Supporting Information

Kijimoto et al. 10.1073/pnas.1118589109

Table S1. Primers, species subjected, sequence (from 5′ to 3′), and use

Primer	Species	Sequence (from 5′ to 3′)	Use
dsxORFF01	*O. taurus*, *O. sagittarius*	ATGTCCGATCAACAGGATTAC	Primer 1 in Fig. 1
dsxORFR01	*O. taurus*, *O. sagittarius*	TCACGCTCTACTTCTAGGTC	Primer 2 in Fig. 1
dsxFspeR01	*O. taurus*	GCATCTATACCTTTATTATACG	Primer 3 in Fig. 1
dsxMspeF01	*O. taurus*	CGTATAGATGAAGGTAGAGATGCT	Primer 4 in Fig. 1
dsxBspeR01	*O. taurus*	AACCTATAATTTACCTTCTATCTCA	Primer 5 in Fig. 1
dsxCF01	*O. taurus*, *O. sagittarius*	TGMTTCCYCAMTCGAAAA	Cloning for RNAi (common region)
DsxCR01	*O. taurus*, *O. sagittarius*	TTTGGCCAATATTGTTRTTYC	Cloning for RNAi (common region)
dsxFspeRNAiF01	*O. taurus*	TTTAATGATTCAAACCTGGCTTTAT	Cloning for RNAi (female-specific)
dsxFspeRNAiR01	*O. taurus*	ATGTAATAATGCCGCAAAGAATAT	Cloning for RNAi (female-specific)
dsxBspeRNAiF01	*O. taurus*	AGGTGTACAACTGAAACTAT	Cloning for RNAi (*dsxb*-specific)
dsxBspeRNAiR01	*O. taurus*	TCAAAACGACATATTGTCA	Cloning for RNAi (*dsxb*-specific)
OtdsxCqPCRF01	*O. taurus*	TGATTCCCCAATCGAAAAGCCC	Quantitative RT-PCR
OtdsxCqPCRR01	*O. taurus*	GGCCAATATTGTTATTCCCCAAATTATCTCTG	Quantitative RT-PCR

Fig. S1. (*A*) Nucleotide sequence alignment of *Onthophagus taurus doublesex* (*dsx*) splice variants. Cloned cDNA sequences from *O. taurus* males (*Otdsxm*), females (*Otdsxf1–5*), and the non–sex-specific isoform (*Otdsxb*) are shown. Putative start and stop codons for each isoform are shown in green and red, respectively. The gray underscore indicates the region used as an RNAi target of all variants; orange, the region used to target *Otdsxf1–5*, and black, the region used to target *Otdsxb* (each region is shown as a solid horizontal bar in Fig. 1*B*). Putative splicing donor and acceptor sequences (GT and AG, respectively) are underlined. (*B*) Alignment of translated sequences of *O. taurus dsx*. Cloned cDNA sequences from *O. taurus* males (OtDsxM, blue), females (OtDsxF1–5), and the non–sex-specific isoform (OtDsxB, orange) were translated and aligned. Inferred oligomerization domain 1 (OD1, also known as DM domain) and oligomerization domain 2 (OD2) are boxed in yellow and green, respectively. A National Center for Biotechnology Information conserved domain search (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) suggested that OtDsxM contains two OD2 domains; the putative second OD2 is underlined. Because OtDsxB contains a region that does not align to any of the other OtDsx sequences, it is not forcefully aligned. (*C*) Protein sequence alignment of *Onthophagus* and lepidopteran *dsx*. The Dsx sequences from *O. taurus* (OtDsxM and OtDsxF1–5) and *Bombyx mori* (BmDsxM, BmDsxF1, and BmDsxF2) are shown. OD1 and OD2 are boxed in yellow and green, respectively; the putative second OD2 in *Onthophagus* is underlined. Because the male-specific region of the gene is diverged, sequences are not forcefully aligned. Given that a DsxB sequence has not been reported in *Bombyx*, OtDsxB is omitted from the alignment. (*D*) Protein sequence alignment of *Onthophagus* and hymenopteran *dsx*. The Dsx sequences from *O. taurus* and *Apis mellifera* are shown. Important domains are labeled as in *B*. Because C terminus sequences of OtDsxM and ApDsxM do not align, they are not forcefully aligned. (*E*) Nucleotide sequence alignment of *dsx* from *O. sagittarius* and *O. taurus*. *O. sagittarius* sequences were cloned and sequenced using the same procedures as for *O. taurus*. Putative start and stop codons are indicated in green and red, respectively. The blue underscore indicates the region used as an RNAi target (shown as a horizontal solid gray bar in Fig. 1*A*). (*F*) cDNA sequences from *O. sagittarius* were translated and aligned with those from *O. taurus*. OD1, OD2, and the putative second OD2 are indicated.

Fig. S1

Fig. S2. Bivariate plot of body size (*x*-axis) and head horn length (*y*-axis) for male (*Left*) and female (*Right*) *O. taurus* injected with *Otdsxb* dsRNA (red triangles) or mock-injected (green squares). Data used to plot mock-injected animals are the same as in Fig. 2 and Figs. S4 and S6. The *dsxb* dsRNA injections did not affect horn development in males ($T_{52} = 0.72$, P = not significant) or females (ANOVA, $F_{1,42} = 0.15$, P = not significant) compared with mock-injected animals.

Fig. S2

Fig. S3. *OtdsxC* dsRNA injections reduce *dsx* transcript abundance and result in incomplete development of male genitalia. (*A*) Quantitative RT-PCR confirms reduction of *Otdsx* transcript abundance in large male (*Left*) and female (*Right*) *O. taurus* after *OtdsxC* dsRNA injection (red) compared with mock injection (green), measured within 24 h after pupation. (*B*) *OtdsxC* dsRNA injection results in malformation of male copulatory organs compared with mock-injected animals. Male copulatory organs of mock-injected animals (*Upper*) and *dsxC* RNAi animals (*Lower*) from *O. taurus* (*Left*) and *O. sagittarius* (*Right*) are shown. Specifically, both phallobase (solid line) and parameres (dashed line) are reduced in length and width, the paramere tip (arrow head) is substantially reduced, and the phallobase and paramere fail to attain their typical perpendicular orientation. (*C*) Quantitative effect of *dsxC*RNAi injection on male copulatory organ development in *O. taurus*. Injection of *dsxC* dsRNA (red filled triangles) significantly reduces the length of both the phallobase (*Left*) and the paramere (*Right*) compared with no treatment (green open squares) and mock injection (green filled squares). Measurements were corrected for body size before analysis (pairwise *t* test, WT vs. mock-injected, P = not significant for both paramere and phallobase; paramere: mock injected *vs. dsxC*RNAi, $P = 0.0056$; all other comparisons, $P < 0.0001$). The animals were selected at random from the pool of animals generated for each treatment group.

Fig. S3

Fig. S4. Bivariate plot of body size (*x*-axis) and head horn length (*y*-axis) for female (*Left*) and male (*Right*) *O. taurus* injected with *Otdsxf* dsRNA (red triangles), injected with *OtdsxC* dsRNA (light-red triangles; females only), or mock-injected (green squares). Data used to plot mock- and *OtdsxC*-injected animals are the same as in Fig. 2 and Figs. S2 and S6. *Otdsxf* knockdown induces horns in females, but does not affect horn development in males.

Fig. S4

Fig. S5. Effects of *dsx* dsRNAi on *O. taurus* pupal thoracic (pronotal) horns. (*A*) Pupal thoracic horn growth is sexually dimorphic and not affected by mock (control) injection. The blue squares and red circles indicate males and females, respectively. In each sex, mock-injected and WT (no injection) animals are designated by open and filled symbols, respectively. Pairwise comparisons between WT and mock-injected animals were nonsignificant for both sexes. (*B*) Injection of *dsx* dsRNA reduces thoracic horn length in males compared with no injection and mock injection (ANOVA, $F_{2,156} = 12.75$, $P < 0.001$). Mock-injected (blue filled squares) and *dsx* RNAi males (yellow squares) are shown. (*C*) Injection of *dsx* dsRNA modestly but significantly increases thoracic horn length in females compared with WT and mock-injected individuals (ANOVA, $F_{2,118} = 13.02$, $P < 0.001$). Mock-injected (red filled circles) and *dsx* RNAi females (yellow circles with red outline) are shown. Pairwise comparisons involving *dsx* dsRNAi individuals were significant ($P < 0.05$) in each case (WT or mock-injected) and for both sexes. Note that the *y*-axis scale in *C* differs from that in *A* and *B*.

Fig. S5

Fig. S6. Mock (control) injection does not reduce head horn expression in *O. taurus*. (*Left*) Representative animals obtained after mock injections. Small individuals are shown on the left and large individuals on the right, with males in the top row and females in the bottom row. Anterior is to the left. (*Right*) Bivariate plot of body size (*x*-axis) and head horn length (*y*-axis) for *O. taurus* male WT (blue open squares) and mock-injected (blue filled squares) individuals and female WT (red open circles) and mock-injected (red filled circles) individuals. Mock injections resulted in slightly increased horn length in males ($T_{90} = 2.97$, $P < 0.05$), but had no effect on horn length in females (P = not significant).

Fig. S6

Fig. S7. *dsx* RNAi-induced horn reduction affects large males disproportionately. Shown is the relative amount of horn length (*y*-axis, %) lost in response to *dsx* dsRNA injection in male *O. taurus* as a function of body size (*x*-axis, mm). Relative horn loss was calculated as the percent difference between horn lengths obtained after dsRNA injection and the horn lengths expected for a given body size (obtained from WT scaling relationships). If dsRNA injection affected males similarly over the entire range of body size, then the percent reduction in horn length would be constant across adult sizes. Instead, *dsx* RNAi-induced horn reduction is modest in smaller males but substantial in larger males.

Fig. S7

Fig. S8. RT-PCR results confirm a sex-specific splicing pattern of *O. sagittarius dsx* similar to that of *O. taurus*. Shown are RT-PCR results obtained from cDNA generated from pupal male and female *O. sagittarius* at an equivalent stage to that used for *O. taurus* in Fig. 1*B*, using the same primers 1 and 2 as in Fig. 1*B*.

Fig. S8