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The role of *doublesex* in the evolution of exaggerated horns in the Japanese rhinoceros beetle

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Male-specific exaggerated horns are an evolutionary novelty and have diverged rapidly via intrasexual selection. Here, we investigated the function of the conserved sex-determination gene *doublesex* (*dsx*) in the Japanese rhinoceros beetle (*Trypoxylus dichotomus*) using RNA interference (RNAi). Our results show that the sex-specific *T. dichotomus dsx* isoforms have an antagonistic function for head horn formation and only the male isoform has a role for thoracic horn formation. These results indicate that the novel sex-specific regulation of *dsx* during horn morphogenesis might have been the key evolutionary developmental event at the transition from sexually monomorphic to sexually dimorphic horns.

Keywords: *doublesex*; *Trypoxylus dichotomus*; exaggerated horn; sexual dimorphism; sexual selection

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INTRODUCTION

The evolution of exaggerated morphological traits in animals is a topic that has captured the interest of biologists for centuries [1,2]. Sexual selection can lead to the rapid evolution of exaggerated traits via two mechanisms: intersexual selection by female choice (for example, peacock's tail) or intrasexual selection by male–male competition (for example, male deer antlers) [3]. The theoretical framework on the roles of sexual selection on the evolution of sexually dimorphic traits has been extensively studied since Darwin first proposed sexual selection as the second force of evolution [1,3]. In contrast, knowledge on the molecular mechanisms underlying the evolution of sexually dimorphic traits by sexual selection is rather limited. To address this topic, we investigated beetle horns; one of the best examples of exaggerated traits evolved via intrasexual selection [1]. To our knowledge, hornedness has been independently acquired in at least 13 families of Coleoptera. The number and location of beetle horns varies and horn morphology shows a diverse range of shapes and sizes. These traits are largely male specific, as horns are used as weapons in male–male combat [4].

How novelties arise and how similar structures are created independently remain central questions in evolutionary developmental biology. Sexually dimorphic beetle horns provide an ideal opportunity to address these questions: they are an evolutionary novelty that is not homologous to any existing trait and have arisen independently in many taxa. Because beetle horns are sexually dimorphic, it is logical to assume that sex-determination genes are involved in the evolution of this novelty. The developmental genetic mechanisms underlying sexual dimorphisms have been well studied in various animal taxa [5,6]. One of the key factors involved in sexual dimorphisms is the Doublesex/Mab-3 related (Dmrt) transcription factor family, which is evolutionarily conserved from worms (*mab-3*) to mammals (*DMRT-1*) [5,6]. The functions of *Dmrt* genes in somatic sexual dimorphism has diverged among taxa but is deeply conserved in gonad development across phyla [5].

In insects, the bottom downstream gene in the sex-determination cascade is *doublesex* (*dsx*) [7]. Sex-specific alternative splicings produce male- and female-specific isoforms. Both Dsx isoforms share a common zinc finger-like DNA-binding motif called the DM domain and act as transcription factors to control all aspects of sex-specific morphologies. Therefore, *dsx* is key to understanding how sexually dimorphic morphologies in insects are acquired during evolution. The roles of *dsx* on the evolution of sexual dimorphisms such as male-specific sex combs and abdominal pigmentation, and the female-specific sex pheromone-producing enzyme in *Drosophila* have been well studied [8–12]. These studies have revealed general principles for the evolution of novel sex-specific traits, namely, *cis*-regulatory changes in

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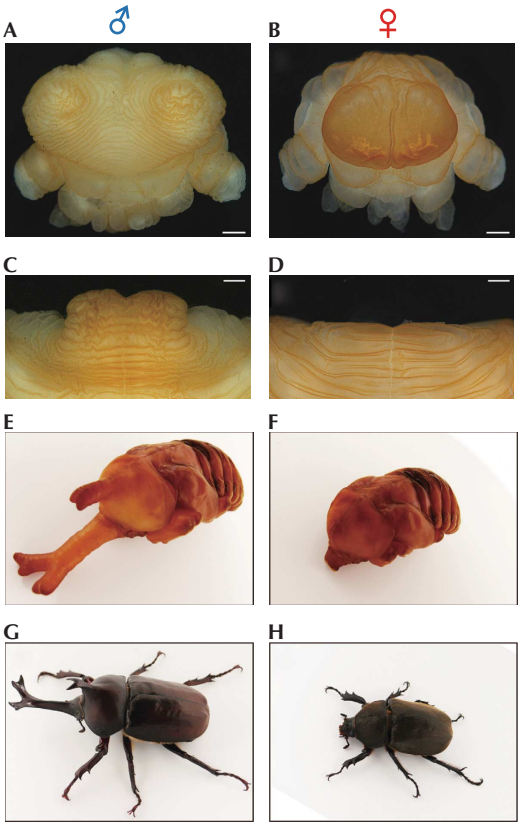

Fig 1 | Sexual dimorphism in developmental horn morphology of *T. dichotomus*. Horn primordia are formed during the prepupal stage. **(A,B)** Frontal view of head primordium of male **(A)** and female **(B)**. **(C,D)** Dorsal view of prothorax horn-forming region of male **(C)** and corresponding region of female **(D)** at day 8 of prepupal stage. Both horn primordia in males are wrinkled to elongate during pupal ecdysis. **(E,F)** Pupal head and prothorax morphologies of male **(E)** and female **(F)**. Male pupae have horns on the head and prothorax, whereas females have a small horn-like structure only on the head. The female pupal head horn degenerates during pupal–adult development. Adult head and prothorax morphologies of male **(G)** and female **(H)**. Only male adults have horns on the head and prothorax. Scale bars, 1 mm **(A–D)**.

dsx regulation that affect the expression domain of *dsx*, and modifications of Dsx-binding sites in downstream *dsx* target genes [5,8,11]. Functional studies on *dsx* have demonstrated that *dsx* modifies sexually monomorphic pre-existing structures, thereby creating sexual dimorphisms [13,14]. However, little is known about the roles of *dsx* in the development of evolutionary novelty. Recently, Kijimoto *et al* [15] have shown that *dsx* has a crucial role in morph-, sex- and species-specific development of horns in *Onthophagus* (Coleoptera, Scarabaeidae, Scarabaeinae).

To gain further insights into the evolution of exaggerated horns, we studied the Japanese rhinoceros beetle (*Trypoxylus dichotomus*; Coleoptera, Scarabaeidae, Dynastinae), which has sexually dimorphic exaggerated horns on the head and prothorax (Fig 1) and has acquired horns independently from *Onthophagus*. These male-specific structures develop from horn primordia that arise during the prepupal larval stage (Fig 1A,C). Behavioural studies have revealed that they are used as weapons in male–male combat for access to female [16]. Therefore, intrasexual selection by male–male competition drives the evolution of the horns of *T. dichotomus*.

We focused on *dsx* to understand male-specific horn formation in *T. dichotomus* and examined its function using RNA interference (RNAi). In *Drosophila*, loss-of-function of *dsx* resulted in two distinct intersexual phenotypes, namely an intermediate phenotype between male and female as is the case for sex combs, or a male-like phenotype as seen in abdominal pigmentation and segment number [13,14]. In *Onthophagus*, *dsx* RNAi resulted in an intersexual phenotype in both sexes, involving reduction of horn size in the horned sex and induction of horn development in the hornless sex [15]. Thus, there are three possibilities for the *dsx* RNAi phenotype concerning horn formation in *T. dichotomus*. (1) If both male and female *dsx* isoforms have a function in horn formation (antagonistic), an intermediate sized horn is expected to develop in both sexes. (2) If only the male *dsx* isoform has a function in horn formation (induction), a hornless male *dsx* RNAi phenotype similar to the wild-type female phenotype is expected. (3) If only the female *dsx* isoform has a function in horn formation (repression), a horn similar to the male wild-type horn is expected to appear in the female *dsx* RNAi phenotype. To our surprise, we found that the sex-specific isoforms of *dsx* have different regulatory functions for the head and prothoracic horns.

RESULTS AND DISCUSSION

Td-dsx produces several sex-specific splicing variants

We isolated full-length complementary DNAs (cDNAs) of the *T. dichotomus dsx* homologue (*Td-dsx*) from horn primordia (Fig 2). Td-Dsx has two evolutionarily conserved functional domains: an amino (N)-terminal DNA-binding domain (DM domain or OD1); and a carboxy (C)-terminal dimerization domain (OD2) [17,18]. When compared with Dsx from other insects, a high level of conservation was found only in these two domains (supplementary Fig S1 online). Further, all zinc-chelating residues in the DM domain are conserved (supplementary Fig S1A online) [19]. Conserved DM domain and OD2 sequences suggests that Td-Dsx binds DNA as a dimer. Thus, Td-Dsx is likely to function as transcription factor as is the case for Dsx in other insects. Sex-specific splicings occur in the 3′ region, and result in different C-terminal amino-acid ends. Unlike *Drosophila dsx*, we could not find a male-specific exon, but rather male-specific isoforms are produced by skipping female-specific exons (DsxM-1 and 2, Fig 2). Furthermore, the same non-sex-specific isoforms were isolated from both sexes (DsxC-1, 2 and 3, Fig 2). Interestingly, all isoforms except DsxC have two forms, either with or without putative exon 2, which is located just next to OD2 on the N-terminal side. This is the first report of a non-sex-specific splicing variation in a common, non-sex-specific region. Notably, *Td-dsx* contains a highly variable 3′ region, unlike *dsx* cloned from other insects except *Antheraea assama*, *Onthophagus taurus* and *O. sagitarius* [15,20].

Recently, it has been shown that precise spatio-temporal regulation of *dsx* is essential for sex-specific development [8,21]. Thus, we subsequently examined the expression profiles of *Td-dsx* in the head and prothoracic horn-forming region of both sexes during prepupal stages using the semi-quantitative reverse-transcription polymerase chain reaction (RT–PCR) with exon-specific primers (Fig 3). Both male- and female-specific isoforms were expressed in horn-forming regions during horn

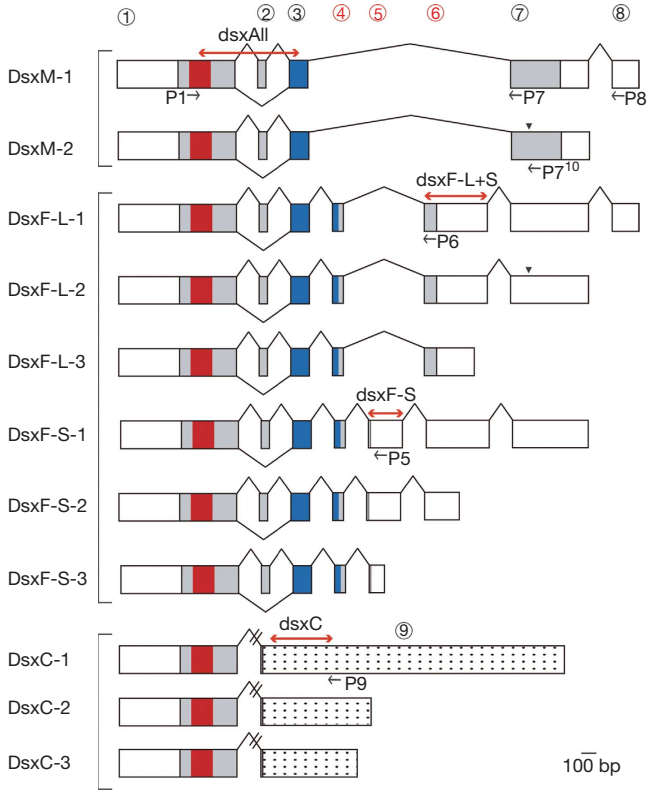

Fig 2 | Schematic representation of putative exon–intron structures of *Td-dsx*. The length of both male (M)-specific and female (F)-specific isoforms are highly variable, and dsxF has longer (L) and shorter (S) open reading frames (ORFs). The isoforms isolated from both sexes are designated common (C). The 3′ untranslated region (UTR) variants are designated 1–3, corresponding to the length from the longer to the shorter variant. The numbers in circles on the upper panel correspond to each putative exon, and the numbers indicated in red are female-specific exons. Boxes and lines indicate putative exons and introns, respectively. Filled and open boxes indicate ORFs and UTRs, respectively. Red and blue boxes are conserved functional domains DM (OD1) and OD2, respectively. The position of exon 9 cannot be estimated, so does not correspond to those of the above-mentioned exons. 3′-UTRs are indicated in the dotted box. Black triangles on exon 7 of DsxM-2 and DsxF-L-2 indicate a 10-base pair (bp) insertion that is rarely present, which creates a stop codon. Arrows indicate the position of primers used for RT–PCR analysis in Fig 3. Red double-ended arrows correspond to the region of dsRNA synthesis for larval RNAi analysis in Fig 4. The scale bar representing 100 bp applies only to the exon region, and the intron regions are arbitrary and not to scale.

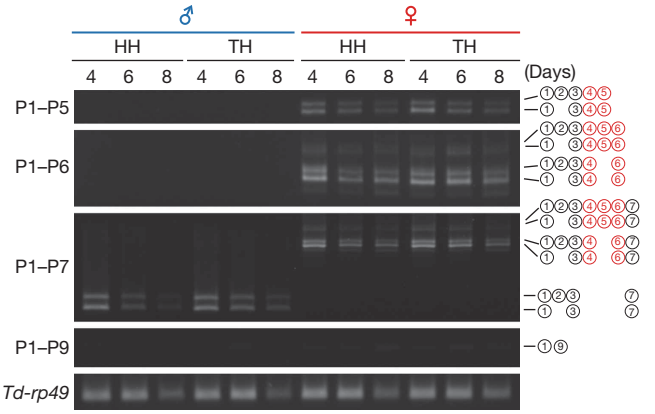

Fig 3 | RT–PCR analysis showing expression profile of *Td-dsx* isoforms in head and prothoracic horn-forming region during the prepupal development of both sexes. The PCR products for *Td-dsx* isoforms using P1–P5, P1–P6, P1–P7 and P1–P9 primer sets were shown. *Td-ribosomal protein 49* (*Td-rp49*) was used as an internal control. The combinations of putative exons corresponding to each PCR band are indicated on the right of each panel. Female-specific exons are indicated in red. Numbers at the top of panels indicate days in prepupal period. HH and TH indicate head horn and prothoracic horn-forming region, respectively. Primer sets P1–P7^{10} (primer P7^{10} was designed to amplify the isoforms with a 10-bp insertion in exon 7) and P1–P8 gave almost undetectable bands (data not shown). No band was detected in the negative control in which cDNA synthesis was performed without reverse transcriptase (data not shown).

development at the prepupal stage. *Td-dsxM* showed relatively higher expression at an early stage of the developing head horn. In contrast, the expression level of *dsxC* was very low. Further, all these isoforms except *dsxC* showed variation regarding the inclusion of putative exon 2. However, the splicing patterns did not change between the head and thoracic horn-forming regions of each sex.

Td-dsx controls sexually dimorphic horn formation

Next, to investigate the function of each *Td-dsx* isoform in horn development, we performed a larval RNAi experiment to knock-down the gene function. Larval RNAi, in which double-stranded RNA (dsRNA) is injected into the larval body cavity, is effective for the analysis of gene function during adult development, especially in beetles [22,23]. We synthesized four different dsRNAs on the basis of the different exons of *Td-dsx* (Fig 2) and injected them into late third-instar larvae of both sexes. Initially, we tested *Td-dsx* dsRNA corresponding to exons 1–3 (dsxAll) to knock-down all isoforms. When we injected the dsRNA for dsxAll into male larvae, the head horn reduced in size and the thoracic horn completely disappeared (Figs 4B,G and 5A,E). Surprisingly, injection of the same dsRNA into female larvae resulted in the formation of a small horn on top of the head (Figs 4L,Q and 5A,E). Unlike in *Onthophagus*, where *dsx* RNAi resulted in intersexual, ectopic or unaffected horn formation [15], our results are a first report that knockdown of *Td-dsx* can cause a complete loss of a horn, although this was only observed for the prothoracic horn. Further, this difference indicates independent recruitment of *dsx* in the horn-forming gene network between *Trypoxylus* and *Onthophagus*. Our results also show that in the absence of functional Td-Dsx, both males and females develop the same intersexual phenotype. Furthermore, the intersexual phenotype suggests that the regulation of *dsx* in horn formation is different between the head horn and the thoracic horn, as they showed different phenotypes by RNAi. Sexual dimorphisms on the prothorax, such as the sex comb in *Drosophila* and horns in *Onthophagus*, are controlled by the *Hox* gene, *Sex combs reduced* (*Scr*) [8,24]. Further, it is proposed that the positive feedback loop between *dsx* and *Scr* might have a role in the diversification of sex comb morphology in *Drosophila* [8]. *Scr* is

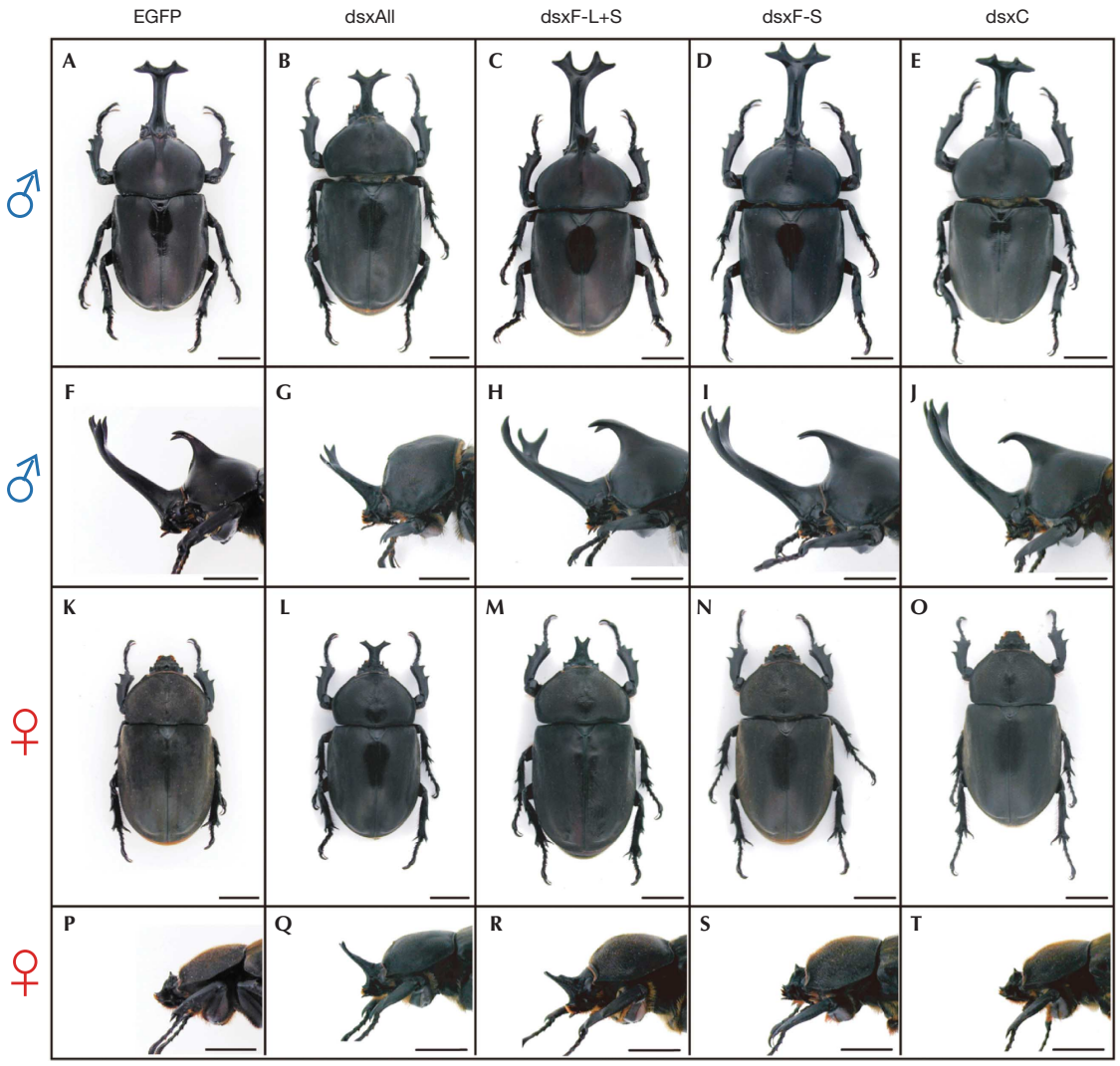

Fig 4 | Phenotypic analysis by larval RNAi of *Td-dsx* in adults. *Td-dsx* dsRNAs synthesized from putative exons 1, 2 and 3 (named dsxAll: **B,G, L,Q**), putative exon 6 (dsxF-L + S: **C,H,M,R**), putative exon 5 (dsxF-S: **D,I,N,S**) and putative exon 9 (dsxC: **E,J,O,T**) were injected into male (**B–E** and **G–J**) and female (**L–O** and **Q–T**) last-instar larvae. Injection of dsxAll dsRNA resulted in an intersexual phenotype in both sexes, whereas that of dsxF-L + S dsRNA showed the same phenotype only in females. dsxF-S and dsxC dsRNAs had no detectable effect. Enhanced green fluorescent protein (*EGFP*) dsRNA (**A,F,K,P**) was injected into male (**A,F**) and female (**K,P**) last-instar larvae as a control with no obvious effect. Dorsal view of adults (**A–E** and **K–O**). Lateral view of head and prothorax of adults (**F–J** and **P–T**). Scale bars, 1 cm.

thus a plausible candidate to be involved not only in differences between head and prothoracic horns but also in rapid diversification of thoracic horns by the positive feedback with *dsx*.

When we injected dsRNA synthesized on the basis of the longest female-specific exon (dsxF-L + S) into male larvae, no aberrant phenotype was observed (Figs 4C,H and 5B,F), although the same intersexual phenotype was observed in females (Figs 4M,R and 5B,F). However, when dsRNA specific to the female short exon (dsxF-S) or common isoform (dsxC) was injected, no aberrant phenotypes were observed in either of the sex (Figs 4D,E,I,J,N,O,S,T and 5C,D,G,H). This suggests that Td-DsxF-S and Td-DsxC have no role in horn formation.

A role for *dsx* in the evolution of male-specific horns

Our RNAi analysis sheds light on the evolution of sexually dimorphic horns. With no input from the sex-determination pathway, the head horn becomes smaller and the thoracic horn does not form in both sexes. Interestingly, this phenotype is reminiscent of the sexually monomorphic small head horn of *Oryctes rhinoceros*. This small head horn is used for burrowing into coconut trunks in both sexes [25]. It might be possible that in *T. dichotomus*, a similar small sexually monomorphic horn (like the RNAi induced intersexual phenotype), was first evolved and was subsequently co-opted to serve as a weapon for male–male combat, after which intersexual selection acted on the function of *dsx* in the horn development gene network to drive a rapid evolution of sexually dimorphic horns. If this proves to be

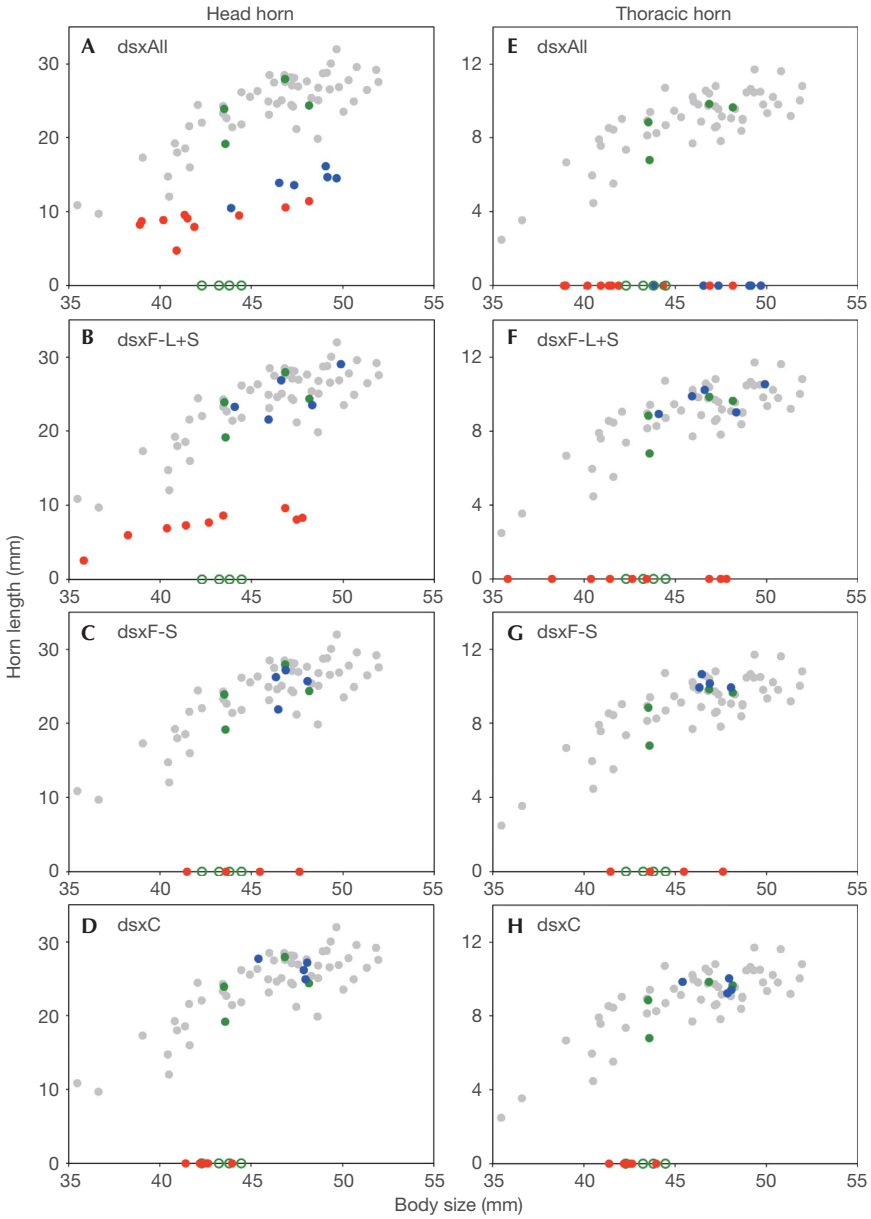

Fig 5 | Allometric analysis of head and thoracic horn following *Td-dsx* RNAi treatment. Horn length and body size from head to tail of RNAi-treated and -untreated wild-type adults were measured. (**A–D**) Head horn. (**E–H**) Thoracic horn. Untreated wild-type males ($n = 53$), enhanced green fluorescent protein (*EGFP*) dsRNA-treated males ($n = 4$) and females ($n = 4$), and *Td-dsx* dsRNA-treated males (dsxAll, $n = 6$; dsxF-L + S, $n = 5$; dsxF-S, $n = 4$; dsxC, $n = 4$) and females (dsxAll, $n = 10$; dsxF-L + S, $n = 9$; dsxF-S, $n = 4$; dsxC, $n = 5$) are shown in grey, filled and open green, blue and red, respectively (supplementary Fig S2 online). Body sizes of wild-type males, *EGFP* dsRNA-treated males and *Td-dsx* dsRNA-treated males are not significantly different (analysis of variance (ANOVA), $F = 0.469$, $P = 0.798$), and those of *EGFP* dsRNA-treated females and *Td-dsx* dsRNA-treated females are also not significantly different (ANOVA, $F = 0.431$, $P = 0.785$). (**A–D**) Head horn lengths of wild type and *EGFP* dsRNA-treated males are not significantly different (ANOVA, $F = 0.004$, $P = 0.948$). (**A**) Head horn lengths of *Td-dsx* dsRNA (dsxAll)-treated males are shorter than those of wild type and *EGFP* dsRNA-treated males (ANOVA, $F = 12.693$, $P < 0.001$). (**B–D**) Head horn lengths of wild-type males, *EGFP* dsRNA-treated males and *Td-dsx* dsRNA-treated males are not significantly different (**B**: dsxF-L + S, ANOVA, $F = 0.081$, $P = 0.923$; **C**: dsxF-S, ANOVA, $F = 0.134$, $P = 0.875$; **D**: dsxC, ANOVA, $F = 0.511$, $P = 0.603$). (**A,B**) *Td-dsx* dsRNA (dsxAll and dsxF-L + S)-treated females are induced head horns (**A**: dsxAll, Mann–Whitney *U*-test, $Z = 2.860$, $P = 0.004 < 0.05$; **B**: dsxF-L + S, Mann–Whitney *U*-test, $Z = 2.816$, $P = 0.005 < 0.05$). The sizes of these induced head horn of *Td-dsx* dsRNA (dsxAll and dsxF-L + S)-treated females are not significantly different (*t*-test, $t = 1.850$, $P = 0.082$). (**E–H**) Thoracic horn lengths of wild type and *EGFP* dsRNA-treated males are not significantly different (ANOVA, $F = 0.014$, $P = 0.905$). (**E**) Thoracic horns of *Td-dsx* dsRNA (dsxAll)-treated males are disappeared (Kruskal–Wallis test, $H = 12.693$, $P < 0.001$). (**F–H**) Thoracic horn lengths of wild-type males, *EGFP* dsRNA-treated males and *Td-dsx* dsRNA-treated males are not significantly different (**F**: dsxF-L + S, ANOVA, $F = 0.512$, $P = 0.602$; **G**: dsxF-S, ANOVA, $F = 0.971$, $P = 0.385$; **H**: dsxC, ANOVA, $F = 0.335$, $P = 0.717$).

the case, novel sex-specific regulation of *dsx* in horn morphogenesis might be the key evolutionary event allowing for the transition from sexually monomorphic to sexually dimorphic horns.

The different phenotypes of *dsx* RNAi in head and prothoracic horns suggest that two types of co-option might have occurred in the evolution of horns in *T. dichotomus*. In the case of the head horn, *dsx* might first acquire an expression domain in the head horn-forming region via *cis*-regulatory changes in *dsx*. Then, *dsx* might be co-opted into a pre-existing horn formation gene network, and the horn becomes sexually dimorphic. If horns are advantageous for male–male combat, the male Dsx isoform will promote horn formation while the female Dsx isoform acts to repress horn formation, much like the antagonistic male and female Dsx isoforms that either activate or repress target genes in *Drosohila* [11,12,26,27]. On the other hand, in the prothoracic horn, the male Dsx isoform initiates horn formation by a co-option of the horn formation gene network into the sex-determination pathway.

In addition to this, horn formation is also controlled by environmental signals, especially nutrition [28,29]. The insulin/IGF pathway, which directly links nutrition to cell growth, has a crucial role in the growth of horns [30,31]. Therefore, an integration of signals from both sex determination and nutrition into the horn-developmental pathway is involved in rapid horn development leading to the extreme trait of the male-specific exaggerated horn.

Td-dsx controls all sexual-dimorphic structures

Previous studies showed that *dsx* has a role in sexually dimorphic morphologies, such as sex combs, abdominal segment number, reproductive organs and genitalia [7,8,11,32,33]. To confirm whether *Td-dsx* controls sexual dimorphisms other than horns, we analysed all known sexually dimorphic structures in *T. dichotomus* by *Td-dsx* RNAi using dsRNA for all isoforms (dsxAll). In the surface structures of the prothorax and elytra (forewing), wild-type males had glossy and smooth surfaces, whereas they were rough and hairy in females. The *Td-dsx* RNAi phenotype revealed the existence of intermediate phenotypes in both sexes (supplementary Fig S2 online), and also affected the development of internal reproductive organs (supplementary Fig S3A–I online). In the gonads, both testes and ovaries resembled an intermediate structure following *Td-dsx* RNAi (supplementary Fig S3G,I online). Furthermore, the male genitalia were severely affected and transformed into female-like genitalia, whereas the female genitalia were less affected (supplementary Fig S3K–M online). Taken together, these findings indicate that *Td-dsx* had crucial roles in all aspects of the sexually dimorphic development that we examined.

In conclusion, our *dsx* RNAi analyses reveal that *dsx* has essential roles in not only pre-existing traits but also horns as an evolutionary novelty, and might also have an important role at the evolutionary transition from sexually monomorphic to sexually dimorphic traits.

METHODS

Larval RNAi analysis. *Td-dsx* cDNAs (dsxAll, 430 bp; dsxF-L + S, 450 bp; dsxF-S, 245 bp; and dsxC, 421 bp) for RNAi analysis were amplified using the primer sets listed in supplementary Table S1 online, and subcloned into the pCR4-TOPO vector. PCR products were confirmed by sequencing. Plasmids containing *Td-dsx* and enhanced green fluorescent protein (*EGFP*) cDNAs were used as templates for dsRNA synthesis. Preparation of dsRNA was performed according to Niimi *et al* [23]. dsRNA (10 or 20 μg) was injected laterally into the T1 segment of each third (last)-instar larva just before the prepupal stage using a 1-ml syringe (Terumo) with a 30-gauge needle (Becton Dickinson). *EGFP* dsRNA was injected as a negative control. As described in Kijimoto *et al* [15], analysis of variance was performed with IBM SPSS Statistics Ver. 21.0 (IBM SPSS Inc., Illinois) to assess the significance of the effect of *Td-dsx* RNAi on horn length.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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