RNAi response was also observed, albeit to a lesser extent, in females. Normally, the posterior ridge on the female head is proportional to body size (Fig. 2). Knocking down *Otdsxf* in large females resulted in the development of conspicuous ectopic horns similar in size to those small WT males and large *Otdsxm* RNAi males. However, the effect in small females was more subtle and involved the induction of distinct lateral points but without altering the overall height of the ridge. Similar results were obtained when female larvae were injected with female isoform-specific *Otdsxf* dsRNA (Fig. S3). These data support the hypothesis that the function of *dsx* in regulating sex-specific horn development is conditional on nutritional conditions.

Tissue-Specific and Nutrition-Dependent Expression of *Otdsxm* May Mediate *dsx*-Dependent Horn Development in Alternative Male Morphs. How does nutrition-dependent regulation of horn development intersect with the *dsx* dependent regulation of horn development? Nutrition-dependent regulation could act independent, or downstream, of *dsx* for example, influencing the sensitivity to *dsx* regulation. Alternatively, nutrition-dependent regulation could act through *dsx*; for example, in well-nourished males, *dsx* expression could be up-regulated in body regions fated to undergo exaggerated growth. To test the latter hypothesis, we examined *dsx* expression in the developing horn (head, thorax) and non-horn (leg, abdomen) tissues of large and small males as well as females. We found that *Otdsxm* expression is by far highest in the head horn primodia of large males (which exhibit the most dramatic growth response to increased nutrition), followed by the thoracic horn primordia of large males (which exhibit a less extreme response), but low in the head and thoracic horn

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primordia of small males and in the legs and abdomen regardless of male size. In contrast, in females *Otdsxf* levels remained more uniform across all tissue types (Fig. 1*C*). These findings are consistent with the model that the development of exaggerated horns in large males is associated with tissue-specific, nutrition-dependent expression of *Otdsxm* in the horn primordia of large males.

Reversed and Novel *dsx* Functions Underlie Recently Evolved Reversed Sexual Dimorphism in the Congener *O. sagittarius* Despite Conservation of *dsx* Expression. We next investigated whether lineage-specific changes in *dsx* function may have mediated the evolution of radically different patterns of sex-specific trait expression, such as reversed sexual dimorphisms. Here we used *O. sagittarius*, a species that has evolved a radically divergent pattern of horn development not seen elsewhere in the genus. Recall that *O. sagittarius* shared a common ancestor with *O. taurus* ~5 Mya, but has since evolved a pattern of horn development radically divergent from the ancestral pattern still seen in *O. taurus*. Most obvious is the expression of a reversed sexual dimorphism, with female *O. sagittarius* expressing single long medial thoracic and head horns that are lacking in males (Fig. 3). Careful analysis of the location of head horn development suggests that the location of female head horn development in *O. sagittarius* is homologous to that of the paired posterior head horns in male *O. taurus* (22). If and how female *O. sagittarius* use their horns in reproduction is presently unknown. In addition,

adult male *O. sagittarius* express a pair of small head horns in a novel location near the front of the head. Anterior head horns present in *O. sagittarius* are not present in *O. taurus* or more basal branches in the phylogeny and thus are believed to have arisen since the divergence of *O. sagittarius* and *O. taurus* (16). Finally, and again in contrast to *O. taurus*, no dimorphism exists within male or female *O. sagittarius*. Instead, in both sexes, large individuals essentially represent proportionally enlarged versions of small individuals (21, 23), which reduces the need for allometric analyses in this species.

We investigated structures and functions of male and female *dsx* isoforms in *O. sagittarius*. Sequencing cDNA clones and RT-PCR experiments revealed that *O. sagittarius* also expresses male- and female-specific *dsx* transcripts (Fig. S8). The inferred splicing patterns and translated protein sequences of male and female *dsx* isoforms are highly similar to those in *O. taurus* (Fig. S1 *E* and *F*). Thus, the reversed sexual dimorphism in horn growth in *O. sagittarius* is not related to reversal of the sex-specific splicing of *dsx* isoforms in developing horns. In contrast to our results for *O. taurus*, we failed to clone a *dsxb* isoform in *O. sagittarius*. However, this does not rule out the possibility that *O. sagittarius* also expresses this isoform.

We investigated possible functions of *Osdsxm and Osdsxf* using dsRNA injections targeting the same transcript region shared across isoforms as used in *O. taurus*. As with *O. taurus*, *dsxC* RNAi resulted in malformation of male genitalia (Fig. S3*B*), but resulted in a more complex suite of changes in horn development. Specifically, *dsx* knockdown in *O. sagittarius* revealed three major effects. First, *dsx* RNAi substantially reduced thoracic horn size in females and induced a small but conspicuous protrusion in the male prothorax (Fig. 3, filled triangles). This indicates that *Osdsxm* functions to inhibit growth of the thoracic horn, whereas *Osdsxf* functions to promote growth. Thus, the sex-specific functions of *dsx* in regulating thoracic horn growth are reversed in *O. sagittarius* compared with *O. taurus*.

Second, *dsx* RNAi had no obvious effect on the development of anterior head horns in males, but caused females to express a pair of ectopic, male-like anterior head horns (Fig. 3, open triangles). This indicates that *Osdsxf* functions to inhibit the growth of anterior head horns in females. Although the direction of the effect was the same as that seen in *O. taurus* females, the specific head region affected differed distinctly.

Third, *dsx* RNAi resulted in the development of surprisingly large, branched ectopic horns in the posterior head in males, and the transformation of the large single horn normally seen in females to a branched horn (Fig. 3, orange triangles). In male *O. sagittarius*, *dsx* thus functions to inhibit the growth of branched posterior head horns. This suggests that similar to the situation for the thoracic horn, the male-specific function of *dsx* in regulating posterior head horn development is reversed in *O. sagittarius* compared with *O. taurus*; *dsxm* promotes the development of paired posterior horns in male *O. taurus*, but inhibits it in *O. sagittarius*. In contrast, *dsx* RNAi in female *O. sagittarius* did not result simply in a reduction of the single medial head horn, as would be predicted if *Osdsxf* functioned exactly opposite to *Otdsxf*, that is, as a promotor of horn growth. Instead, *Osdsxf* promotes transformation of a branched into an unbranched posterior head horn. All of the phenotypes described above were observed in 100% of *dsx* RNAi individuals ($n = 15$ per sex), and in no control-injected individuals ($n = 98$).

Taken together, these findings suggest that *dsx* RNAi phenotypes observed in *O. sagittarius* reflect a mosaic of (*i*) reversed *dsx* functions relative to *O. taurus* and the presumed common ancestor, as seen in the regulation of thoracic horns (both sexes) and posterior head horns (males), and (*ii*) novel *dsx* functions, as seen in the regulation of anterior head horn size and posterior head horn shape in females.
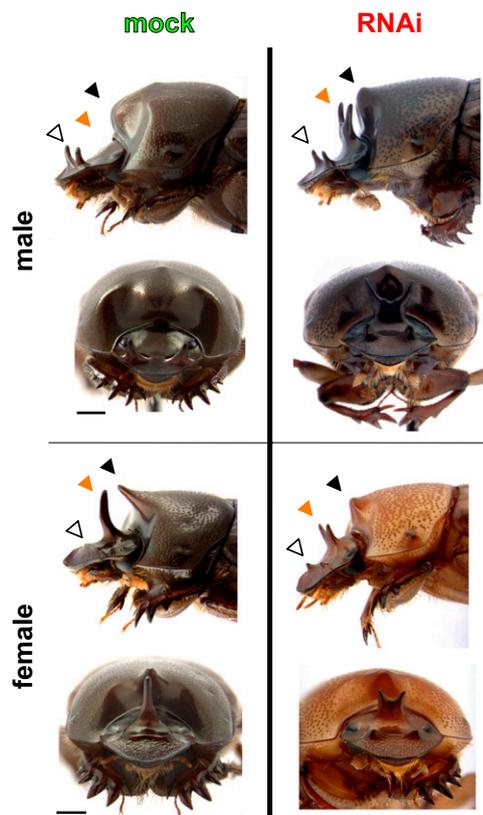
Fig. 3. Effects of *dsx* dsRNA injection on horn development in *O. sagittarius* males (*Upper*) and females (*Lower*). Shown are lateral (*Upper* row) and frontal (*Lower* row) views of representative animals, with mock-injected individuals on the left and dsRNA-injected individuals on the right. Horn development did not differ between mock-injected individuals and WT individuals. Open triangles indicate anterior head horns in mock-injected males and the corresponding region in females. Orange triangles indicate posterior head horns in mock-injected females and the corresponding region in males. Filled triangles indicate thoracic horns. (Scale bars: 1 mm.)

EVOLUTION

dsx and the (Co)evolution and Diversification of Sexual and Male Dimorphism. Our results significantly expand our knowledge of the range of evolutionary changes mediated by *dsx*. First, our findings add beetle horns to the list of secondary sexual traits whose development is regulated by sex-specific *dsx* splice forms. Moreover, our results suggest that the location, direction, and nature of *dsx*-mediated regulation of horn development have diversified rapidly among closely related species, enabling in at least one instance the evolution of a dramatic reversal of sexually dimorphic trait expression. For instance, whereas in *O. taurus*, *Otdsxm* functions to promote posterior head horns in males and *Otdsxf* inhibits them in females, their orthologs in *O. sagittarius* function to inhibit posterior head horns in males but promote their fusion into a single horn in females.

Second, our results suggest that, at least in *O. taurus*, *Otdsx* expression may be influenced by nutritional conditions, and that differential expression of *Otdsxm* may underlie the development of alternative, nutritionally-cued male morphs. These observations parallel in part earlier findings in *Nasonia* wasps, in which differential *dsx* expression in males has been proposed to underlie species-specific differences in male wing size (13). However, *dsx* expression in male *Nasonia* differs constitutively between species, whereas in male *O. taurus* it appears to be altered facultatively in response to nutrition. If correct, the use of *dsx* as a regulator of both sexual and male dimorphism also may explain the tight co-evolution of both patterns of phenotype expression in *Onthophagus* reported by earlier phylogenetic studies (24), which concluded that 19 of 20 instances of change in sexual dimorphism (gain or loss) were paralleled by a corresponding gain or loss of male dimorphism. Whereas these earlier studies suggested shared endocrine mechanisms as the underlying cause of this striking covariation, our findings offer an alternative, or additional, explanation: in *O. taurus*, a species typical for the genus, *Otdsxm* appears to execute a dual function, promoting sex-specific horn development in males and facilitating the dimorphic development of horns in males of different body sizes. Thus, concerted gain and loss of sexual and male dimorphism may be a consequence of lineage-specific gain and loss of the role of *dsx* in the promotion of male horns.

More generally, our findings illustrate a phenomenon considered of critical importance in the evolution and diversification of novel traits: the lability of regulatory inputs, such as sexual identity or nutritional conditions, and target traits, such as genitalia or different horn types, linked through an otherwise highly conserved switch mechanism, *dsx*. This phenomenon, referred to as "interchangeability" (25) or "weak linkage" (26), is thought to facilitate the origin and diversification of form and function because it enables novel combinations of inputs and outputs without requiring their de novo evolution, while simultaneously producing functional and integrated phenotypes through the use of a conserved regulatory and developmental machinery. In the context of horned beetles, such interchangeability not only might have facilitated the evolution of sexual and male dimorphisms, but also might have occurred repeatedly and convergently for different horn types, further facilitating their subsequent diversification. For instance, both functional and phylogenetic studies suggest that head and thoracic horns developed, and have evolved, largely independent of one another (16, 24).

Despite this independent history, based on the present study, sex- and morph-specific development of both horn types appears to be regulated by *dsx* in extant onthophagine species, consistent with convergent cooption of *dsx* in the developmental regulation of different horn types. Furthermore, comparative morphological studies have shown that head and thoracic horns in the same species can differ widely in the degree to which their development is affected by sex and nutrition, suggesting that sex- and nutrition-dependent horn development can diversify independently for different body regions (21, 23, 24, 27). Taken together,

our results paint an extraordinarily dynamic picture of *dsx* evolution and its role in the origin and diversification of sexual and male dimorphism in horned beetles.

Materials and Methods

Beetle Care. Beetles used in this study were collected and reared essentially as described previously (28). In brief, *O. taurus* was collected near Bloomington, IN and Chapel Hill, NC, and *O. sagittarius* was collected near Kaneohe, Hawaii. Adults were kept at 25 °C (*O. taurus*) and 28 °C (*O. sagittarius*) in a sand/soil mixture at a 16:8 h light:dark cycle and fed homogenized cow manure twice a week. To obtain larvae, five females and three males were selected and kept in a small plastic container with packed, moist sand/soil mixture with cow manure and allowed to produce brood balls. Brood balls were collected after 8 d, and larvae were transferred to 12-well plates as described previously (29).

Cloning and Sequencing of *Onthophagus dsx*. The *dsx* sequence information was first obtained from a previously published EST sequence (5). RT-PCR was used to amplify *dsx* cDNA fragments from both male and female RNA samples; primer sequences are listed in Table S1. Fragments were subsequently cloned into pSC-A vector using a StrataClone PCR Cloning Kit (Agilent). Sequencing reactions from cloned fragments were carried out using BigDye cycle sequencing chemistry (Life Technologies). All 12 cDNA fragments isolated in this work were sequenced and submitted to GenBank (accession nos. JN165757–JN165768).

dsRNA Generation and Injection. dsRNA to knock down *dsx* splice variants was generated as described previously (30). In brief, the region of interest (Fig. 1) was cloned using a StrataClone PCR Cloning Kit, followed by BigDye sequencing. The vector containing the fragment was purified using a QIAprep Spin Miniprep Kit (Qiagen). The vector was then subjected to PCR using M13 forward and reverse primers, and the PCR product was used as a template for in vitro transcription. Forward and reverse RNA strands were produced using MEGAscript T7 and T3 Kits (Life Technologies), treated by DNase I to remove DNA template, precipitated by ethanol, denatured at 75 °C for 10 min, chilled on ice, and mixed at a 1:1 ratio by weight. The resulting mixture was incubated in a water bath at 80 °C until room temperature was reached. The concentration of the annealed RNA was measured, confirmed by gel electrophoresis, and stored at −80 °C until injection. Injections into *Onthophagus* larvae were carried out as described previously (30), with 0.5 μg of dsRNA injected into larvae during the first 5 d of the final, third instar. Approximately 200 *O. taurus* larvae and 150 *O. sagittarius* larvae were injected with dsxRNA constructs over the course of the experiment. The same procedure was performed to knock down female-specific variants, as well as the splice variant present in both sexes.

Rapid Amplification of cDNA Ends for *dsx*. Rapid amplification of cDNA ends (RACE) was performed to obtain possible additional *dsx* splice variants in *Onthophagus*, using the FirstChoice RLM-RACE Kit (Life Technologies) in accordance with the manufacturer's instructions. Specifically, the aim was to determine the existence of possible variants whose expression is shared between both sexes. Primers were designed for the same region used for *dsxC* dsRNA generation; primer sequences are listed in Table S1; 3′- and 5′-RACE were performed, and the products from males and females were cloned and sequenced as described above. Then primers were designed to amplify fragments containing the sequences obtained from both 3′- and 5′-RACE, to confirm that both RACE products were derived from the same splice variant.

Mock Injections. Mock (control) injections were carried out as described previously (30). In brief, animals were reared under the same conditions as RNAi-injected animals, but were injected with dsRNA from a 167-bp PCR product derived from a pBluescript SK vector. Transcription reactions, DNaseI treatment, and transcript annealing were performed as descrived above. A total of 1 μg of dsRNA was injected into larvae during the first 5 d of the final, third instar. Approximately 70 *O. taurus* and 80 *O. sagittarius* larvae received mock injections.

Allometric Measurements. RNAi-treated and control pupae and adults were measured using a 2D image analysis setup consisting of a dissecting microscope (Leica) mounted with a digital camera (Scion) and ImageJ software (31). Thorax width served as a measure of pupal and adult body size. Head length and thoracic horn length were measured as described previously (21). Measurements were recorded to the nearest 0.01 mm.

Imaging. Beetle images were collected using the same setup as used for allometric measurements. In most cases, 5–10 images with different focal planes were aligned and merged using Adobe Photoshop Creative Suite 4.

Statistical Analysis. Allometric scaling relationships of head and pronotal horns were analyzed similarly to previous studies (21, 30, 32, 33). In brief, because female head horns and pupal thoracic (pronotal) horns of both sexes exhibit linear scaling relationships, effects of treatment (*dsx* dsRNA injections, mock injections, and WT) were compared using ANOVA, with horn length as the response variable and body size and treatment as model effects. Analyses were repeated for each sex, followed by pairwise comparisons using Tukey's HSD test. In males, in contrast, head horns exhibit a strongly sigmoidal allometry, and thus horn length residuals were compared across treatment groups. Residuals were calculated as the difference between horn lengths observed in a given individual and those expected for the individual's body size given the WT scaling relationship. Two-tailed *t* tests were used for pairwise comparisons.

Quantitative RT-PCR. *Quantification of* dsx *in WT animals.* Quantitative RT-PCR (qRT-PCR) was performed to detect *dsx* expression levels in *O. taurus* in different body regions. Head horns, pronotal horns, legs, and abdominal epithelium of ~24-h-old pupae were dissected, and total RNA was purified using a Qiagen RNeasy Kit, followed by on-column genomic DNA digestion (Qiagen). Primers to detect *dsx* were dsxqPCRF01 and dsxqPCRR01 (Integrated DNA Technology); sequences are listed in Table S1. *dsx* expression level was normalized using actin as an internal control. The same primer set was used for actin as in a previous study (34). Two-step qRT-PCR was performed using a QuantiTect Reverse-Transcription Kit and QuantiTect SYBR Green PCR Kit (Qiagen) on a Stratagene MX3000P system (Agilent). Three independent sets of tissue samples from large males, small males, and females (i.e., 4 tissues × 3 morph/sex × 3 biological replicates) were harvested and subjected to qRT-PCR, with two technical replicates for each sample.

Equal volumes of fractions of all RNA samples were pooled and vacuum-concentrated, then treated along with all remaining samples. The mixed sample was serially diluted to obtain a dilution series ranging from 0.01 ng to 10 ng of template RNA, which was subsequently subjected to PCR to confirm that both genes amplified with similar PCR efficiency (28, 29). A 40-ng RNA sample was used in each PCR, with a final concentration of 0.3 μM for primer sets. Reaction conditions were 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. After PCR, the temperature was raised to 95 °C to observe dissociation curves. The ΔΔCt method was used to obtain relative expression levels of *dsx* across tissues, morphs, and sexes (35, 36). Relative *dsx* expression values were obtained in reference to the abdomen of large males. The same procedure was repeated three times with different sets of RNA samples, to confirm reliability of the experiments.

Quantification of dsx *in control-injected and* dsx *dsRNA-injected animals.* Approximately 24-h-old pupae were used for validation of *dsx* knockdown by qRT-PCR. Total RNA was extracted from whole bodies of six large *dsx* dsRNA-injected males and females, as well as three large WT males and females, using TRI Reagent (Life Technologies), following the manufacturer's standard protocol. qRT-PCR was performed separately on each independent set of samples. Each reaction was duplicated during the PCR step. A total of 100 ng of total RNA was treated and analyzed as described above. Knockdown efficiency was assessed relative to *dsx* expression levels detected in WT individuals. The experiment was repeated three times with different RNA samples.

1. Emlen DJ (2008) The evolution of animal weapons. *Annu Rev Ecol Evol Syst* 39: 387–413.
2. Andersson MB (1994) *Sexual Selection* (Princeton Univ Press, Princeton), pp 1–599.
3. Moczek AP, Emlen DJ (1999) Proximate determination of male horn dimorphism in the beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *J Evol Biol* 12(1):27–37.
4. Moczek AP, Emlen DJ (2000) Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: Do alternative reproductive tactics favour alternative phenotypes? *Anim Behav* 59(2):459–466.
5. Kijimoto T, Costello J, Tang Z, Moczek AP, Andrews J (2009) EST and microarray analysis of horn development in *Onthophagus* beetles. *BMC Genomics* 10:504.
6. Snell-Rood EC, et al. (2011) Developmental decoupling of alternative phenotypes: Insights from the transcriptomes of horn-polyphenic beetles. *Evolution* 65(1):231–245.
7. Sánchez L (2008) Sex-determining mechanisms in insects. *Int J Dev Biol* 52(7):837–856.
8. Shukla JN, Nagaraju J (2010) *Doublesex*: A conserved downstream gene controlled by diverse upstream regulators. *J Genet* 89(3):341–356.
9. Williams TM, Carroll SB (2009) Genetic and molecular insights into the development and evolution of sexual dimorphism. *Nat Rev Genet* 10(11):797–804.
10. Cline TW, Meyer BJ (1996) Vive la différence: Males vs females in flies vs worms. *Annu Rev Genet* 30:637–702.
11. Shirangi TR, Dufour HD, Williams TM, Carroll SB (2009) Rapid evolution of sex pheromone-producing enzyme expression in *Drosophila*. *PLoS Biol* 7(8):e1000168.
12. Williams TM, et al. (2008) The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. *Cell* 134(4):610–623.
13. Loehlin DW, et al. (2010) Non-coding changes cause sex-specific wing size differences between closely related species of *Nasonia*. *PLoS Genet* 6(1):e1000821.
14. Tanaka K, Barmina O, Sanders LE, Arbeitman MN, Kopp A (2011) Evolution of sex-specific traits through changes in HOX-dependent *doublesex* expression. *PLoS Biol* 9(8):e1001131.
15. Robinett CC, Vaughan AG, Knapp JM, Baker BS (2010) Sex and the single cell, II: There is a time and place for sex. *PLoS Biol* 8(5):e1000365.
16. Emlen DJ, Marangelo J, Ball B, Cunningham CW (2005) Diversity in the weapons of sexual selection: Horn evolution in the beetle genus *Onthophagus* (Coleoptera: Scarabaeidae). *Evolution* 59(5):1060–1084.
17. Ohbayashi F, Suzuki MG, Mita K, Okano K, Shimada T (2001) A homologue of the *Drosophila doublesex* gene is transcribed into sex-specific mRNA isoforms in the silkworm, *Bombyx mori*. *Comp Biochem Physiol B Biochem Mol Biol* 128(1):145–158.
18. Cho S, Huang ZY, Zhang J (2007) Sex-specific splicing of the honeybee *doublesex* gene reveals 300 million years of evolution at the bottom of the insect sex-determination pathway. *Genetics* 177(3):1733–1741.
19. Kijimoto T, Andrews J, Moczek AP (2010) Programed cell death shapes the expression of horns within and between species of horned beetles. *Evol Dev* 12(5):449–458.
20. Moczek AP (2009) The origin and diversification of complex traits through micro- and macroevolution of development: Insights from horned beetles. *Current Topics in Developmental Biology*, ed Jeffrey WR (Academic, New York), Vol 86, pp 135–162.
21. Moczek AP (2006) Pupal remodeling and the development and evolution of sexual dimorphism in horned beetles. *Am Nat* 168(6):711–729.
22. Balthasar V (1963) *Monographie der* Scarabaeidae *und* Aphodiidae *der palaearktischen und orientalischen Region; Coleoptera: Lamellicornia* (Tschechoslowakische Akademie der Wissenschaften, Prague).
23. Shelby JA, Madewell R, Moczek AP (2007) Juvenile hormone mediates sexual dimorphism in horned beetles. *J Exp Zoolog B Mol Dev Evol* 308(4):417–427.
24. Emlen DJ, Hunt J, Simmons LW (2005) Evolution of sexual dimorphism and male dimorphism in the expression of beetle horns: Phylogenetic evidence for modularity, evolutionary lability, and constraint. *Am Nat* 166(Suppl 4):S42–S68.
25. West-Eberhard MJ (2003) *Developmental Plasticity and Evolution* (Oxford Univ Press, New York).
26. Gerhart J, Kirschner M (2007) The theory of facilitated variation. *Proc Natl Acad Sci USA* 104(Suppl 1):8582–8589.
27. Moczek AP (2007) Pupal remodeling and the evolution and development of alternative male morphologies in horned beetles. *BMC Evol Biol* 7:151.
28. Moczek AP, Nagy LM (2005) Diverse developmental mechanisms contribute to different levels of diversity in horned beetles. *Evol Dev* 7(3):175–185.
29. Shafiei M, Moczek AP, Nijhout HF (2001) Food availability controls the onset of metamorphosis in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). *Physiol Entomol* 26(2):173–180.
30. Moczek AP, Rose DJ (2009) Differential recruitment of limb patterning genes during development and diversification of beetle horns. *Proc Natl Acad Sci USA* 106(22):8992–8997.
31. Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics Int* 11(7):36–42.
32. Emlen DJ (1996) Artificial selection on horn length body size allometry in the horned beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Evolution* 50(3):1219–1230.
33. Emlen DJ (2001) Costs and the diversification of exaggerated animal structures. *Science* 291(5508):1534–1536.
34. Snell-Rood EC, Moczek AP (2012) Insulin signaling as a mechanism underlying developmental plasticity: The role of FOXO in a nutritional polyphenism. *PLoS ONE* 7(4):e34857.
35. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25(4):402–408.
36. Winer J, Jung CK, Shackel I, Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem* 270(1):41–49.

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