SUPPLEMENTARY METHODS

Insects. Third-instar larvae of *Trypoxylus dichotomus septentrionalis* were purchased from Kuwagata Koubo Mushikichi (Fukuoka, Japan). After sexing the larvae according to the presence (male) or absence (female) of the Herold's organ, they were reared individually in a container (140 mm in height and 95-mm in diameter for males; 130 mm in height and 75 mm in diameter for females) filled with humus as a food supply at 25 °C under darkness.

The beginning of the prepupal stage was defined as the completion of the pupation chamber. A 4-day-old prepupa of either sex had horn primordium regions that were white, opaque, and soft. In male horn primordia, there were fewer concentric wrinkles than at a later stage. In females, no wrinkles are observed. Faint pigmentation was observed in the male horn primordia of a 6-day-old prepupa. In females of the same age, faint pigmentation was only observed at the anterior most tip of the head region. Just before pupation, wrinkles and pigmentation of both horn primordia in both sexes were visible in 8-day-old prepupa.

Cloning and Sequencing. Total RNA was extracted from the prepupal head or prothoracic horn primordia of male and female *T. dichotomus* using Trizol (Gibco BRL) according to the manufacturer's instructions. First-strand cDNA was synthesized with the SMART PCR cDNA Amplification Kit (Clontech) using 1 μg total RNA. *Td-dsx* and *Td-ribosomal protein 49* (*Td-rp49*) cDNA fragments were amplified using

degenerate primer sets (supplementary Table SI online). PCR reactions were performed using 2.5 µl of the 10-fold diluted first-strand cDNA, a pair of primers, and AmpliTaq Gold (Perkin Elmer). To obtain full-length cDNA, 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE were performed with the gene-specific primers (supplementary Table SI online), using the SMART PCR cDNA Amplification Kit according to the manufacturer's instructions. The nucleotide sequences of the PCR products, which were inserted into the pCR4-TOPO vector (TOPO TA Cloning Kit, Invitrogen), were determined using an automated DNA sequencer 3130 genetic analyzer (Applied Biosystems). Sequence analysis was carried out using a DNASIS system (Hitachi Software Engineering). The DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank accession numbers for *Td-dsx* are AB744665~AB744675, and for *Td-rp49* is AB744676.

Semi-quantitative RT-PCR Analysis. Prepupal head and prothoracic horn primordia of 4-, 6-, and 8-day-old *T. dichotomus* prepupa were dissected in ice-cold phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, and 10.14 mM Na2HPO4, pH 7.2), and frozen in liquid nitrogen soon after dissection. Total RNA was extracted and treated with 10 U DNase I for 30 min at 37 °C. DNase I-treated total RNA (1 µg) was used for first-strand cDNA synthesis as described above. A cDNA synthesis reaction without reverse transcriptase was performed as a negative control for the RT-PCR experiment. The primers for RT-PCR analysis are listed in supplementary Table SI

online. PCR was carried out with 35 cycles for *Td-dsx* and 28 cycles for *Td-rp49*. *Td-rp49* was used as an internal control. The results represent a combination of three independent samplings.

Phylogenetic Analysis.

Multiple sequence alignment (Blosum) was carried out using CLUSTAL W ver. 1.83 (http://clustalw.ddbj.nig.ac.jp/), and adjusted manually to determine amino-acid sequence identities (supplementary Fig S1 online). Phylogenetic-tree reconstruction (Kimura) was carried out using CLUSTAL W.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Comparisons of conserved domains of *T. dichotomus* (Td) Dsx with that from several insect species. (A) DM domain (OD1). (B) OD2. (C) Female-specific domain contains the C-terminal amino acids of OD2. The amino acid sequences of these domains are based on Oliveira *et al.* [18]. OD2 is enclosed in a blue line. OD1 and OD2 of (B) are common to both sexes. Asterisks indicate identical amino acids among all species compared. Amino acids with conservative substitutions and semi-conservative substitutions based on physiochemical criteria are marked with double dots (:) and single dots (.), respectively. Abbreviations and accession numbers are as follows: Td, *Trypoxylus dichotomus*, AB744667 and AB744670; Ot,

Onthophagus taurus, JN165757–JN165768; Osa, *Onthophagus sagittarius*, JN165763–JN165762; Tc, *Tribolium castaneum*, XM_001807396; Bm, *Bombyx mori*, NM_001111345 and NM_001043406; Aas, *Antheraea assama*, GU930278, GU930283 and GU930284; Osc, *Ostrinia scapulalis*, AB548678 and AB548680; Ag, *Anopheles gambiae*, AY903307 and AY948420; Aae, *Aedes aegypti*, GU121162, GU121163, GU121164 and GU121161; Ms, *Megaselia scalaris*, AF283696 and AF283695; Md, *Musca domestica*, AY461854 and AY461853; Lc, *Lucilia cuprina*, GU784833 and GU784834; Dm, *Drosophila melanogaster*, AE014297 and AE014297; Bo, *Bactrocera oleae*, AJ547622 and AJ547621; Cc, *Ceratitis capitata*, AF434935 and AF435087; Ao, *Anastrepha obliqua*, AY948421 and AY948421; Am, *Apis mellifera*, EU236954 and EU236957; Nv, *Nasonia vitripennis*, NM_001162517 and NM_001162518. (D) Dsx neighbor-joining (NJ) tree of common region between males and females based on the deduced sequences of 136 amino acids. Numbers on the nodes indicate NJ bootstrap values (%) (>60%) from 1,000 replicates. Horizontal branch lengths show the amino-acid distances. Abbreviations and accession numbers for insect Dsx are the same as in (A–C). Dsx sequences of *Daphnia magna* were used as an outgroup. Accession numbers for *Daphnia magna* Dsx1 (Dapma1-α and -β) and Dsx2 (Dapma2) are AB569296, AB569297, and AB569298, respectively.

Supplementary Figure S2. Phenotypic analysis of surface structure following larval RNAi of *Td-dsx* in adults. *Td-dsx* dsRNAs synthesized from putative exons 1, 2, and 3

(dsxAll) were injected into male (C) and female (D) last-instar larvae. We analyzed the resulting six males and 10 females. All male and female *Td-dsx* dsRNA-treated beetles showed the same phenotype (C) and (D), respectively. *EGFP* dsRNA was injected into male (A) and female (B) last-instar larvae as a control with no obvious effect. Left panels are dorsal views of adults. Middle and right panels are enlarged pictures of respective prothorax and elytra-surface structures in yellow boxes of each left panel. Black scale bars in left panels and white scale bars in middle and right panels represent 1 cm and 200 μm, respectively.

Supplementary Figure S3. (A–I) Phenotypic analysis of internal reproductive organs in adults following larval *Td-dsx* RNAi. Male and female internal reproductive organs from an untreated wild-type male (A) and female (C), and a *Td-dsx* dsRNA-treated male (B) and female (D and E) are shown. Testis from an untreated male (F), undeveloped ovary from an untreated female (H), and gonad-like organs from a *Td-dsx* dsRNA-treated male (G) and female (I) are shown. *Td-dsx* dsRNA synthesized from putative exons 1, 2, and 3 (dsxAll) were injected into male (B and G) and female (D, E and I) last-instar larvae. We analyzed the resulting eight males and eight females. All male and female *Td-dsx* dsRNA-treated beetles showed a similar phenotype (G) and (I), respectively. (J–N) Phenotypic analysis of genitalia in adults following larval *Td-dsx* RNAi. Genitalia from untreated wild-type male (J), *Td-dsx* dsRNA-treated male (K and L) and female (M), and untreated wild-type female (N) are shown. We injected *Td-dsx*

dsRNA (dsxAll) into eight larvae for each sex. Six and two males treated with *Td-dsx* dsRNA showed mild (K) and severe (L) phenotypes, respectively, whereas eight females showed a similar phenotype (M). *Td-dsx* dsRNA synthesized from putative exons 1, 2, and 3 (dsxAll) were injected into male (K and L) and female (M) last-instar larvae. Upper, middle, and lower panels are dorsal, ventral, and lateral views, respectively. Scale bars in all panels are 1 mm. Abbreviations: ae, aedeagus; ag, accessory gland; ed, ejaculatory duct; fg, female genitalia; he, hemisternite; mg, male genitalia; ova, ovary; ovd, oviduct; ovo, ovariole; pa, paramere; sp, spermatheca; te, testis; tf, testicular follicle; vd, vas deferens.

A DM domain (OD1)

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Td      RTPPNCARCRNHRLKIALKGHKRYCKYRHCKCEKCRLTSERQRVMAMQ
Ot      RTPPNCARCRNHRVKVPLKGHKRYCKYRHCKCEKCRLTSERQRVMAMQ
Osa     RTPPNCARCRNHRVKVPLKGHKRYCKYRHCKCEKCRLTSERQRVMAKQ
Tc      RTPPNCARCRNHRLKIALKGHKRYCKYRTCKCEKCRLTTERQRVMAMQ
Bm      RAPPNCARCRNHRLKIELKGHKRYCKYQHCTCEKCRLTADRQRVMAKQ
Aas     RAPPNCARCRNHRLKVELKGHKRYCKYRYCNCEKCRLTADRQRVMALQ
Osc     RAPPNCARCRNHRLKVELKGHKRYCKYRYCTCEKCRLTADRQRVMALQ
Ag      RTPPNCARCRNHGLKIGLKGHKRYCKYRTCHCEKCCLTAERQRVMALQ
Aae     RTPPNCARCRNHGHKIGLKGHKRYCKYRNCTCEKCCLTAERQRVMALQ
Ms      RTPPNCARCRNHSLKIALKGHKRYCKYRYCDCEKCRLTADRQKIMAAQ
Md      RTKPNCARCHNHGLKIKLKGHKRYCKYRFCNCEKCRLTADRQRVMALQ
Lc      RTKPNCARCHNHGFKIKLKGHKRYCKYRNCNCEKCRLTADRQRVMALQ
Dm      RTPPNCARCRNHGLKITLKGHKRYCKFRYCTCEKCRLTADRQRVMALQ
Bo      RTPPNCARCRNHGLKITLKGHKRYCKFRYCTCEKCRLTADRQRVMALQ
Cc      RTPPNCARCRNHGLKITLKGHKRYCKFRYCTCEKCRLTADRQRVMALQ
Ao      RTPPNCARCRNHGLKITSKGHKRYCKFRYCTCEKCRLTADRQRVMALQ
Am      RA-RNCARCLNHRLEITLKSHKRYCKYRTCTCEKCKITANRQQVMRQN
Nv      RI-PKCTRCQNHGKKVQVKFHKRECEFRYCLCEMCILTTKRQQIMKVQ
        *   :*:** **  ::  * *** *::: * ** * :*:.**::*  :
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B OD2

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Td      SVQKDLLQDCHKLLDKFQYPWEMMPLLYAIMKLA-PNLEEASKRIDE
Ot      IGQKDLIQESLQLLEKFRYSWEMMPLIYAIVKDT-PDLEEASKRIDE
Osa     IGQKDLIQESLQLLEKFRYSWEMMPLIYAIVKDT-PDLEEASKRIDE
Tc      AQSTDLLEDCQKLLERFKYPWEMMPLMYAILKDARADLEEASRRIDE
Bm      VPSETLVENCHRLLEKFHYSWEMMPLVLVIMNYARSDLDEASRKIYE
Aas     VSLETLVENCHKLLEKFHYSWEMMPLVLVILNYAGSDLEEASRKIDE
Osc     VSLETLVENCHKLLEKFHYSWEMMPLVLVILNYAGSDLDEASRKIDE
Ag      LPDDELVKRAQWLLEKLGYPWEMMPLMYVILKSADGDVQKAHQRIDE
Aae     GTDDELVKRSQWLLEKLRYPWEMMPLMYVILKGADGDVNKARQRIDE
Ms      IQKESLLDCCHRLLEQFRFPFEMMPLMYVILKSV-DDEEEASRLISE
Md      MSIDLILDYCQKLIEKFGYPWEMMPLMYVILKDAGVDIDEASKRIEE
Lc      MSIDLILDYCQKLIEKFGYPWEMMPLMYVILKDAGVDIDEASKRIEE
Dm      LGQDVFLDYCQKLLEKFRYPWELMPLMYVILKDADANIEEASRRIEE
Bo      LAQDVFLEHCQKLLEKFRYPWEMMPLMYVILKDAGADIEEASRRIEE
Cc      LAQDVFLEHCQKLLEKFRYPWEMMPLMYVILKDAGADIEEASRRIEE
Ao      LAQDVFLEHCQKLLEKFRYPWEMMPLMYVILKDAGADIEEASRRIEE
Am      ENVEILLEHSSKLVELFQYPWEALLLMYINLKYAGANPEEVVRRMVD
Nv      RLVEELLGYSVKLLQRFGYHWQSLTLMYVILKDSRADVEVAMRRITQ
            ::  .  *:: : : :: : *:   ::    : : . : : :
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C Female-specific domain

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Td-F-L      GKQMVYEYSQRYNLNMYDGGELRKSTHKENREKNNFPKAT-LTSVNLQLKCNLLMPY
Td-F-S      GKQMVYEYSQRYNLNMYDGGELRKSTRQYG
Ot-F1       GQRAVKEYSIINNLNMYDGGELRYPTRQYG
Ot-F2       GQRAVKEYSIINNLNMYDGGELRYPTHKRAQERRSSGTFKPIGGVNLQVKRNLHVK
Ot-F3       GQRAVKEYSIINNLNMYDGGELRYPTHKRAQERRSSGTFKPIGGVNLQLKLDF
Ot-F4       GQRAVKEYSIINNLNMYDGGELRYPTHKRAQERRSSGNFKPIGGVNLQVKGNLHVK
Ot-F5       GQRAVKEYSIINNLNMYDGGELRYPTRQYG
Osa-F1      GQRAVKEYSIINNLNMYDGGELRYPTHKRAQERRSSGNFKPIGGVNLQRLTIQTWLYHKKNDPALTKSIITINLK
OSa-F2      GQRAVKEYSIINNLNMYDGGELRYPTHKRAQERRSSGNFKPIGGVNLQVKDF
Osa-F3      GQRAVKEYSIINNLNMYDGGELRYPTHKRAQERRSSGNFKPIGGVNLQVKGNLHRLTIQTWLY
Osa-F4      GQRAVKEYSIINNLNMYDGGELRYPTHKRAQERRSSGNFKPIGGVNLQVKGNLHVK
Osa-F5      GQRAVKEYSIINNLNMYDGGELRYPTRQYG
Bm          GKMIVDEYARKHNLNVFDGLELRNSTRQKML--EINNISGVLSSSMKLFCE
Aas-1       GKMIINEYARKHNLNVFDGLELRNSTRQKML--EINNISGVLSSSMKLFCE
Aas-2       GKMIINEYARKHNLNVFDGLELRNSTRQYGL
Osc         GKMIINEYARKHNLNIFDGLELRNSTRQKMLQSEINNISGVLSSSMKLFCE
Ag          GQAVVNEYSRLHNLNMFDGVELRNTTRQSG
Aae-F-1     GQAVVNEYSRLHNLNMFDGVELRSTTRQSG
Ms          GQYAVNEYSRQHNLNIFDGGELRSQSRQCG
Md          GQHVVNEYSRQHNLNIYDGCELRCATRQCG
Lc          GQHVVNEYSRQHNLNIYDGCELRCATRQCG
Dm          GQYVVNEYSRQHNLNIYDGGELRNTTRQCG
Bo          GQHVVNEYSRQHNLNIYDGGELRSTTRQCG
Cc          GQHVVNEYSRQHNLNIFDGGELRSTTRQCG
Ao          GQHVVNEYSRQHNLNIYDGGELRSTTRQCG
            *:  : **:   ***::** ***  :
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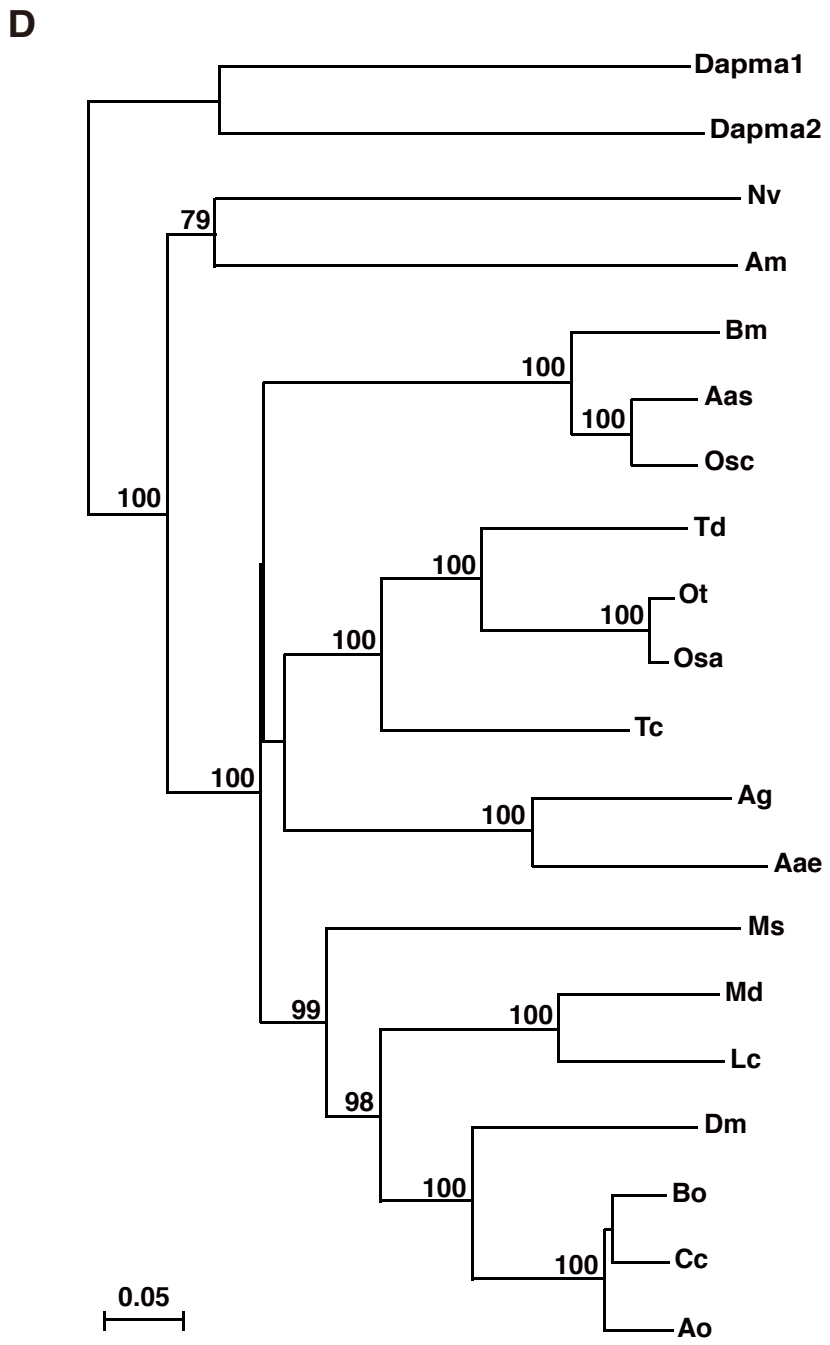
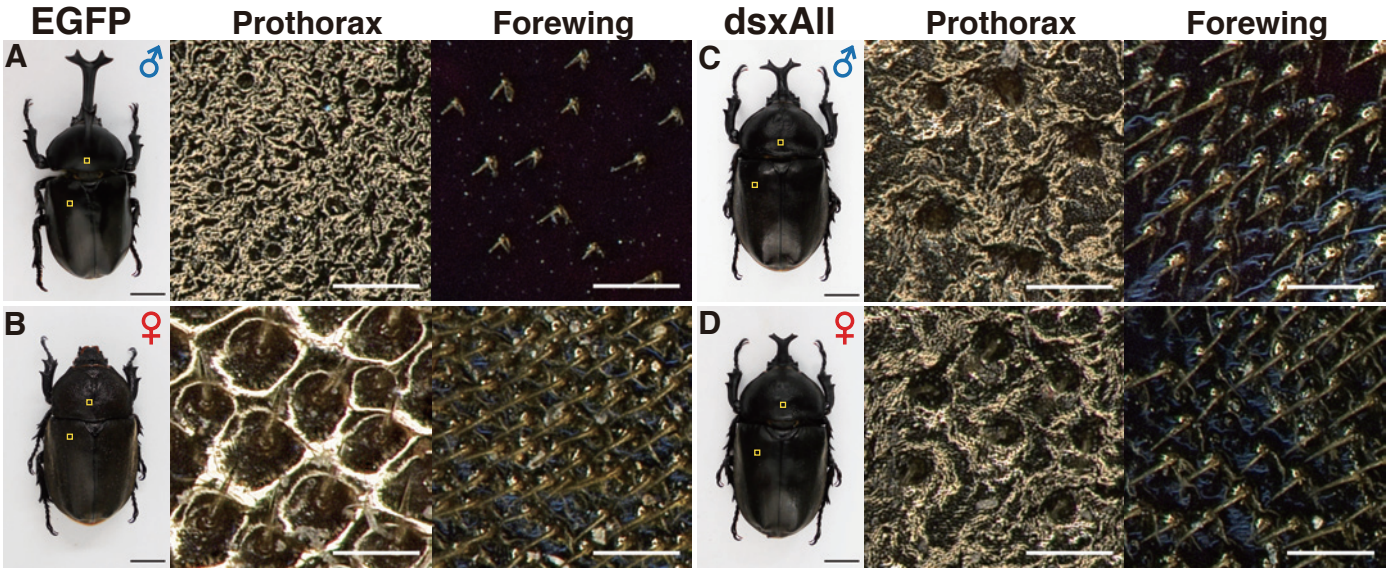
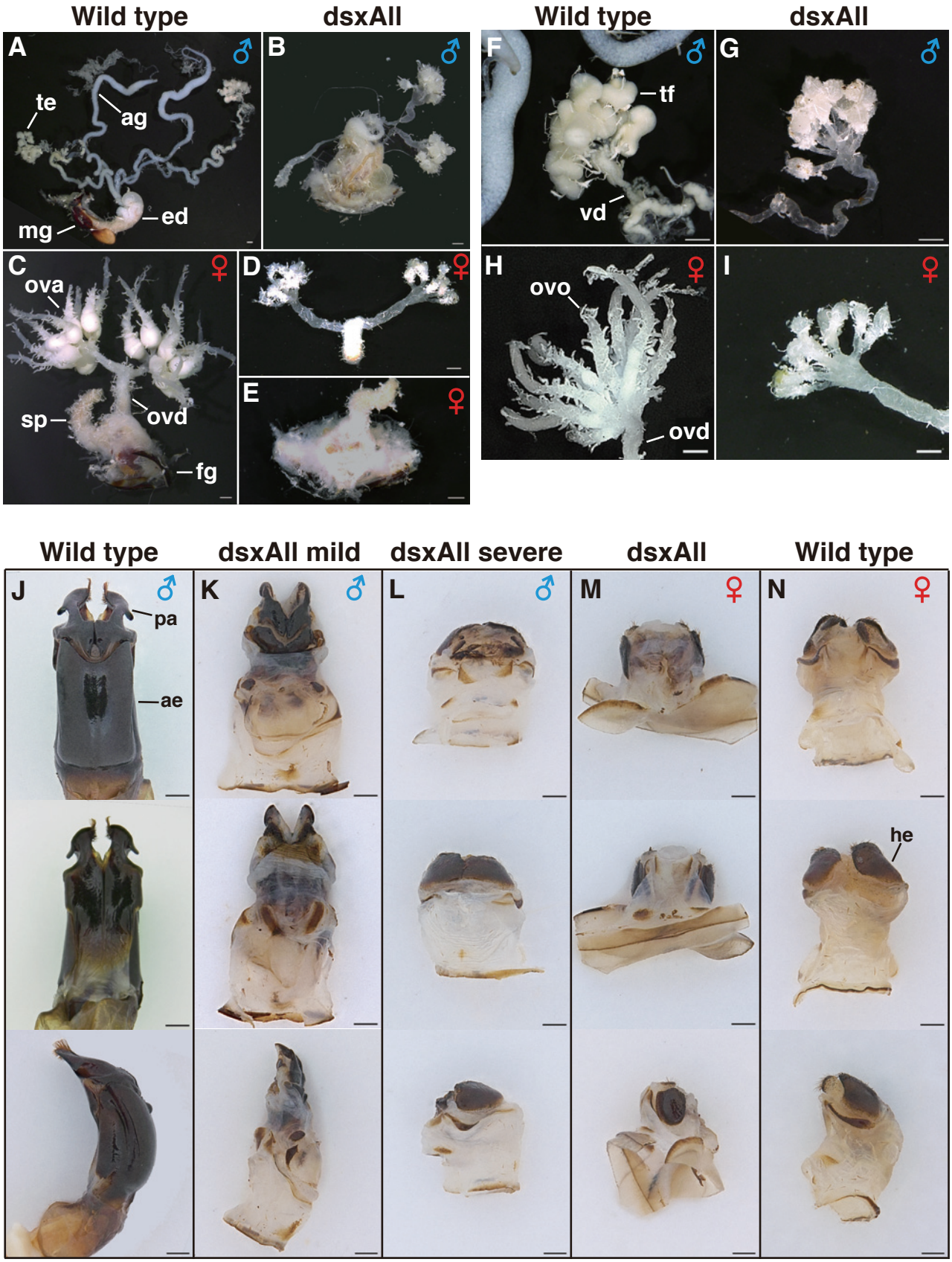


Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3

Supplementary Table SI. Primers used in this study

Degenerate primer for gene cloning

dsx-F	sense	5'-AAYTGYGCIMGITGYMGIAAYCA-3'
dsx-R	antisense	5'-TACACTIARIGGCATYTCCCA-3'
rp49-F	sense	5'-AARAARMGIACIAARMAITTYAT-3'
rp49-R	antisense	5'-TGIGCIATYTCISCRCARTA-3'

(M=A+C, R=A+G, Y=T+C, S=C+G, I=inosine)

RACE

Td-dsx-1	antisense	5'-CCGACGTTAGGCCGATTTCTCGC-3'
Td-dsx-2	antisense	5'-GCCCTTCGCAAAGCCGTTTGCATGGCC-3'
Td-dsx-3	sense	5'-CCGGCACCGATTCACGTACCGGATCGG-3'
Td-dsx-4	sense	5'-CCCGGATCTTCGTCCACTCCTTCC-3'
Td-dsx-RACE-01	antisense	5'-CGGTTTCCCGATTGTTGACAGGTTCTAC-3'
Td-dsx-RACE-02	antisense	5'-GGCCATAACTCTTTGACGTTCCGACGTT-3'
Td-dsx-RACE-03	antisense	5'-GGCCTTGTCGCAGCATCGCCTCATCC-3'
Td-dsx-RACE-04	antisense	5'-ACGTGATGCGGGGAGCTATGAAAAC-3'

Semiquantitative PCR

P1	sense	5'-TCGTCTAAAGATCGCCTTGAAAGG-3'
P5	antisense	5'-AAGAAACTATACGTATACGG-3'
P6	antisense	5'-TGTTCTTCTCTCGGTTCTCC-3'
P7	antisense	5'-AACTGCTCAGCACCTTTACC-3'
P7[10]	antisense	5'-TCCAGGAAAGTTTTACTTAC-3'
P8	antisense	5'-ACAATAATCACTTACGACCC-3'
P9	antisense	5'-CTTTTGGTATATCACCAACGGG-3'
Td-rp49-1	sense	5'-CTGACCGTTATGGAAAACTT -3'
Td-rp49-2	antisense	5'-TACGATTTTGCATCAACAAT-3'

dsRNA synthesis

dsx-F	sense	5'-AAYTGYGCIMGITGYMGIAAYCA-3'
dsx-R	antisense	5'-TACACTIARIGGCATYTCCCA-3'
Td-dsxF-L-F	sense	5'-ATAAGGAGAACCGAGAGAAG-3'
Td-dsxF-L-R	antisense	5'-CTTAATCTTAGATTTTCAAT-3'
Td-dsxF-S-F	sense	5'-GTCAGTATGGATGATTGAAA-3'
Td-dsxF-S-R	antisense	5'-CTAATTAGATAAAAAAGATA-3'
Td-dsxC-F	sense	5'-CCTAGATCAGTATCTGGTGATGGTG-3'
Td-dsxC-R	antisense	5'-CTTTTGGTATATCACCAACGGG-3'

Supplementary Table SII. Effect of *Td-dsx* dsRNA on horn formation

dsRNA	Amount of injected dsRNA (μg)	sex	Number of injected larvae	Number of adults	Number of adults with specific phenotypes	Efficiency (%)
EGFP	10	♂	2	2	0	0
		♀	2	2	0	0
	20	♂	3	2	0	0
		♀	3	2	0	0
dsxAll	10	♂	6	3	3	100
		♀	6	6	6	100
	20	♂	3	3	3	100
		♀	5	4	4	100
dsxF-L+S	10	♂	3	3	0	0
		♀	6	6	6	100
	20	♂	3	2	0	0
		♀	3	3	3	100
dsxF-S	10	♂	2	2	0	0
		♀	2	2	0	0
	20	♂	3	2	0	0
		♀	2	2	0	0
dsxC	10	♂	3	2	0	0
		♀	3	3	0	0
	20	♂	3	2	0	0
		♀	2	2	0	0