doublesex regulates sexually dimorphic beetle horn formation by integrating spatial and temporal developmental contexts in the Japanese rhinoceros beetle *Trypoxylus dichotomus*

Authors
Shinichi Morita[1], Toshiya Ando[1,2], Akiteru Maeno[3], Takeshi Mizutani[1], Mutsuki Mase[1,4], Shuji Shigenobu[2,5], Teruyuki Niimi[1,2,4]*

Affiliations
[1] Division of Evolutionary Developmental Biology, National Institute for Basic Biology, 38 Nishigonaka, Myodaiji, Okazaki 444-8585, Japan
[2] Department of Basic Biology, School of Life Science, SOKENDAI (The Graduate University for Advanced Studies), 38 Nishigonaka, Myodaiji, Okazaki 444-8585, Japan
[3] Mammalian Genetics Laboratory, Genetic Strains Research Center, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan.
[4] Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan.
[5] NIBB Core Research Facilities, National Institute for Basic Biology, 38 Nishigonaka Myodaiji, Okazaki 444-8585, Japan

Keywords
Trypoxylus dichotomus, Sex determination, Sex differentiation, *doublesex*, beetle horn, sexual dimorphism.

Abstract

Many scarab beetles have sexually dimorphic exaggerated horns that are an evolutionary novelty. Since the shape, number, size, and location of horns are highly diverged within Scarabaeidae, beetle horns are an attractive model for studying the evolution of sexually dimorphic and novel traits. In beetles including the Japanese rhinoceros beetle *Trypoxylus dichotomus*, the sex determination gene *doublesex* (*dsx*) plays a crucial role in sexually dimorphic horn formation during larval-pupal development. However, knowledge of when and how *dsx* drives the gene regulatory network (GRN) for horn formation to form sexually dimorphic horns during development remains elusive. To address this issue, we identified a *Trypoxylus*-ortholog of the sex determination gene, *transformer* (*tra*), that regulates sex-specific splicing of the *dsx* pre-mRNA, and whose loss of function results in sex transformation. By knocking down *tra* function at multiple developmental timepoints during larval-pupal development, we estimated the onset when the sex-specific GRN for horn formation is driven. In addition, we also revealed that *dsx* regulates different aspects of morphogenetic activities during the prepupal and pupal developmental stages to form appropriate morphologies of pupal head and thoracic horn primodia as well as those of adult horns. Based on these findings, we discuss the evolutionary developmental background of sexually dimorphic trait growth in horned beetles.

1. Introduction

Beetle horns are used as weapons for intraspecific combats between males. Beetle horns display sexual dimorphism in many Scarab species, and their shapes, numbers, sizes and forming regions are highly diverged even among closely related species (Eberhard, 1980; Emlen et al., 2007a; Busey et al., 2016). Interestingly, the diversified horn forms are associated with their fighting styles, such as scooping up, piercing and throwing (McCullough et al., 2014). Furthermore, beetle horns are thought to be an evolutionary novelty. They are an outgrowth structure derived not from an appendage but from an epidermal sheet. Elucidating how these novel traits were acquired in Scarab species will lead to better understanding the mechanisms of morphological diversification during evolution. Therefore, beetle horns are an attractive model for studying not only the evolution of novel traits but also the association of trait novelty with sexually dimorphic development.

Beetle horn development has been investigated in several horned beetles including the Japanese rhinoceros beetle, *Trypoxylus dichotomus* (Coleoptera, Scarabaeidae, Dynastinae). *T. dichotomus* has sexually dimorphic exaggerated horns on the head and prothorax, which are used in combats among conspecific males as weapons (Hongo, 2003; Siva-Jothy, 1987).The head horn is shaped like a plow with a long stalk and bifurcated twice at the distal tip, while the prothoracic horn is not only shorter than the head horn but also bifurcated once at the distal tip. In *T. dichotomus,* horns are first formed as epidermal primordia during the prepupal period. *T. dichotomus* male adults have exaggerated horns at the head and prothoracic regions, whereas female *T. dichotomus* are lacking these structures. However, *T. dichotomus* females do have a small head horn at pupal stage (Fig. 1B, Fig. S1). Sexual dimorphism of horns can be first observed at prepupal stage (Emlen et al., 2007a; Ito et al., 2013). Immediately after pupation in males, the prepupal horn primordia are elongated to the same length as an adult horn. However, the pupal horn shape is rounded, and slightly larger than an adult horn (Fig. 1B, Fig. S1) (Emlen et al., 2007b; Matsuda et al., 2017). In other horned beetles, the pupal horn is reported to

be remodeled during the pupal period to form the final adult horn (Emlen et al., 2007a; Moczek et al., 2006, 2007).

In insects, orthologs of a transcription factor gene *doublesex* (*dsx*) play a pivotal role in sexual differentiation, and are involved in formation of sexually dimorphic traits by expressing sex-specific isoforms (*dsxM* and *dsxF*) (Cline and Meyer, 1996; Gempe and Beye, 2011; Hasselmann et al., 2008; Hediger et al., 2010; Ito et al., 2013; Kijimoto et al., 2012; Shukla and Palli, 2012a; Suzuki et al., 2005). In addition, tissue-specific higher expression of *dsx* is detected where sexually dimorphic structures are formed to induce their development (Tanaka et al., 2011; Rice et al., 2018; Kunte et al., 2014; Bhardwaj et al., 2018). In horned beetles including *T. dichotomus*, the *dsx* orthologs also regulate sexually dimorphic horn formation during larval-pupal development. RNA interference (RNAi) targeting *dsx* results in intersexual phenotypes both in males and females (Ito et al., 2013; Kijimoto et al., 2012).

In *Drosophila melanogaster*, sex-specific splicing of *dsx* is regulated by the sex determination cascade (Cline and Meyer, 1996; Schütt and Nöthiger, 2000). In *D. melanogaster*, *Sex-lethal* (*Sxl*) is the master sex determination gene, which encodes an RNA-binding protein with two RNA recognition motifs (RRM) that directly bind to the target RNAs. Since functional Sxl protein is translated only in females (Erickson and Cline, 1993; Wrischnik et al., 2003), Sxl controls sex-specific splicing of *transformer* (*tra*) pre-mRNA and produces functional Tra protein only in females (Inoue et al., 1990). Female-specific Tra has a short conserved motif (CAM: C, *Ceratitis*; A, *Apis*; M, *Musca*) (Hediger et al., 2010), which is conserved in all *tra* orthologs identified in other insects except for those in *Drosophila* species and the sandfly (*Phlebotomus papatasi*) (Geuverink and Beukeboom, 2014). Tra forms a heterodimer with Transformer2 (Tra2) in females. Tra2 has an RRM domain that directly binds to target RNAs including the *dsx* pre-mRNA. Then, the Tra/Tra2 heterodimer regulates the sex-specific alternative splicing of *dsx* by directly binding to *dsx* transcripts in females (Inoue et al., 1992: Lynch et al., 1996). *dsx* regulates a battery of downstream genes to form sexually dimorphic traits by binding to Dsx

binding motif sequences (Erdman et al., 1996; Luo et al., 2011), and activating or repressing their transcription (Verhulst and van de Zande, 2015). Intersex (Ix) functions as a female-specific co-activator by directly binding to female-specific isoforms of the DsxF protein, and regulates development of female-specific traits (Garrett-Engele et al., 2002). Ix has a conserved motif, the Mediator complex subunit 29 (Med29). The *Sxl*-[*tra/tra2*]-[*dsxF/ix*] pathway is turned on only in females, and default splicing in males results in expression of the male-specific splicing variant, *dsxM* (Cline and Meyer, 1996; Schütt and Nöthiger, 2000). Loss of function of the sex determination genes in females, and gain of function of those in males result in sex transformation (Cline and Meyer, 1996; Schütt and Nöthiger, 2000).

Orthologous genes corresponding to the *D. melanogaster* sex determination genes described above are conserved among many holometabolous insects (Hoshijima et al., 1991; Hediger et al., 2010; Pane et al., 2005; Concha and Scott, 2009; Verhulst et al., 2010a; Liu et al., 2015; Shukla and Palli, 2012b, 2013). However, whether these genes are involved in sex determination pathways to form sexually dimorphic horns remains elusive. In addition, when and how *dsx* interacts with the gene regulatory network (GRN) for horn formation to drive cellular activity such as cell growth, cell death and cell movement to form sexually dimorphic is also unknown.

To understand the developmental and genetic mechanisms needed to form sexually dimorphic horns in *T. dichotomus* we first described a precise time course of the morphogenetic changes of male and female horn primordia during larval-pupal development using time-lapse photography. Next, we examine the function of putative sex determination genes in *T. dichotomus* using larval RNAi by focusing on orthologs of known *D. melanogaster* sex determination genes. Based on the results, we selected *Tdic-tra* to investigate the initiation timing of GRN for horn formation by knocking down at multiple timepoints during larval-pupal development. We demonstrate that the GRN for horn formation, which is supposed to be modified by *dsxM* and *dsxF* functions in males and females, is driven at a

very early stage of larval-pupal development before clear morphological changes in horn primordia can be detected. In addition, we show that *dsxM* has different functions in both the prepupal and pupal stages during the formation of appropriate morphologies in pupal and adult horns in males. Based on these findings, we discuss the evolutionary developmental background of sexually dimorphic horn formation in horned beetles.

2. Materials and methods

2.1. Insects

We purchased *T. dichotomus* larvae from Loiinne (Japan), and Urakiso Tennen Kabuto no Sato (Japan). The last instar larvae were sexed as described previously (Ito et al., 2013), individually fed on humus in plastic containers, and kept at 10 °C until use. Larvae were moved to room temperature at least 10 days, and reared at 28 °C.

2.2. Micro-CT analysis

A male head tissue at 24 hours after pupal-chamber formation (24 h APF) was fixed in Carnoy solution at room temperature overnight, washed in 70% ethanol and stored in 70% ethanol. The sample was rehydrated through a graded ethanol series, and stained with 25% Lugol solution (Degenhardt et al., 2010; Metscher, 2009a, 2009b) for 5 days. The stained sample was scanned using an X-ray micro-CT device (ScanXmate-E090S105, Comscantechno Co., Ltd., Japan) at a tube voltage peak of 60 kVp and a tube current of 100 μA. The sample was rotated 360 degrees in steps of 0.24 degrees, generating 1500 projection images of 992 × 992 pixels. The micro-CT data were reconstructed at an isotropic resolution of 13.3 × 13.3 × 13.3 μm, and converted into an 8-bit tiff image dataset using coneCTexpress software (Comscantechno Co., Ltd., Japan). Three-dimensional tomographic images were obtained using the OsiriX MD software (version 9.0, Pixmeo, SARL, Switzerland) and Imaris software (version 9.1, Carl Zeiss Microscopy Co., Ltd., Japan). Supplemental videos were edited using Adobe Premiere Pro CC (Adobe Systems Co., Ltd., Japan).

2.3. Time-lapse photography

To monitor the precise time course of the morphogenetic changes of male and female horn primordia during larval-pupal development, time-lapse photography was performed at 28 °C every 30 minutes until they had developed into adults using a CMOS camera (VCC-HD3300, SANYO, Co., Japan).

2.4. Observation of horn primordia development based on stable prepupal staging

We found that the head-rocking behavior observed at the end of pupal-chamber formation can be an unambiguous developmental marker for initiation of the prepupal stage (0 h APF). Using this marker, sampling of horn primordia for staging was performed every 12 hours until they had developed into pupae (120 h APF) using time-lapse photography. Head and thoracic horn primordia were dissected out from larvae in 0.75% sodium chloride, and fixed for 90 minutes at room temperature with 4% paraformaldehyde in phosphate buffered saline (PBS). After being washed twice in PBS, photographic images were obtained with a digital microscope (VHX-900, KEYENCE, Co., Japan).

2.5. BLAST search for sex determination genes

We searched for orthologs of the sex determination genes in the *T. dichotomus* RNA-seq database (Ohde et al., in preparation) using full-length complementary DNA (cDNA) sequences of *D. melanogaster* genes (*Sxl*, *tra*, *tra2*, *ix*) as query sequences using the tblastn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). We evaluated orthology of those genes by performing reciprocal tblastx searches against the *D. melanogaster* cDNA database (r6.06) using the identified *T. dichotomos* genes as queries. We deposited cloned partial cDNA sequences of *T. dichotomus* genes at the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank Accession number for *Tdic-Sxl* is LC385009, for *Tdic-tra* are LC385010 - LC385012, for *Tdic-tra2* are LC385013 - LC385018, and for *Tdic-ix* is LC385019.

2.6. cDNA library construction, reverse transcription-polymerase chain reaction (RT-PCR), and sequencing

Total RNA was extracted from each of head or thoracic horn primordia in wild type and RNAi-treated individuals (*EGFP*, *Sxl*, *tra*, *tra2*, *ix*, *dsx* and *dsxF*) using TRI Reagent (Molecular Research Center, Inc., USA) according to the manufacturer's instructions. First-stranded cDNA was

synthesized with the SuperScript III Reverse Transcriptase (Life Technologies Japan Ltd., Japan) using 1 μg total RNA as a template. Primer sets for cloning and double stranded RNA (dsRNA) synthesis were designed based on the cDNA sequences identified above (Table S1). PCR was performed using Ex Taq DNA polymerase (Takara Bio Inc., Japan) according to the manufacturer's protocol. Amplified PCR products were purified using MagExtractor (TOYOBO, Co., Ltd., Japan), and subcloned into the pCR4-TOPO vector using the TOPO TA cloning Kit (Life Technologies Japan Ltd., Japan). Sequences of the inserts were determined by a DNA sequencing service at FASMAC Co. Ltd., Japan.

2.7. Gene expression analysis by quantitative RT-PCR (qRT-PCR)

Primer sets for qRT-PCR were designed using Primer3Plus program (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Table S1). qRT-PCR was performed using the cDNA libraries synthesized above and THUNDERBIRD SYBR qPCR Mix (TOYOBO, Co., Ltd., Japan) according to the manufacturer's instructions. The relative quantification in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.8. Larval RNAi

Primer pairs for dsRNA synthesis were designed to be inside a common region shared by male and female isoforms (Fig. 2A, Table S1). Partial sequences of the target sequences were amplified by PCR using primers flanked with the T7 promoter sequence in the 5'-ends. dsRNAs were synthesized using AmpliScribe T7-Flash Transcription Kit (Epicentre Technologies, Corp., USA), and the purified PCR products used as templates. Injections of dsRNA were performed as described previously (Ito et al., 2013). dsRNA was injected into each late last instar larva or prepupa under the conditions described in Table S2 and Table S3. *Enhanced green fluorescent protein* (*EGFP*) dsRNA was injected as a negative control.

2.9. Horn and body length measurement

Horn length was measured by extracting the contour of a horn in the lateral view using the SegmentMeasure plugin for ImageJ 64 developed by Hosei Wada. We defined a body length as the length between the anterior tip of the clypeus in the head to the posterior most region of the abdomen, and was directly measured using a digital caliper (DN-100, Niigata seiki, Co., Ltd., Japan)

3. Results

3.1. Development of sexual dimorphism in *T. dichotomus* horn primordia

To identify the developmental timepoint when sexual dimorphism of horns first appears in *T. dichotomus*, we described morphological changes of head and thoracic horn primordia during the prepupal period. Micro-CT analysis of a head horn primordium at an early stage (24 h APF) in the prepupa revealed that a head horn primordium was formed in the clypeolabral region during the prepupal stage as investigated in other beetles so far (Fig. 1A,B, Movie 1a,b) (Posnien et al., 2010, 2011; Busey et al., 2016; Ohde et al., in preparation). In addition, head horn primordia were formed above the clypeus in the clypeolabrum (Fig. 1A, Movie 1a,b). To determine the exact timepoint when protrusion of the primordium is initiated, we established a time-lapse photography system (Fig. 1C). Until recently, developmental staging of *T. dichotomus* prepupae had been difficult because they form pupation chambers underground and pupate in them. Using our time-lapse photography system, we found that the head-rocking behavior at the end of pupal-chamber formation can be an unambiguous marker for the initiation of the prepupal stage (Movie 2). We could minimize the developmental deviation between individuals within 12 hours using this precise marker (Fig 1C, Movie 2). The average prepupal period was 5.5 ± 0.19 days (131 ± 4.7 hours) in males and 5.4 ± 0.17 days (129 ± 4.3 hours) in females (Fig. 1D). Based on this staging paradigm, we manually dissected out horn primordia every 12 h after pupal-chamber formation (APF). We found that morphological sexual dimorphism of horn primordia appeared at 36 h APF (Fig. 1B). Therefore, we concluded that the GRN driving horn sexual dimorphism formation would be activated before 36 h APF in *T. dichotomus*. In addition, we also found another morphological character that appears at 36 h APF, and can be an unambiguous developmental marker to know the onset of sexual dimorphism formation. In insect, mandibular tendons tightly connect mandibular muscles and apodemes during larval stages (Movie 1b) (Manton and Harding, 1964; Soler et al., 2016), but we found that the tight connections were physically separated at 36 h APF by apolysis occurring at every body part including the surrounding ocular in the head.

Therefore, this apolysis can be an unambiguous developmental marker to distinguish between 24 h APF and 36 h APF.

3.2. Identification of genes regulating the sex-specific splicing of *Tdic-dsx* during *T. dichotomus* horn formation

In order to estimate the onset of the developmental program for sexually dimorphic horn formation, we tried to shut down this program at various developmental stages by knocking down *Tdic-dsx* in males or females. If the timing of *Tdic-dsx* RNAi is early enough, *Tdic-dsx* expression levels can be repressed in horn primordium from the onset of sexual dimorphic developmental program, and resulted in short head horn formation (Ito et al., 2013). On the other hand, the later the timing of *Tdic-dsx* RNAi treatment becomes, the more the initial phases of the male-specific horn formation program driven by *Tdic-dsx* are trimmed, and a slightly shorter horn in males, or a slightly protruded prothorax in females would be formed. However, because horn size deviates depending on the nutrition conditions in *T. dichotomus*, it was technically difficult to determine the exact time point when the developmental program for sexually dimorphic horn formation initiates by assessing whether the horn is slightly shortened in males or slightly protruded in females when the gene function is inhibited by RNAi before or after *Tdic-dsx* begins to function. In order to get around this problem, we focused on knocking down the sex determination genes which would result in sex transformation, which accompanies more drastic morphological change, through switching of the sex-specific splicing variant of *dsx*. We predicted that such drastic morphological change in RNAi-treated individuals would make morphological scoring of horn formation technically easier. As candidate sex determination genes, we focused on *T. dichotomus* orthologs of *D. melanogaster* sex determination genes (*Sxl*, *tra*, *tra2* and *ix*) (Cline and Meyer, 1996; Schütt and Nöthiger, 2000), and tested whether these genes might have function during sex determination in *T. dichotomus*.

First, we investigated whether these genes produce sex-specific splicing variants in *T. dichotomus* by RT-PCR. All of these genes were

expressed in male and female prepupal head horns (Fig. 2A light green bars, Fig. 2B, Table S1). Among these genes, sex-specific splicing variants were detected only in *Tdic-tra* (Fig. 2B). Next, to test whether these genes function as sex determination genes, we performed larval RNAi experiments using dsRNA targeting the common regions between sexes (Fig. 2A, black bars, Table S1, Table S2) (Ito et al., 2013).

In females, the morphological sex transformation was observed in the RNAi treatments targeting *Tdic-tra* and *Tdic-ix*, whereas no morphological changes were observed in males (Fig. 3A). Such female-specific sex transformation phenotypes were comparable with the mutant phenotypes of *tra*, *tra2* and *ix* in *D. melanogaster* (Cline and Meyer, 1996; Schütt and Nöthiger, 2000). Concerning *Tdic-tra2,* we could not observe its adult phenotype because of the prepupal lethality in both sexes. The phenotypes in *Tdic-tra* RNAi females and *Tdic-ix* RNAi females were different and the *Tdic-ix* RNAi phenotype was similar to the effects of the*Tdic-dsx* RNAi phenotype (Fig. 3A). In the *Tdic-tra* RNAi females, ectopic horn formation was observed both the head and prothorax (Fig. 3A), whereas in *Tdic-ix* RNAi females, ectopic horns were formed only in the head, and these horns were significantly shorter than the ectopic head horns in *Tdic-tra* RNAi females (Fig. 3A,B). Such a difference in morphology was also observed in another sexually dimorphic structure, the intercoxal process of prosternum (IPP). Male IPPs are generally larger than female IPPs (Fig. 3A). Although both the *Tdic-tra* and *Tdic-ix* RNAi females have larger IPPs than female controls (*EGFP* RNAi), *Tdic-tra* RNAi females have much larger IPPs than *Tdic-ix* RNAi females (Fig. 3A). These results combined with the intermolecular interactions reported in *D. melanogaster* led us to predict that *Tdic-tra* may also regulate the sex-specific splicing of *Tdic-dsx* in *T. dichotomus*. To test this, we investigated the splicing patterns of *Tdic-dsx* in the above RNAi-treated males and females. We designed PCR primer sets to amplify the region including the whole female specific exon, which is spliced out in males (Fig. 2A, light green bars, Table S1). We found that the sex-specific splicing pattern observed in wild type (Fig. 2B) was switched in *Tdic-tra* and *Tdic-tra2* RNAi treatments, whereas splicing patterns were not

changed by *Tdic-Sxl* and *Tdic-ix RNA*i treatments (Fig. 3C). These data indicate that *Tdic-tra* and *Tdic-tra2* also regulate female-specific splicing of *Tdic-dsx* in *T. dichotomus*. The switching of sex-specific splicing of *Tdic-dsx* and the resultant morphological sex transformation in *Tdic-tra* RNAi females implies that the sex-specific splicing regulation of *Tdic-dsx* by *Tdic-tra* is also conserved in *T. dichotomus* as in other holometabolous insects investigated so far (*Drosophila*; Hoshijima et al., 1991, *Musca*; Hediger et al., 2010, *Ceratitis*; Pane et al., 2005, *Lucilia*; Concha and Scott, 2009, *Nasonia*; Verhulst et al., 2010a, *Bactrocera*; Liu et al., 2015 and red flour beetle *Tribolium*; Shukla and Palli, 2012b, 2013). Taken together, we concluded that *Tdic-tra* functions as a sex determination gene during horn formation in *T. dichotomus*.

3.3. Estimation of the onset of the developmental program for sexually dimorphic horn formation using *Tdic-tra* RNAi

As sexual dimorphism of horn primordia appeared at 36 h APF (Fig. 1B), we can estimate that the onset of developmental program for sexually dimorphic horn formation would be a stage before 36 h APF. To estimate this timepoint more accurately, we performed *Tdic-tra* RNAi in females at multiple developmental timepoints during pupal chamber formation periods and prepupal periods and evaluated the extent of sexual transformation in horns.

If the timing of *Tdic-tra* RNAi in females is early enough, the ectopic *Tdic-dsxM* can be produced in female horn primordium from the onset of sexual dimorphic developmenal program, and full sexual transformation can be achieved. On the other hand, the later the timing of *Tdic-tra* RNAi treatment becomes, the more the initial phases of the male-specific horn formation program driven by ectopically expressed *Tdic-dsxM* are trimmed, and at the same time repressed by normally expressed *dsxF*. Therefore, by determining the latest RNAi injection timing when a full sexual transformation phenotype is observed, we can estimate the onset of the sexually dimorphic horn formation program mediated by the ectopic *Tdic-dsxM* expression.

In this experiment, fully matured female last instar larvae and unstaged female prepupae were injected with *EGFP* or *Tdic-tra* dsRNA (Table S3). We performed time-lapse photography until pupation using these larvae, and retrospectively estimated the exact timing of injection before pupation. As we expected, morphological scoring of horn formation became technically easier than that in *Tdic-dsx* RNAi treatments. *Tdic-tra* RNAi females treated later than the timepoint at 130 hours before pupation showed either no morphological changes or incomplete sex transformation of head or horn, if any phenotype was observed at all (Fig. 4A(ii) and (iii), B green dot). On the other hand, *Tdic-tra* RNAi females treated earlier than the timepoint at 134 hours before pupation formed fully developed male horns (Fig. 4A(i), B magenta dot). In this experiment, the prepupal period of wild type females and *Tdic-tra* RNAi females were comparable (Fig. 1D). Therefore, these RNAi experiments suggested that the estimated timepoint of the onset of the developmental program for sexually dimorphic horn formation is around 134 hours before pupation.

Since this timepoint was estimated by means of RNAi, we predicted that there should be time lag between the timing of injection and the timing of the decrease of the functional protein after the mRNA degradation. Thus, we also quantified expression dynamics of *Tdic-tra* mRNA after RNAi treatment by qRT-PCR. Because the pupal-chamber formation period before the head-rocking behavior is reversible, it is technical difficulty to determine 134 hours before pupation (7 hours before pupal chamber formation). As the prepupal period after pupation is irreversible, RNAi treatments were performed at 24 h APF in this experiment, and the expression levels of *Tdic-tra* and *Tdic-dsxF* were quantified every 12 hours up to 36 hours after injection (Fig. 2A, green bars, Fig. 4C). As a result, the expression levels of *Tdic-tra* and *Tdic-dsxF* were decreased at after 36 hours after injection (Fig. 4C). Thus, we corrected the estimated timepoint for onset of the developmental program for sexually dimorphic horn formation driven by *Tdic-dsx* as -7 h APF (127 minus 134) (Fig. 4D), because the mean prepupal period of *Tdic-tra* RNAi females was slightly (4 hours) shorter than that of normally developed females (127 hours in *Tdic-tra RNAi*) (Fig. 1D). Since

mRNA started to be degraded 36 hours after RNAi treatments (Fig. 4C), we estimated that this timepoint corresponded to 29 hours APF (-7 plus 36) (Fig. 4D). Interestingly, taking morphological data into account, this timepoint was 7 hours before the initial sexual dimorphism appears in horn primordia (Fig.1B, Fig. 4D).

3.4. Sexually dimorphic morphogenesis in male and female horn primordia

T. dichotomus male adults have exaggerated horns at the head and prothoracic regions whereas female adults only have three small protrusions at the clypeolabral region (Fig. 5A, magenta arrowhead). We wondered whether the male head horn and three female head protrusions were homologous or not. Since horn primordium forms in both males and females and occupies the almost entire head segment in clypeolabrum during prepupal stages, head horn primordia formation in males and females can be interpreted as homologous between each other (Fig. 1B). However, due to the lack of clear morphological landmarks indicating homology between male head horn and female head protrusion, their homology remained elusive. Unexpectedly, however, we obtained an intermediate sexual transformation phenotype of *Tdic-tra* RNAi to solve this problem. When we injected *Tdic-tra* dsRNA in small amounts (2.5 μg), we obtained adult beetles possessing both the three small protrusions specific to females and a small ectopic protrusion anterior to the three protrusions, seemingly analogous to a male head horn (Fig. 5B, C). This result suggests that at least the distal tip of a male head horn is not homologous to female head protrusions.

The male and female horn primordia were formed above the clypeus in clypeolabrum during larval-pupal development (Fig. 1A, B), but the adult male head horn and adult female protrusions formed from different regions in a clypeolabrum (Fig. 5A-C). The shape of pupal horn is rounded and slightly larger than that of male adult horns in *T. dichotomus,* and the male and female pupal epithelia in the head horn primordia are remodeled during the pupal stage to form respective adult head structures (Fig. 5D, Fig. S1) (Emlen et al., 2007a). In males, both a head horn and a thoracic horn become slimmer during pupal remodeling, whereas in females, almost all of the

pupal head horn disappears to form small three protrusions (Fig. S1). Thus, we also investigated whether such sexually dimorphic remodeling during pupal periods within horn primordia were also regulated by *Tdic-dsx*. We injected dsRNA targeting *Tdic-dsx* into male larvae at several developmental stages (Table S3) and found that adult males treated with *Tdic-dsx* dsRNA at 13 hours before pupal-chamber formation (13 h BPF) showed thickened horns similar to a pupal thoracic horn (Fig. 5E). In addition, when we injected *Tdic-dsx* dsRNA at 85 h BPF, males did not form adult thoracic horns although the small and short pupal thoracic horn were formed (Fig. 5F). These results indicate that during the prepupa, DsxM is required for pupal horn primordium formation both in the head and in the thorax horn. On the other hand, during pupation, DsxM is required for horn remodeling only in the thorax and is dispensable in the head horn remodeling.

Finally, we also tested whether *Tdic-dsx* is upregulated region-specifically in a sexually dimorphic head horn primordum as reported in sexually dimorphic structure formation in other insects (Kopp, 2011; Tanaka et al., 2011; Rice et al., 2018; Kunte et al., 2014; Bhardwaj et al., 2018). We performed qRT-PCR at 36 h APF, and found that *Tdic-dsxM* actually showed significantly higher expression in head primordium epidermis than in the surrounding head epidermis (Fig. 5G,H). On the other hand, *Tdic-dsxF* did not show significantly higher expression in the horn primordium at this stage.

4. Discussion

Here, we have described development of sexually dimorphic horn in a Japanese rhinoceros beetle, *T. dichotomus*, from both a morphological and genetic point of view. We focused on *Tdic-dsx* as the major regulatory gene for sexually dimorphic horn formation and investigated its time of action by knocking down sex determination genes in multiple developmental timepoints during horn morphogenesis period through the use of RNAi. To manipulate sex-specific alternative splicing of *Tdic-dsx*, we identified *Tdic-tra* as a key regulator for the sex-specific splicing of *Tdic-dsx*. We discuss below the genetic regulatory framework of sex determination in *T. dichotomus*, the detailed actions of *Tdic-dsx* in males and females to form sexually dimorphic horns, and evolutionary background realizing the exaggerated horn formation in horned beetles.

4.1. Genetic regulatory mechanisms of sex determination in *T. dichotomus*

Previous studies focusing on horned beetle revealed that *dsxM* promotes male-specific traits including exaggerated horn formation whereas *dsxF* promotes female-specific traits including suppression of horn formation (*Trypoxylus*; Ito et al., 2013, *Onthophagus*; Kijimoto et al., 2012). Our RNAi experiments targeting the orthologs of *D. melanogaster* sex determination genes revealed that Tdic-Tra and Tdic-Tra2 regulate sex-specific splicing of the *Tdic-dsx* pre-mRNA, and control downstream sex differentiation mediated by *Tdic-dsx*, whereas *Tdic-Sxl* is dispensable for sex differentiation during larval-pupal development (Fig. 3C).

Concerning Tdic-Tra and Tdic-Tra2, their conserved function as splicing factors targeting *Tdic-dsx* pre-mRNA as in *D. melanogaster,* the housefly *Musca domestica, the* Mediterranean fruit fly *Ceratitis capitata,* the Australian sheep blowfly *Lucilia cuprina*, the parasitic wasp *Nasonia vitripennis,* the oriental fruit fly *Bactrocera dorsalis* and *T. castaneum*, implies that regulatory mechanisms of the sex-specific splicing of *dsx* is similar to those in *D. melanogaster* (Concha and Scott, 2009; Hediger et al., 2010; Hoshijima et al., 1991; Liu et al., 2015; Pane et al., 2005; Shukla and Palli, 2012b, 2013; Verhulst et al., 2010a). In addition, the domaing

structures of Tdic-Tra and Tdic-Tra2 are conserved, and the RNA binding motif (RRM) is present only in Tra2. Thus, as in *D. melanogaster*, Tdic-Tra and Tdic-Tra2 might form a heterodimer that directly binds to *Tdic-dsx* pre-mRNA, and regulates the sex-specific splicing (Inoue et al., 1992: Lynch et al., 1996; Martín et al., 2011). In addition, the RNAi phenotypes of *tra* and *tra2* orthologs (i.e. the viable masculinized phenotype in *Tdic-tra* RNAi females, and the lethal phenotype in *Tdic-tra2* RNAi females) were comparable with those reported in other beetles (*Tribolium*; Shukla and Palli, 2013, *Cyclommatus*; Gotoh et al., 2016). Therefore, the function of Tra and Tra2 as sex determination factors targeting *dsx,* and the function of Tra2 as an essential factor progressing normal development during larval-pupal development seem to be conserved among Polyphaga, an insect suborder including the above three beetles.

In *D. melanogaster,* Ix directly binds to DsxF but not to DsxM, and function as a co-activator to facilitate transcription activity of target genes of DsxF (Garrett-Engele et al., 2002). The intersexual phenotype observed only in *Tdic-ix* RNAi-treated females (Fig. 3A) implies that Tdic-Ix might also interact with Tdic-DsxF but not with Tdic-DsxM to regulate female-specific sex differentiation as in *D. melanogaster*. Although we did not investigate the detailed molecular interaction between Ix and DsxF in *T. dichotomus*, a female-specific intersexual transformation phenotype reported in a stag beetle *Cyclommatus* (Gotoh et al., 2016) suggests that the female-specific function of *ix* may be conserved among Polyphaga as well.

Contrasting with the above functionally conserved genes, our RNAi experiments implied that Tdic-Sxl did not regulate the sex-specific alternative splicing of *Tdic-tra*, which is a direct regulatory target of Sxl in *D. melanogaster*. In fact, splicing of *tra* orthologs are not regulated by *Sxl* orthologs even in other Dipteran species (Meise et al., 1998; Saccone et al., 1998), and the sex determination systems of insects are instead regulated by various species-specific genes (e.g. *Sxl* in *D. melanogaster, Mdmd* in *Musca domestica*, *Nix* in *Aedes aegypti, Yob* in *Anopheles gambiae* and *Fem* in *Bombyx mori*) (Cline and Meyer, 1996; Schütt and Nöthiger, 2000; Hall et al., 2015; Kiuchi et al., 2014; Krzywinska et al., 2016; Sharma et al., 2017). Thus,

the sex-specific alternative splicing of *Tdic-tra* would also be regulated by species-specific genes other than *Tdic-Sxl*.

To summarize the regulatory mechanisms of *T. dichotomus* sex determination discussed above, functional Tdic-Tra is first expressed female-specifically through species-specific unknown mechanisms. Then, a Tdic-Tra/Tdic-Tra2 heterodimer would be formed only in females and produce *Tdic-dsxF* mRNA and Tdic-DsxF to promote female differentiation by interacting with Tdic-Ix (Fig. 6). On the other hand, in males, functional Tdic-Tra is not expressed, and default splicing of *Tdic-dsx* would result in *Tdic-dsxM* mRNA and Tdic-DsxM production to promote male differentiation. This genetic regulation, in which Tra/Tra2 controls the sex-specific alternative splicing of *dsx*, is shared with other holometabolous insects investigated so far, except for some holometabolous insects in which *tra* ortholog is lost (Geuverink and Beukeboom, 2014). Therefore, *T. dichotomus* seems to also utilize conserved core regulatory mechanisms shared among holometabolous insects to regulate sex-specific splicing of *dsx* and expression of *dsx*-mediated sexually dimorphic traits.

4.2. The gene regulatory network driving horn dimorphism formation in *T. dichotomus*

A previous study revealed that *Tdic-dsx* RNAi in both males and females does not result in a total loss of horns, but results in intermediate-lengthened head horn formation and loss of a thoracic horn (Fig. 3) (Ito et al., 2013). The head horn phenotype suggests that there exists a GRN for head horn formation that is operated independently of *Tdic-dsx*. *Tdic-dsxM* seems to enhance this GRN whereas *Tdic-dsxF* seems to suppress it. On the other hand, the thoracic horn phenotypes suggest that thoracic horn formation seems to be totally dependent on *Tdic-dsxM* function, and that action of *Tdic-dsxM* on horn formation might be different between head and thoracic horns during prepupa and/or pupa. However, in the course of our *Tdic-dsx* RNAi experiments, we noticed that thoraces of severely intersexually transformed individuals are always slightly curved at the thoracic horn formation region (Fig. 3A). This implies that *Tdic-dsxF*

expression in the female thorax still has suppressive activity against the GRN for thoracic horn formation. If this is the case, the regulatory relation between *Tdic-dsx* and GRN for horn formation has partial similarity between the head and the thorax. The phenotypic difference previously described in head and thoracic horns would be due to difference in length of horn formed in each body region. In the sections below, we mainly discuss the GRN for horn formation based on our head horn data in which interpretation of phenotypes is less ambiguous.

Our knockdown experiment of *Tdic-tra* revealed that the onset of *Tdic-dsx* modulating GRN for head horn formation is as early as 29 h APF. This is approximately 7 hour before the appearance of sexual dimorphism of head horn primordium. Therefore, *Tdic-dsx* seems to modulate patterning of a head horn primodium just before the onset of tissue growth, following tissue morphogenesis during prepupal stages.

Several genes involved in the GRN for horn formation have been identified in horned beetle species other than *Trypoxylus*, primarily by focusing on *Drosophila* appendage patterning genes (Moczek and Nagy, 2005; Moczek and Rose, 2009; Zattara et al., 2016). These studies propose an attractive evolutionary model in which large portions of the GRN for proximodistal appendage patterning were recruited to acquire beetle horns in the head and thoracic regions. Still, the overall framework of GRN for horn formation including the key regulatory gene sets involved, spatiotemporal expression dynamics and regulatory relation among those genes, has not been unveiled so far. Therefore, investigating regulatory relations between appendage genes and other head/thoracic patterning genes expressed in horn primordia focusing on this developmental timepoint will lead to verification of this model in the future studies. In addition, because candidates of the *dsx* target genes involved in GRN for horn formation were identified recently in a horned beetle, *Onthophagus taurus* (Ledón-Rettig et al., 2017), functional analyses focusing on orthologs of those genes expressed in *Trypoxylus* horn primordia at this timepoint will also lead to understanding of conserved and divergent aspects of sexually dimorphic horn formation in horned beetles.

We found that *Tdic-dsx* was upregulated region-specifically in head horn primordia at 36 h APF in both sexes (Fig. 5H). Such upregulation of the *dsx* ortholog during development is also reported in male-specific sex comb formation in *Drosophila* species (Kopp, 2011; Tanaka et al., 2011; Rice et al., 2018), in female-specific mimetic wing color pattern of *Papilio polytes* butterfly (Kunte et al., 2014), and male-specific pheromone producing organs in *Bicyclus anynana* buttefly wing (Bhardwaj et al., 2018). Intense studies focusing on regulatory mechanisms of sex comb formation in *Drosophila* species revealed that such region-specific upregulation of *dsx* is mediated by a positive feedback loop between *dsx* and a Hox gene, *sex combs reduced* (Kopp, 2011; Tanaka et al., 2011). Therefore, region-specific upregulation of *Tdic-dsx* in a head horn primordium might be mediated by an analogous positive feedback loop. Whether this feedback loop is composed of the autoregulation of *Tdic-dsx* or regulation among several regulatory genes, and how this regulatory loop was acquired during evolution will be an important question to reveal the molecular mechanisms of evolution of sexually dimorphic horns in horned beetles.

4.3. *Tdic-dsx*-mediated sexually dimorphic morphogenesis in head and thoracic horn formation during larval-pupal development

Our results of the short head horn formation via *Tdic-dsx* RNAi (Fig. 3A) clarified that during the prepupal stage *Tdic-dsxM* promoted the growth of head and thoracic horn primordium, whereas *Tdic-dsxF* suppressed growth of head and thoracic horn primordium (Fig. 6, prepupal stage) (Ito et al., 2013). In addition, during the pupal stage, *Tdic-dsxM* also regulates remodeling of a thoracic horn primordium from a rounded shape to a slender hooked shape (Fig. 5E,F, Fig. 6, pupal stage), whereas *Tdic-dsxM* is dispensable for head horn remodeling during pupal-adult development. These results indicate that *Tdic-dsxM* and *Tdic-dsxF* regulate different aspects of morphogenesis at both prepupal and pupal stages. Furthermore, to what extent *Tdic-dsx* is required during larval-pupal development is also finely regulated during male head formation, male thoracic horn formation, female head protrusion formation, and female flat prothorax formation.

Importantly, in contrast to the previous study that suggested that functions of *Tdic-dsxM* and *Tdic-dsxF* in *T. dichotomus* head and thoracic horn formation are to some extent analogous as described in the previous section (that is, in both of the head and the thoracic horn formation, *Tdic-dsxM* functions as a positive regulator whereas *Tdic-dsxF* functions as a negative regulator) (Ito et al., 2013), our experiments clarified that both spatial cues (i.e. different developmental contexts in head and prothorax) and temporal cues (i.e. different developmental contexts in prepupa and pupa) modulate the actions of *Tdic-dsxM* and *Tdic-dsxF* to drive appropriate morphogenetic activity in each horn primordium at each developmental stage.

A notable feature of *Tdic-dsx* during sexually dimorphic development is that the onset of action during horn development seems to be categorized earlier (i.e. during theprepupal period) compared to that of many other sexually dimorphic adult traits in holometabolous insects reported so far (i.e. the pupal period). In *D. melanogaster* sex comb formation, *Papilio* mimetic wing morph formation, and *Bicyclus* wing pheromone gland formation, tissue-specific high expression of *dsx* orthologs are detected after pupation (Tanaka et al., 2011; Rice et al., 2018; Kunte et al., 2014; Bhardwaj et al., 2018). Such a difference in *dsx*'s onset of action seems to be due to the requirement of drastic growth during sexually dimorphic structure formation. In either case mentioned above, the finally formed structure accompanies little to no tissue-level drastic growth during development. On the other hand, as in *T. dichotomus* horn formation, *D. melanogaster* genital organ formation, whose sexually dimorphic morphogenesis is regulated by *dsx*, accompanies drastic sexually dimorphic growth during larval-pupal development (Sanchez et al., 2001; Keisman et al., 2001; Ahmad and Baker, 2002), and its onset of action is as early as the prepupal stage. Association between *dsx*'s earlier onset of action and requirement of drastic growth during sexual dimorphism formation suggest that recruitment of the *dsx* function in the earlier stage may be one of the prerequisites to form exaggerated sexual dimorphism in insects.

The intermediate head horn formation via *Tdic-tra* RNAi in females (Fig. 5A-C) revealed that the distal region of male head horn and female

small protrusions are not homologous and are instead formed from different regions in the clypeolabrum. Such a distinct developmental origin of head horn within the clypeolabram is also clearly demonstrated in *Onthophagus* species of dung beetles (Busey et al., 2016), in which the *dsx* orthologs regulate the formation of distinct sexually dimorphic horns in either the anterior or the posterior region of the head. Analogy of multiple horn formation regions within the clypeolabrum in distinct horned beetle species implies a shared origin of beetle horns among these distantly related species. A comprehensive understanding of the GRN for horn formation in both *Trypoxylus* and *Onthophagus* alongside comparative developmental studies in the future will enable verification of this hypothesis.

5. Conclusion

Here we described the accurate developmental time course of horn primordial morphogenesis during the prepupal stage in the horned beetle *T. dichotomus* using a time-lapse photography system. In addition, we functionally characterized both *Tdic-tra* and *Tdic-tra2,* genes that regulate the sex-specific splicing of *Tdic-dsx*. By manipulating expression levels of *Tdic-tra* and *Tdic-dsx* during different developmental time points, and by quantifying the extent of sex transformation, we revealed the following three crucial features of *Tdic-dsx* function during the development of sexually dimorphic horn formation: (1) *Tdic-dsx* modulates the GRN for horn formation as early as 29 h APF, a timepoint which corresponds to 7 h before sexual dimorphisms of horn primordia first appears; (2) *Tdic-dsx* regulates different aspects of the tissue growth, tissue death and tissue movement of horn primordia depending on both spatial (head/prothorax) and temporal (prepupa/pupa) contexts; (3) *Tdic-dsxM* and *Tdic-dsxF* promotes the formation of outgrowth structure in distinct regions within the clypeolabrum. These findings inform our understanding of the patterning mechanisms at play during *T. dichotomus* horn formation, as well as provide information regarding regulatory shifts in these mechanisms during the evolution of sexually dimorphic traits in horned beetles. The present study provides a good starting point to elucidate such issues.

References

Ahmad, S.M., Baker, B.S., 2002. Sex-specific deployment of FGF signaling in *Drosophila* recruits mesodermal cells into the male genital imaginal disc. *Cell* 109, 651–661. https://doi.org/10.1016/S0092-8674(02)00744-4

Bhardwaj, S., Prudic, K.L., Bear, A., Dasgupta, M., Wasik, B.R., Tong, X., Cheong, W.F., Wenk, M.R., Monteiro, A., 2018. Sex differences in 20-hydroxyecdysone hormone levels control sexual dimorphism in *Bicyclus anynana* wing patterns. *Mol. Biol. Evol.* 35, 465–472. https://doi.org/10.1093/molbev/msx301

Busey, H.A., Zattara E.E., Moczek A.P., 2016. Conservation, innovation, and bias: Embryonic segment boundaries position posterior, but not anterior, head horns in adult beetles. *J. Exp. Zool. B. Mol. Dev. Evol.* 326, 271–279. https://doi.org/10.1002/jez.b.22682

Cline, T.W., Meyer, B.J., 1996. Vive la différence: males vs females in flies vs worms. *Annu. Rev. Genet.* 30, 637–702. https://doi.org/10.1146/annurev.genet.30.1.637

Concha, C., Scott, M.J., 2009. Sexual development in *Lucilia cuprina* (Diptera, Calliphoridae) is controlled by the transformer gene. *Genetics* 182, 785–798. https://doi.org/10.1534/genetics.109.100982

Degenhardt, K., Wright, A.C., Horng, D., Padmanabhan, A., Epstein, J.A., 2010. Rapid 3D phenotyping of cardiovascular development in mouse embryos by micro-CT with iodine staining. *Circ. Cardiovasc. Imaging* 3, 314–322. https://doi.org/10.1161/CIRCIMAGING.109.918482

Eberhard, W.G., 1980. Horned beetles. *Sci. Am.* 242, 166–183. http://www.jstor.org/stable/24966285

Emlen, D.J., Corley Lavine, L., Ewen-Campen, B., 2007a. On the origin and evolutionary diversification of beetle horns. *Proc. Natl. Acad. Sci. U.S.A.* 104 Suppl 1, 8661–8668. https://doi.org/10.1073/pnas.0701209104

Emlen, D.J., Marangelo, J., Ball, B., Cunningham, C.W., 2007b. Diversity in the weapons of sexual selection: horn evolution in the beetle genus *Onthophagus*

(coleoptera: scarabaeidae). *Evolution* 59, 1060–1084. https://doi.org/10.1111/j.0014-3820.2005.tb01044.x

Erdman, S.E., Chen, H.J., Burtis, K.C., 1996. Functional and genetic characterization of the oligomerization and DNA binding properties of the *Drosophila doublesex* proteins. *Genetics* 144, 1639–1652. http://www.genetics.org/content/144/4/1639.long

Erickson, J.W., Cline, T.W., 1993. A bZIP protein, sisterless-a, collaborates with bHLH transcription factors early in *Drosophila* development to determine sex. *Genes Dev.* 7, 1688–1702. https://doi.org/10.1101/gad.7.9.1688

Garrett-Engele, C.M., Siegal, M.L., Manoli, D.S., Williams, B.C., Li, H., Baker, B.S., 2002. *intersex*, a gene required for female sexual development in *Drosophila*, is expressed in both sexes and functions together with *doublesex* to regulate terminal differentiation. *Development* 129, 4661–4675. http://dev.biologists.org/content/129/20/4661.long

Gempe, T., Beye, M., 2011. Function and evolution of sex determination mechanisms, genes and pathways in insects. *Bioessays* 33, 52–60. https://doi.org/10.1002/bies.201000043

Geuverink, E., Beukeboom, L.W., 2014. Phylogenetic distribution and evolutionary dynamics of the sex determination genes *doublesex* and *transformer* in insects. *Sex. Dev.* 8, 38–49. https://doi.org/10.1159/000357056

Gotoh, H., Zinna, R.A., Warren, I., DeNieu, M., Niimi, T., Dworkin, I., Emlen, D.J., Miura, T., Lavine, L.C., 2016. Identification and functional analyses of sex determination genes in the sexually dimorphic stag beetle *Cyclommatus metallifer*. *BMC Genomics* 17, 250. https://doi.org/10.1186/s12864-016-2522-8

Hall, A.B., Basu, S., Jiang, X., Qi, Y., Timoshevskiy, V.A., Biedler, J.K., Sharakhova, M.V., Elahi, R., Anderson, M.A.E., Chen, X.-G., Sharakhov, I.V., Adelman, Z.N., Tu, Z., 2015. A male-determining factor in the mosquito *Aedes aegypti. Science* 348, 1268–1270. https://doi.org/10.1126/science.aaa2850

Hasselmann, M., Gempe, T., Schiøtt, M., Nunes-Silva, C.G., Otte, M., Beye, M., 2008.

Evidence for the evolutionary nascence of a novel sex determination pathway in honeybees. *Nature* 454, 519–522. https://doi.org/10.1038/nature07052

Hediger, M., Henggeler, C., Meier, N., Perez, R., Saccone, G., Bopp, D., 2010. Molecular characterization of the key switch *F* provides a basis for understanding the rapid divergence of the sex-determining pathway in the housefly. *Genetics* 184, 155–170. https://doi.org/10.1534/genetics.109.109249

Hongo, Y., 2003. Appraising behaviour during male-male interaction in the Japanese horned beetle *Trypoxylus Dichotomus Septentrionalis* (Kono). *Behaviour* 140, 501–517. https://doi.org/10.1163/156853903322127959

Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H., Shimura, Y., 1991. Control of *doublesex* alternative splicing by *transformer* and *transformer-2* in *Drosophila*. *Science* 252, 833–836. https://doi.org/10.1126/science.1902987

Inoue, K., Hoshijima, K., Sakamoto, H., Shimura, Y., 1990. Binding of the *Drosophila Sex-lethal* gene product to the alternative splice site of *transformer* primary transcript. *Nature* 344, 461–463. https://doi.org/10.1038/344461a0

Inoue, K., Hoshijima, K., Higuchi, I., Sakamoto, H., Shimura, Y., 1992. Binding of the *Drosophila transformer* and *transformer-2* proteins to the regulatory elements of *doublesex* primary transcript for sex-specific RNA processing. *Proc. Natl. Acad. Sci. U.S.A.* 89, 8092–8096. https://doi.org/10.1073/pnas.89.17.8092

Ito, Y., Harigai, A., Nakata, M., Hosoya, T., Araya, K., Oba, Y., Ito, A., Ohde, T., Yaginuma, T., Niimi, T., 2013. The role of *doublesex* in the evolution of exaggerated horns in the Japanese rhinoceros beetle. *EMBO Rep.* 14, 561–567. https://doi.org/10.1038/embor.2013.50

Keisman, E.L., Christiansen, A.E., Baker, B.S., 2001. The sex determination gene *doublesex* regulates the A/P organizer to direct sex-specific patterns of growth in the *Drosophila* genital imaginal disc. *Dev. Cell* 1, 215–225. https://doi.org/10.1016/S1534-5807(01)00027-2

Kijimoto, T., Moczek, A.P., Andrews, J., 2012. Diversification of *doublesex* function underlies morph-, sex-, and species-specific development of beetle horns. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20526–20531. https://doi.org/10.1073/pnas.1118589109

Kiuchi, T., Koga, H., Kawamoto, M., Shoji, K., Sakai, H., Arai, Y., Ishihara, G., Kawaoka, S., Sugano, S., Shimada, T., Suzuki, Y., Suzuki, M.G., Katsuma, S., 2014. A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature* 509, 633–636. https://doi.org/10.1038/nature13315

Kopp, A., 2011. *Drosophila* sex combs as a model of evolutionary innovations. *Evol. Dev.* 13, 504–522. https://doi.org/10.1111/j.1525-142X.2011.00507.x

Krzywinska, E., Dennison, N.J., Lycett, G.J., Krzywinski, J., 2016. A maleness gene in the malaria mosquito *Anopheles gambiae. Science* 353, 67–69. https://doi.org/10.1126/science.aaf5605

Kunte, K., Zhang, W., Tenger-Trolander, A., Palmer, D.H., Martin, A., Reed, R.D., Mullen, S.P., Kronforst, M.R., 2014. *doublesex* is a mimicry supergene. *Nature* 507, 229–232. https://doi.org/10.1038/nature13112

Ledón-Rettig, C.C., Zattara, E.E., Moczek, A.P., 2017. Asymmetric interactions between *doublesex* and tissue- and sex-specific target genes mediate sexual dimorphism in beetles. *Nat. Commun.* 8, 14593. https://doi.org/10.1038/ncomms14593

Liu, G., Wu, Q., Li, J., Zhang, G., Wan, F., 2015. RNAi-mediated knock-down of *transformer* and t*ransformer 2* to generate male-only progeny in the oriental fruit fly, *Bactrocera dorsalis* (Hendel). *PLOS ONE* 10, e0128892. https://doi.org/10.1371/journal.pone.0128892

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Luo, S.D., Shi, G.W., Baker, B.S., 2011. Direct targets of the *D. melanogaster* DsxF protein and the evolution of sexual development. *Development* 138, 2761–2771.

https://doi.org/10.1242/dev.065227

Lynch, K.W., Maniatis, T., 1996. Assembly of specific SR protein complexes on distinct regulatory elements of the *Drosophila doublesex* splicing enhancer. *Genes Dev.* 10, 2089–2101. https://doi.org/10.1101/gad.10.16.2089

Manton, S.M., Harding, J.P., 1964. Mandibular mechanisms and evolution of arthropods. *Phil. Trans. R. Soc. Lond. B* 247, 1–183. https://doi.org/10.1098/rstb.1964.0001

Martín, I., Ruiz, M.F., Sánchez, L., 2011. The gene *transformer-2* of *Sciara* (Diptera, Nematocera) and its effect on *Drosophila* sexual development. *BMC Dev. Biol.* 11, 19. https://doi.org/10.1186/1471-213X-11-19

Matsuda, K., Gotoh, H., Tajika, Y., Sushida, T., Aonuma, H., Niimi, T., Akiyama, M., Inoue, Y., Kondo, S., 2017. Complex furrows in a 2D epithelial sheet code the 3D structure of a beetle horn. *Sci. Rep.* 7, 13939. https://doi.org/10.1038/s41598-017-14170-w

McCullough, E.L., Tobalske, B.W., Emlen, D.J., 2014. Structural adaptations to diverse fighting styles in sexually selected weapons. *Proc. Natl. Acad. Sci. U.S.A* 111, 14484–14488. https://doi.org/10.1073/pnas.1409585111

Meise, M., Hilfiker-Kleiner, D., Dübendorfer, A., Brunner, C., Nöthiger, R., Bopp, D., 1998. *Sex-lethal*, the master sex-determining gene in *Drosophila*, is not sex-specifically regulated in *Musca domestica. Development* 125, 1487–1494. http://dev.biologists.org/content/125/8/1487.long

Metscher, B.D., 2009a. MicroCT for developmental biology: a versatile tool for high-contrast 3D imaging at histological resolutions. *Dev. Dyn.* 238, 632–640. https://doi.org/10.1002/dvdy.21857

Metscher, B.D., 2009b. MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC Physiol.* 9, 11. https://doi.org/10.1186/1472-6793-9-11

Moczek, A.P., Nagy, L.M., 2005. Diverse developmental mechanisms contribute to different levels of diversity in horned beetles. *Evol. Dev.* 7, 175–185.

https://doi.org/10.1111/j.1525-142X.2005.05020.x

Moczek, A.P., Cruickshank, T.E., Shelby, A., 2006. When ontogeny reveals what phylogeny hides: gain and loss of horns during development and evolution of horned beetles. *Evolution* 60, 2329. https://doi.org/10.1554/06-260.1

Moczek, A.P., 2007. Pupal remodeling and the evolution and development of alternative male morphologies in horned beetles. *BMC Evol. Biol.* 7, 151. https://doi.org/10.1186/1471-2148-7-151

Moczek, A.P., Rose, D.J., 2009. Differential recruitment of limb patterning genes during development and diversification of beetle horns. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8992–8997. https://doi.org/10.1073/pnas.0809668106

Pane, A., De Simone, A., Saccone, G., Polito, C., 2005. Evolutionary conservation of *Ceratitis capitata transformer* gene function. *Genetics* 171, 615–624. https://doi.org/10.1534/genetics.105.041004

Posnien, N., Schinko, J.B., Kittelmann, S., Bucher, G., 2010. Genetics, development and composition of the insect head--a beetle's view. *Arthropod Struct. Dev*. 39, 399–410. https://doi.org/10.1016/j.asd.2010.08.002

Posnien, N., Koniszewski, N.D.B., Hein, H.J., Bucher, G., 2011. Candidate gene screen in the red flour beetle *Tribolium* reveals *six3* as ancient regulator of anterior median head and central complex development. *PLOS Genet* 7. https://doi.org/10.1371/journal.pgen.1002416

Rice, G., Barmina, O., Hu, K., Kopp, A., 2018. Evolving *doublesex* expression correlates with the origin and diversification of male sexual ornaments in the *Drosophila* immigrans species group. *Evol. Dev.* 20, 78–88. https://doi.org/10.1111/ede.12249

Richter, S., Stein, M., Frase, T., Szucsich, N., 2013. The Arthropod Head, in: *Arthropod Biology and Evolution: Molecules, Development, Morphology*. 223–240. https://doi.org/10.1007/978-3-642-36160-9_10

Saccone, G., Peluso, I., Artiaco, D., Giordano, E., Bopp, D., Polito, L.C., 1998. The *Ceratitis capitata* homologue of the *Drosophila* sex-determining gene sex-lethal is structurally conserved, but not sex-specifically regulated. *Development* 125,

1495–1500.
http://dev.biologists.org/content/125/8/1495.long

Sanchez, L., Gorfinkiel, N., Guerrero, I., 2001. Sex determination genes control the development of the *Drosophila* genital disc, modulating the response to Hedgehog, Wingless and Decapentaplegic signals. *Development* 128, 1033–1043.
http://dev.biologists.org/content/128/7/1033.long

Schütt, C., Nöthiger, R., 2000. Structure, function and evolution of sex-determining systems in Dipteran insects. *Development* 127, 667–677.
http://dev.biologists.org/content/127/4/667.long

Sharma, A., Heinze, S.D., Wu, Y., Kohlbrenner, T., Morilla, I., Brunner, C., Wimmer, E.A., van de Zande, L., Robinson, M.D., Beukeboom, L.W., Bopp, D., 2017. Male sex in houseflies is determined by Mdmd, a paralog of the generic splice factor gene CWC22. *Science* 356, 642–645.
https://doi.org/10.1126/science.aam5498

Shukla, J.N., Palli, S.R., 2012a. Doublesex target genes in the red flour beetle, *Tribolium castaneum*. *Sci. Rep.* 2, 948.
https://doi.org/10.1038/srep00948

Shukla, J.N., Palli, S.R., 2012b. Sex determination in beetles: production of all male progeny by parental RNAi knockdown of *transformer*. *Sci. Rep.* 2, 602.
https://doi.org/10.1038/srep00602

Shukla, J.N., Palli, S.R., 2013. *Tribolium castaneum* Transformer-2 regulates sex determination and development in both males and females. *Insect Biochem. Mol. Biol.* 43, 1125–1132.
https://doi.org/10.1016/j.ibmb.2013.08.010

Siva-Jothy, M.T., 1987. Mate securing tactics and the cost of fighting in the Japanese horned beetle, *Allomyrina dichotoma* L. (Scarabaeidae). *J. Ethol.* 5, 165–172.
https://doi.org/10.1007/BF02349949

Soler, C., Laddada, L., Jagla, K., 2016. Coordinated development of muscles and tendon-like structures: early interactions in the *Drosophila* leg. *Front. Physiol.* 7, 22.
https://doi.org/10.3389/fphys.2016.00022

Suzuki, M.G., Funaguma Shunsuke, Kanda Toshio, Tamura Toshiki, Shimada Toru,

2005. Role of the male BmDsx protein in the sexual differentiation of *Bombyx mori*. *Evol. Dev.* 7, 58–68. https://doi.org/10.1111/j.1525-142X.2005.05007.x

Tanaka, K., Barmina, O., Sanders, L.E., Arbeitman, M.N., Kopp, A., 2011. Evolution of sex-specific traits through changes in HOX-dependent *doublesex* expression. *PLOS Biol.* 9, e1001131. https://doi.org/10.1371/journal.pbio.1001131

Verhulst, E.C., Beukeboom, L.W., van de Zande, L., 2010. Maternal control of haplodiploid sex determination in the wasp *Nasonia*. *Science* 328, 620–623. https://doi.org/10.1126/science.1185805

Verhulst, E.C., van de Zande, L., 2015. Double nexus—Doublesex is the connecting element in sex determination. *Brief. Funct. Genomics* 14, 396–406. https://doi.org/10.1093/bfgp/elv005

Wrischnik, L.A., Timmer, J.R., Megna, L.A., Cline, T.W., 2003. Recruitment of the proneural gene scute to the *Drosophila* sex-determination pathway. *Genetics* 165, 2007–2027. http://www.genetics.org/content/165/4/2007.long

Zattara, E.E., Busey, H.A., Linz, D.M., Tomoyasu, Y., Moczek, A.P., 2016. Neofunctionalization of embryonic head patterning genes facilitates the positioning of novel traits on the dorsal head of adult beetles. *Proc. Biol. Sci.* 283. https://doi.org/10.1098/rspb.2016.0824

Author contributions

S.M. and T.N. designed the research. S.M. established the time-lapse photography system. A.M. performed the micro CT analysis. S.M. performed all of the molecular biological and biometrical experiments with the help of T.M. and M.M.. S.M. performed statistical analysis of the obtained data. S.S. carried out bioinformatics analyses. S.M., T.A. and T.N. wrote the manuscript. All authors approved the final manuscript.

Acknowledgments

We thank Hosei Wada for developing the "SegmentMeasure" plug-in of ImageJ, Takahiro Ohde, Hiroki Gotoh, and Yasuhiko Chikami for helpful discussion, Robert Zinna for critical reading of the manuscript, the Model Plant Research Facility/NIBB BioResource Center and the Functional Genomics Facility/NIBB Core Research Facilities for technical assistance. TN was supported by MEXT KAKENHI (23128505, 25128706, 16H01452 and 18H04766) and NIBB Cooperative Research Programs (18-433).

Figure legends

Fig. 1 Morphological change of horn primordia and the prepupal period in *Trypoxylus dichotomus.*

(A) (a) Schematic of the frontal view of larval head from *T. dichotomus*. Green, ocular (OC); Magenta, clypeolabrum; CL, clypeus; LB, labrum. (b),(c) Micro-CT images of prepupal head at 24 h APF from *Trypoxylus*. (b) The frontal view. Green arrowheads indicate the position of a sagittal section in (c). (c) The sagittal section of head. Head horn primordia were formed above the clypeus in the clypeolabrum (Movie 1a). (B) Morphological changes of head and thoracic horn primordia during larval-pupal development revealed by the time-lapse photography system. Both in males (the upper panels in the upper half) and in females (the upper panels in the lower half), horn primordia were formed in the clypeolabrum. In males, the frontal views are presented concerning the head horn primordia from 12 h APF to 36 h APF, and the anterodorsal views are presented concerning the head horn primordia from 48 h APF to 120 h APF. In females, the frontal views are presented demonstrated the head horn primordia from 12 h APF to 48 h APF, and the anterodorsal views are presented demonstrating the head horn primordia from 60 h APF to 120 h APF. The dorsal views are presented demonstrating thoracic horn primordia. APF, after puapal-chamber formation; HH, head horn primordia; TH, thoracic horn primordia; Orange arrowheads, the firstly bifurcated distal tips of a head horn; Green arrowheads, the secondly bifurcated distal tips of a head horn; Blue arrowheads, the long column shaped stalk of a head horn; Purple arrowheads, the bifurcated distal tips of a thoracic horn; Green, ocular; Magenta, labrum. (C) Snapshots of a last-instar larva, prepupa and pupa under a time-lapse photography (Movie 2). (D) The durations of the prepupal periods in males and females estimated using the time-lapse photography system. Median, the upper/lower quantile, and minimum/maximum values were presented by a box- and whisker- plot. The median duration of prepupal periods in males and females were 131 hours and 129 hours, respectively. The mean duration of prepupal period in *Tdic-tra* RNAi females was 127

hours.

Fig. 2

Schematic representation of putative exon–intron structures and sex-specific splicing patterns of the sex determination genes..

(A) Schematics of putative exon-intron structures of the sex determination genes in *T. dichotomus*. Orange box, RRM (RNA binding motif); Green box, CAM domain; Light blue box, Med29 (mediator complex subunit 29) domain; Brown box, DM (Doublesex/Mab-3 DNA-binding) domain; Yellow box, OD2 (Oligomerization dmain2); Black bars, template sequences for dsRNA synthesis; Green bars, the amplified regions in qRT-PCR analysis; Light green bars, the amplified region to test sex-specific splicing variants; Magenta lines, translation start sites; Purple lines, stop codons. (B) Evaluation of expression and sex-specific splicing patterns in the sex determination genes in male and female prepupal head horn primordia. Template cDNAs were derived from prepupal horn primordia at 72 h APF. *Tdic-RpL32* was used as an internal control for RT-PCR. Blue arrows, PCR products with male specific splicing variants; Magenta arrows, PCR products with female specific splicing variants; Black arrows, PCR products amplified in both sexes.

Fig. 3

RNAi-mediated loss-of-function phenotypes of the sex determination genes.

(A) Representative individuals in each RNAi treatment in males (the left half) and females (the right half). Each dsRNA was injected into last-instar larvae. The negative control RNAi treatment (*EGFP* dsRNA) showed no morphological defects. The upper row, the dorsal views of adults; the second row, the lateral views of a head and a prothorax in adults; the third row, the ventral views of adults; the forth row, magnified views of orange squares (IPP, intercoxal process of prosternum) in the third row. Scale bars are 1 cm in the upper three rows and 5 mm in the fourth row.

(B) Quantification of the relative head and thoracic horn length in RNAi-treated individuals. Relative head and thoracic horn length in

RNAi-treated males and females are plotted in the blue dots and the magenta dots, respectively. The relative horn lengths were standardized by dividing the horn length by the body size in each RNAi treated individual and by the mean horn length of the *EGFP* RNAi-treated males. Differences in horn length among individual RNAi treatments was compared using student's t-test with Bonferroni correction. Asterisks denote significance: n.s., $p > 0.05$; *, $p < 0.05$. (C) Sex-specific splicing of *Tdic-dsx* in RNAi treatments targeting *Tdic-Sxl, Tdic-tra, Tdic-tra2* and *Tdic-ix*. Blue arrowheads, male specific splicing patterns. Magenta arrowheads, female specific splicing patterns. *Tdic-RpL32* was used as an internal control for RT-PCR

Fig. 4 Estimation of the onset of the *Tdic-dsx* dependent horn developmental program by *Tdic-tra* RNAi.
(A) Relationship between the timepoints of *Tdic-tra* dsRNA injection and the extent of sex transformation. The extent of the sex transformation (masculinization) phenotypes of *Tdic-tra* RNAi females were categorized into three classes: (i) prominent HH and TH were formed (< -134 h APF); (ii) only small HH was formed (-130 h APF ~ 54 h APF); (iii) neither the HH nor TH was formed as in normal females (> -130 h APF). HH, head horn primordia; TH, thoracic horn primordia. (B) Timepoints of *Tdic-tra* dsRNA injection before pupation in each class were plotted. The green dot, the pink dot, and the blue dot indicate 130 h APF, 134 h APF, and 54 h APF, respectively. (C) Time course of relative *Tdic-tra* and *Tdic-dsxF* mRNA expression level after *Tdic-tra* dsRNA injection at 24 h APF. mRNA expression levels at each timepoint were quantified by qRT-PCR. The expression levels of *Tdic-tra* were significantly decreased at 36 hours after injection. Asterisks denote significance: n.s., $p > 0.05$; *, $p < 0.05$. (D) Summary of sex transformation phenotypes and the estimated onset of the *Tdic-dsx* dependent horn developmental program. The magenta, green and blue lines are the boundary timepoints, which divide developmental stages in each of which the same phenotypic category was observed. Magenta arrowheads indicate the timepoints corrected by the mRNA degradation delay (36 hours), which estimated by qRT-PCR. 0h, onset of prepupa; APF, after puapal-chamber

formation; Magenta arrowhead, the estimated onset of the developmental program for sexually dimorphic horn formation driven by *Tdic-dsx*.

Fig. 5

Horn formation phenotypes induced by *Tdic-tra* or *Tdic-dsx* RNAi at late prepupal stages.

(A-C) Comparison of wild type female head and ectopic intermediate sexual transformation head horn in female heads induced by *Tdic-tra* RNAi treatments. (A) A wild type female head. (B) Small ectopic head horn formation in a female head. (C) Middle-sized ectopic head horn formation in a female head. Three small protrusions are formed in clypeolabrum on the wild type female head (A-C, magenta arrowheads), whereas ectopic small- or middle-sized ectopic head horns were formed in the region anterior to the three small protrusions (B, C, green arrowheads) in *Tdic-tra* RNAi treatments. (D-F) Thoracic horn remodeling during pupal-adult development in males. (D) A wild type male thoracic horn. (E) Unremodelled thoracic horn formed by a late *Tdic-dsx* RNAi treatment (13 BPF). (F) Almost complete reduction of a thoracic horn with an early *Tdic-dsx* RNAi treatment (85 BPF). (F') dorsolateral view. (G) The epidermal regions used for qRT-PCR. The 3D volume image was reconstructed from sequential micro CT images. HH, head horn primordium; Blue, head horn primordium (HH); Papule, the head epidermis (non-HH). (H) The relative *Tdic-dsx* expression level in head horn primordia and head epidermis at 36 h APF in both sexes. Asterisks denote significance: n.s., $p > 0.05$; *, $p < 0.05$. BPF, before pupal-chamber formation; HH, head horn primordium epidermis; non-HH, the head epidermis.

Fig. 6

A regulatory model for the formation of sexually dimorphic horns in *T. dichotomus*.

In males, default splicing generates *Tdic-dsxM*, whereas in females, *Tdic-tra* (a female isoform, *traF*) and *Tdic-tra2*, generates *Tdic-dsxF* by female-specific splicing of *Tdic-dsx* pre-mRNA. Prepupal *Tdic-dsxM* expression in males positively regulates the growth of head and thoracic

horn primordia, whereas prepupal *Tdic-dsxF* in females suppresses the horn growth activity. In pupa, *Tdic-dsxM* regulates thoracic horn remodeling, but is dispensable for remodeling of head horn. GRN, gene regulatory network.

Movie 1

Micro-CT analysis of a male prepupal head at 24 h APF.

(a) Head horn primordium formation regions within clypeolabrum. A small epidermal protrusion in the clypeolabral, which seems to be a head horn primordium, was observed above a clypeal primordium (Fig. 1A). The head capsule is indicated in blue (Richter et al., 2013). (b) The morphological characterof the ocular and the mandibular apodeme at 24 h APF. Apolysis was incomplete in the ocular and the mandibular apodeme at 24 h APF (Manton and Harding, 1964; Soler et al., 2016). After this stage, prepupal horn primordia can be readily dissected out due to apolysis at the ocular and the mandibular apodeme (Fig. 1B). This morphological character is unambiguous developmental marker to know the onset of sexual dimorphism formation (36 h APF).

Movie 2

Time-lapse photography of last instar larvae.

A head-rocking behavior was observed at the end of pupal chamber formation.

Fig. S1

Morphological comparison of pupal horn primordia and adult horns in *T. dichotomus.*

Pupal horn primordia are rounded, and slightly larger than adult horns. Light blue in pupal horn primordia shows the morphologies of adult horn or three small protrusions.

Table S1

Primers used in this study.

Table S2

RNAi treatment conditions in Fig.3.

Table S3

RNAi treatment conditions in Fig.4 and Fig.5.

Fig. 1

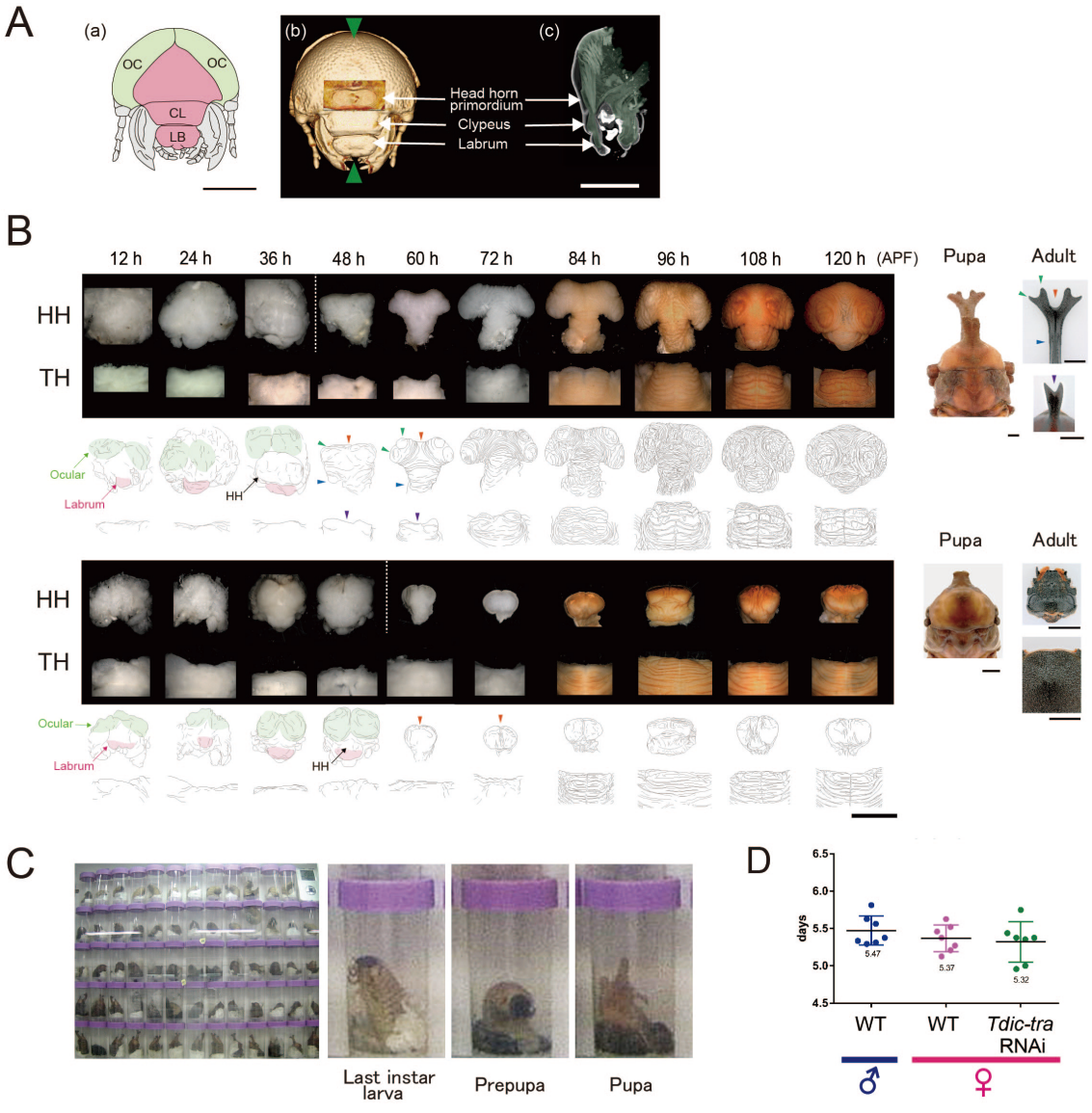
A
(a)
OC
OC
CL
LB
(b)
(c)
Head horn primordium
Clypeus
Labrum
B
12 h
24 h
36 h
48 h
60 h
72 h
84 h
96 h
108 h
120 h (APF)
Pupa
Adult
HH
TH
Ocular
Labrum
HH
Pupa
Adult
HH
TH
Ocular
Labrum
HH
C
Last instar larva
Prepupa
Pupa
D
days
6.5
6.0
5.5
5.0
4.5
5.47
5.37
5.32
WT
WT
Tdic-tra RNAi
♂
♀

Fig. 2

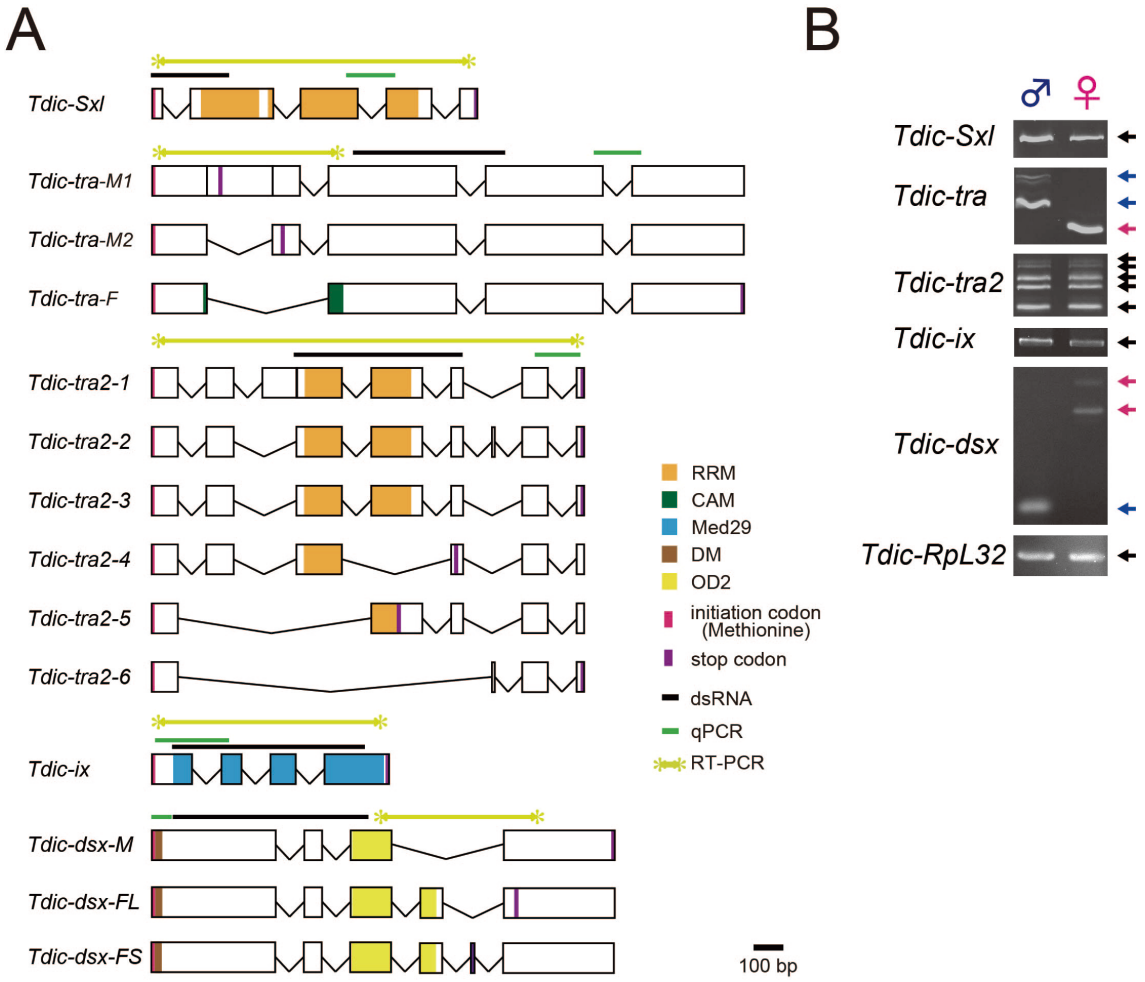
A
Tdic-Sxl
Tdic-tra-M1
Tdic-tra-M2
Tdic-tra-F
Tdic-tra2-1
Tdic-tra2-2
Tdic-tra2-3
Tdic-tra2-4
Tdic-tra2-5
Tdic-tra2-6
Tdic-ix
Tdic-dsx-M
Tdic-dsx-FL
Tdic-dsx-FS
RRM
CAM
Med29
DM
OD2
initiation codon (Methionine)
stop codon
dsRNA
qPCR
RT-PCR
100 bp
B
♂
♀
Tdic-Sxl
Tdic-tra
Tdic-tra2
Tdic-ix
Tdic-dsx
Tdic-RpL32

Fig. 3

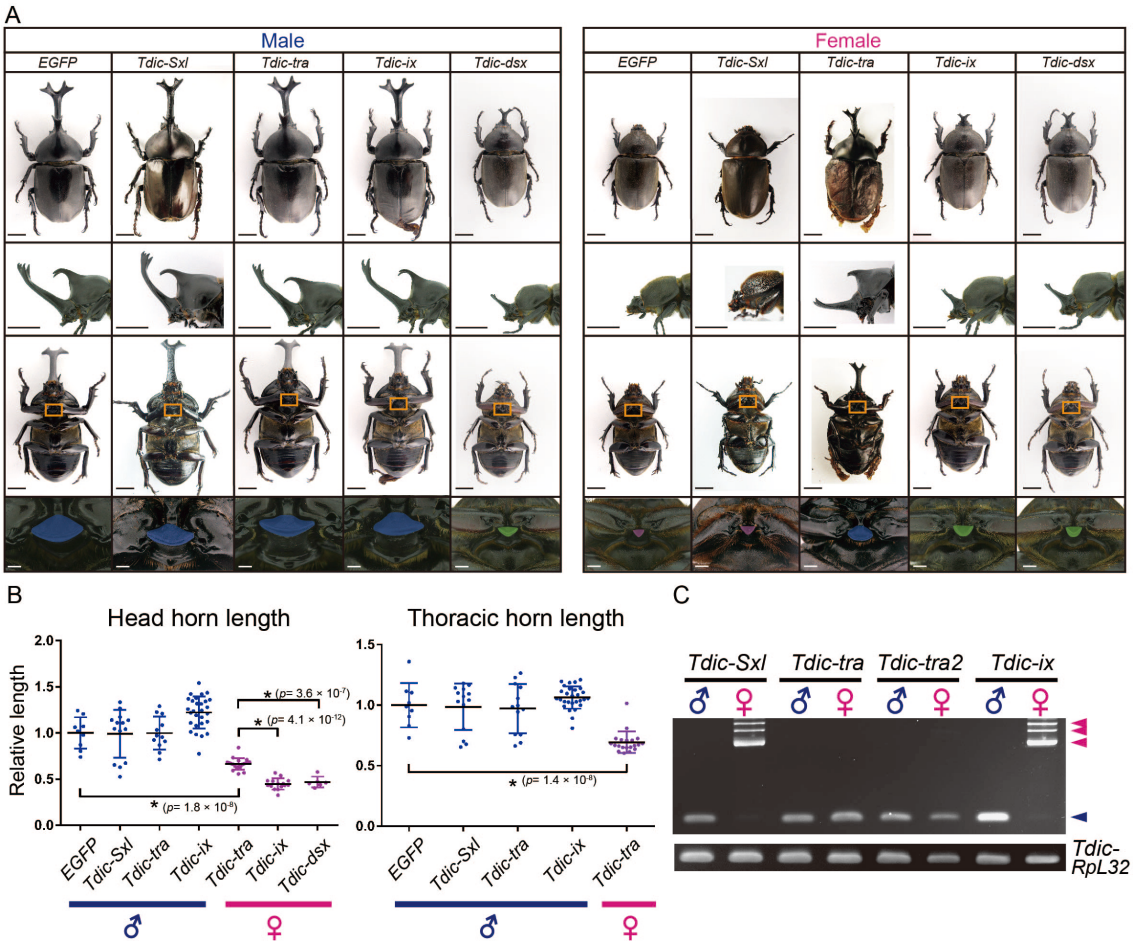
A
Male
EGFP
Tdic-Sxl
Tdic-tra
Tdic-ix
Tdic-dsx
Female
EGFP
Tdic-Sxl
Tdic-tra
Tdic-ix
Tdic-dsx
B
Head horn length
Relative length
2.0
1.5
1.0
0.5
0.0
* (p= 3.6 × 10-7)
* (p= 4.1 × 10-12)
* (p= 1.8 × 10-8)
EGFP
Tdic-Sxl
Tdic-tra
Tdic-ix
Tdic-tra
Tdic-ix
Tdic-dsx
♂
♀
Thoracic horn length
1.5
1.0
0.5
0.0
* (p= 1.4 × 10-8)
EGFP
Tdic-Sxl
Tdic-tra
Tdic-ix
Tdic-tra
♂
♀
C
Tdic-Sxl
Tdic-tra
Tdic-tra2
Tdic-ix
♂
♀
♂
♀
♂
♀
♂
♀
Tdic-RpL32

Fig. 4

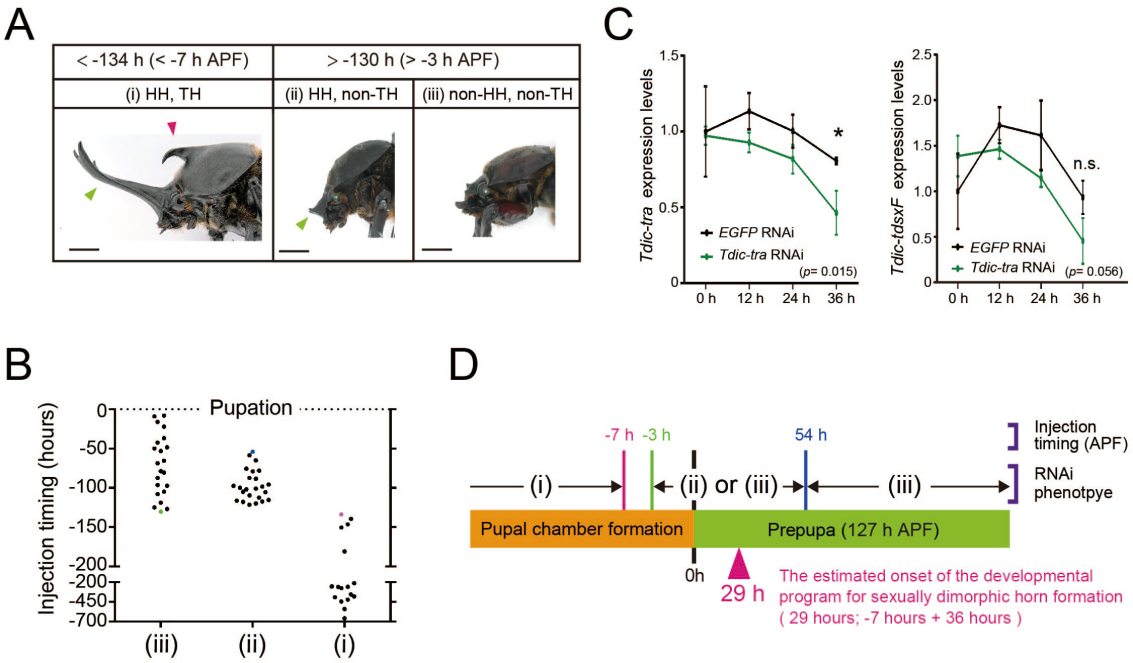
A
< -134 h (< -7 h APF)
> -130 h (> -3 h APF)
(i) HH, TH
(ii) HH, non-TH
(iii) non-HH, non-TH
C
Tdic-tra expression levels
EGFP RNAi
Tdic-tra RNAi
(p= 0.015)
*
0 h
12 h
24 h
36 h
Tdic-tdsxF expression levels
n.s.
(p= 0.056)
B
Injection timing (hours)
Pupation
(iii)
(ii)
(i)
D
-7 h
-3 h
54 h
(i)
(ii) or (iii)
(iii)
Injection timing (APF)
RNAi phenotpye
Pupal chamber formation
Prepupa (127 h APF)
0h
29 h
The estimated onset of the developmental program for sexually dimorphic horn formation
(29 hours; -7 hours + 36 hours)

Fig. 5

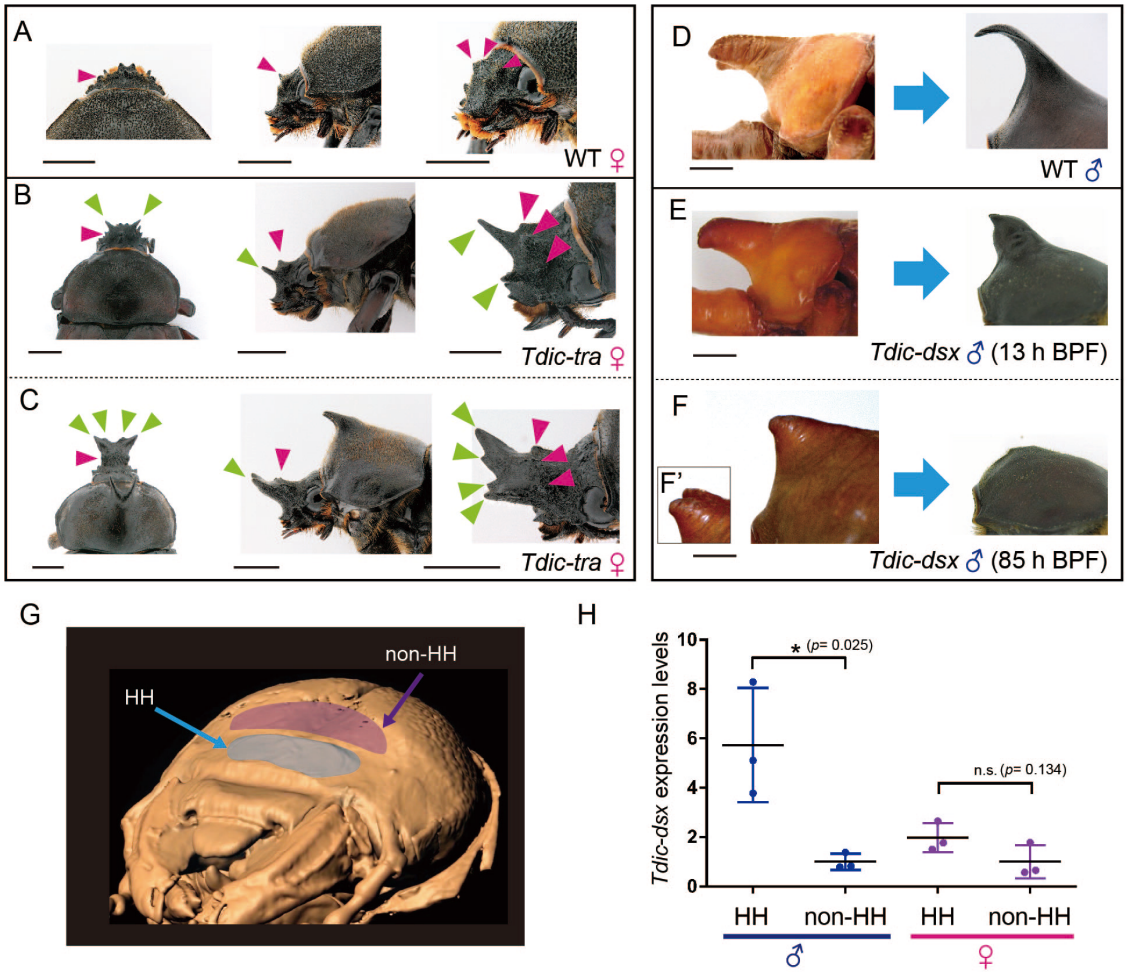
A
WT ♀
B
Tdic-tra ♀
C
Tdic-tra ♀
D
WT ♂
E
Tdic-dsx ♂ (13 h BPF)
F
F'
Tdic-dsx ♂ (85 h BPF)
G
non-HH
HH
H
Tdic-dsx expression levels
10
8
6
4
2
0
* (p= 0.025)
n.s. (p= 0.134)
HH
non-HH
HH
non-HH
♂
♀

Fig. 6

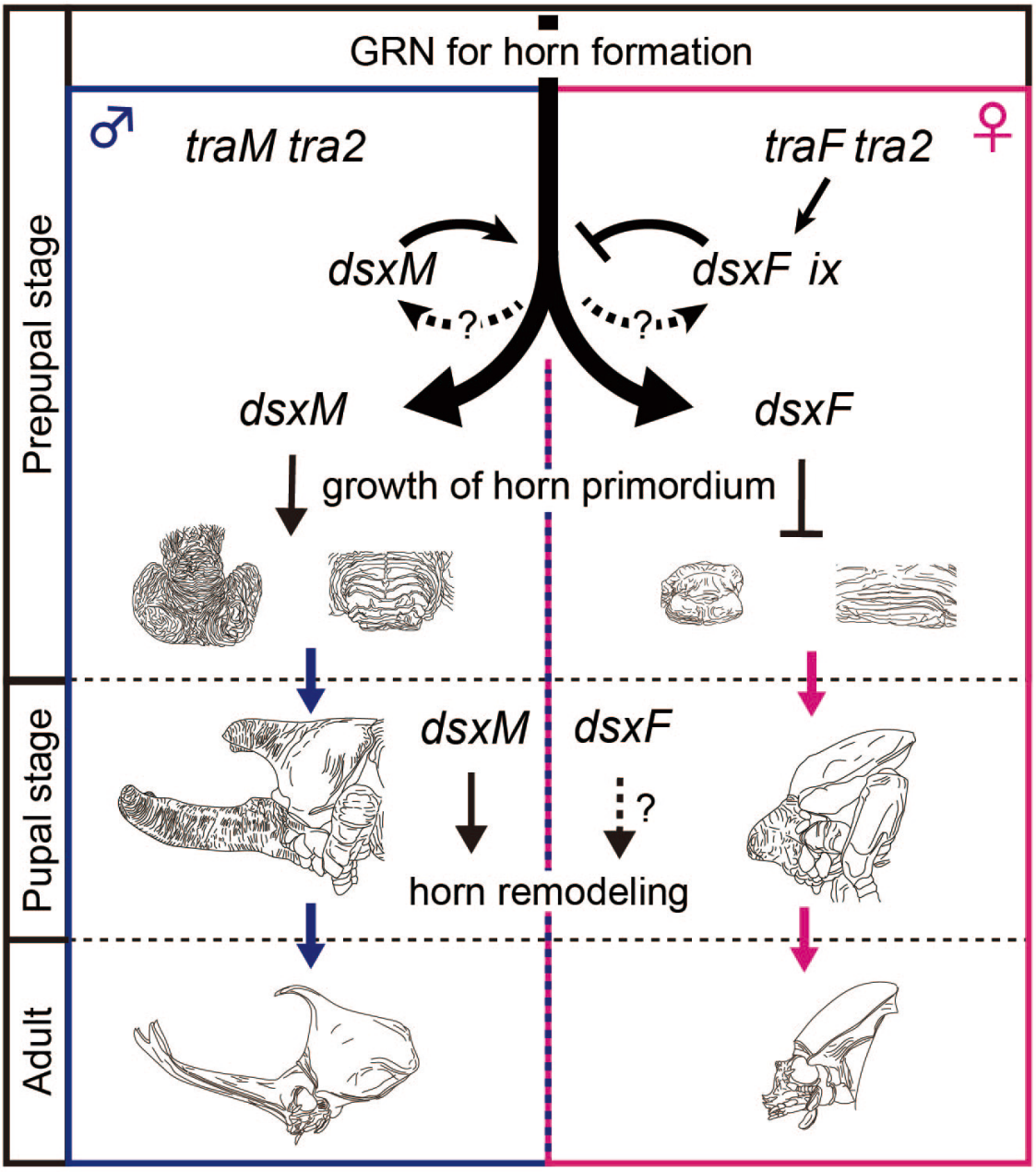
GRN for horn formation
♂
traM tra2
traF tra2
♀
dsxM
dsxF ix
?
?
dsxM
dsxF
growth of horn primordium
Prepupal stage
Pupal stage
Adult
dsxM
dsxF
?
horn remodeling

Fig. S1

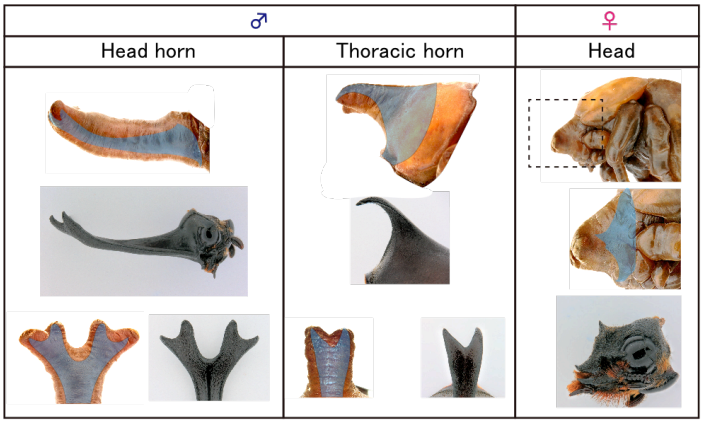

Table S1

	Gene	Forward (F) / Reverse (R)	Sequence (5' to 3')
Clonning	*Tdic-Sxl*	F	taatacgactcactatagggATGTTCGCTAATAAACG
		R	CTAATCATGCTGACAATATTGGC
	Tdic-tra	F	ATGAGTAAGAGACTTCCACC
		R	CTAATCAGTAGTTTTAATGATCTCTTG
	Tdic-tra2	F	CCGCATACTATGAGTGATAGAGAG
		R	TCATCCAATACCACGTGTTC
	Tdic-ix	F	ATGAATGTTGTTGGTATGGG
		R	TCATTCTGATGGTGATATATTC

	Gene	Forward (F) / Reverse (R)	Sequence (5' to 3')
dsRNA	*EGFP*	F	taatacgactcactatagggAATCATGGCCGACAAGCAGAA
		R	taatacgactcactatagggAAACTCCAGCAGGACCATGTG
	Tdic-Sxl	F	taatacgactcactatagggATGTTCGCTAATAAACG
		R	taatacgactcactatagggTCGATTGGGCCGATCTGTGAG
	Tdic-tra	F	taatacgactcactatagggAAGGGTTGTGGTTGCTACG
		R	taatacgactcactatagggCCGGCGAGATCTTCTATCTG
	Tdic-tra2	F	taatacgactcactatagggTCGCAGGCGCCACATGGGCA
		R	taatacgactcactatagggTCTCTGTCTCTATCTCGGCG
	Tdic-ix	F	taatacgactcactatagggAGCAACCTCAAGCATTGGAT
		R	taatacgactcactatagggGTGCATTTGCAGTCGCTAAA

	Gene	Forward (F) / Reverse (R)	Sequence (5' to 3')
qRT-PCR	*Tdic-Sxl*	F	ATTACTGGGTTGCCGAGGGG
		R	GTCCAAGTTCGCGATGGCAG
	Tdic-tra	F	ATCAAAGAAGGCCTCCGCGT
		R	ATCTAGGCGTTGGAGCAGGC
	Tdic-tra2	F	GTACAGCCATCGCAGATCGC
		R	CACGTGTTCTTAACCGTGGTGA
	Tdic-ix	F	GCAACAGCCTCAGCAACCTC
		R	CACAATCAAGACTGCAGCGC
	Tdic-dsx	F	TAGTTAGTGATGTCCGACTCGCA
		R	GTTCGGGGATTCGTCGACGT
	Tdic-dsxF	F	AGGCAACCCTTACGTCTGTCA
		R	ACGATTTCTTCATGTGGCGCA
	Tdic-RpL32	F	AAGACACGCCATATGCTTCC
		R	CACCATGTGCAATCTCTCCA

	Gene	Forward (F) / Reverse (R)	Sequence (5' to 3')
RT-PCR	*Tdic-Sxl*	F	taatacgactcactatagggATGTTCGCTAATAAACG
		R	CTAATCATGCTGACAATATTGGC
	Tdic-tra	F	ATGAGTAAGAGACTTCCACC
		R	TTCGGACGTAGCAACCACAACC
	Tdic-tra2	F	CCGCATACTATGAGTGATAGAGAG
		R	AGCTCGTCCTCGGTGGTGTAGACGC
	Tdic-ix	F	ATGAATGTTGTTGGTATGGG
		R	TCATTCTGATGGTGATATATTC
	Tdic-dsx	F	TATGCCATTATGAAGCTAGCGCC
		R	TATGACTTGGACTAATGAGATCATC
	Tdic-RpL32	F	CTGACCGTTATGGAAAACTT
		R	TACGATTTTGCATCAACAAT

T7 promoter sequence are written in lowercase

Table S2

dsRNA	Sex	Injected dsRNA (μg)	Number of injected larvae	Eclosed to adult	Number of adults with sex specific phenotypes	Efficiency(%)
EGFP	♂	10	8	6	0	0
		20	8	7	0	0
	♀	10	8	7	0	0
		20	8	8	0	0
Tdic-Sxl	♂	2.5	8	2	0	0
		5	8	4	0	0
		10	8	3	0	0
		15	8	4	0	0
		20	8	6	0	0
		50	8	7	0	0
	♀	2.5	8	7	0	0
		5	8	5	0	0
		10	8	3	0	0
		15	8	6	0	0
		20	8	7	0	0
Tdic-tra	♂	2.5	8	5	0	0
		5	15	9	0	0
		10	10	7	0	0
		15	8	5	0	0
	♀	2.5	8	5	5	100
		5	23	17	15	88
		10	12	8	7	88
		15	8	5	5	100
Tdic-tra2	♂	0.5	8	2	0	0
		1	18	0	0	-
		5	18	0	0	-
		10	18	0	0	-
	♀	0.5	8	0	0	-
		1	18	0	0	-
		5	18	0	0	-
		10	18	0	0	-
Tdic-ix	♂	5	13	11	0	0
		10	17	15	0	0
		15	8	8	0	0
		20	8	8	0	0
	♀	5	20	15	14	93
		10	18	14	14	100
		15	8	6	6	100
		20	8	5	5	100
Tdic-dsx	♂	15	8	5	5	100
	♀	15	8	6	6	100

Table S3

dsRNA	Sex	Injected dsRNA (μg)	Number of injected prepupa	Eclosed to adult
EGFP	♂	15	25	20
	♀	15	35	26
Tdic-tra	♀	15	91	63
Tdic-dsx	♂	15	43	29
	♀	15	26	19