Chapter 1
Spectrum of Sex in a Horn of the Japanese Rhinoceros Beetle

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Abstracts Sex has been traditionally considered to be classified into two categories, male and female. However, numerous reports have shown examples of insects unfamiliar to this traditional binary sex view. Recently, the view of the sex spectrum has been proposed as a revised version. In this view, sex is recognised as a continuum from male to female (or female to male), and maleness or femaleness of any sexual traits is quantitatively interpreted as 'a position on the continuum of sex'. This chapter discusses the molecular genetic mechanism defining a position on the continuum of sex based on the knowledge about the Japanese rhinoceros beetle *Trypoxylus dichotomus*.

Keywords Horned beetle · Trypoxylus dichotomus · RNAi · Sex differentiation

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1.1 Introduction

Most insect species show sexual dimorphism, and their appearances are distinctively different between males and females. For example, in some beetles, males have magnificently developed mandibles, while females do not develop well. Based on the facts like this, sex has been traditionally considered to be classified into two categories, male and female. However, to date, numerous individuals cannot be classified as male or female by their appearances. For example, in damselflies, some females show male-like coloration though they produce typical female traits except for the coloration (Gossum and Sherratt 2008). Other examples are the sexually mosaic phenotypes (gynandromorph) and sexually intermediate phenotypes (intersex) accidentally produced by developmental abnormalities (Narita et al. 2010). These examples are not included in the traditionally binary view of sex, which needs to be revised (Nong et al. 2020).

The sex spectrum is a view of recognising sex as a continuum from male to female (or from female to male) (Nong et al. 2020; Preface). In this view, the gynandromorph and the intersex are interpreted to be placed between male and female in the sex spectrum. Furthermore, male-like female coloration in damselflies may be placed between midpoint and female. Thus, almost all differences among sexual traits in insects can be explained as differences in the location on the sex spectrum.

How is the location of the sex spectrum defined in insects? In the fruit fly *Drosophila melanogaster*, a major sex-determining gene *doublesex* (*dsx*) mutation yields the gynandromorph and the intersex in both females and males (Hildreth 1965). It is well known that *dsx* is essential for sexual differentiation not only in *D. melanogaster* but also in other holometabolous insects. These facts indicate that genetic mechanisms for sex determination have a considerable effect on determining insects' location on the sex spectrum.

Here, we first overview the sex-determining mechanism in holometabolous insects. Then, based on an example of the Japanese rhinoceros beetle, we discuss a molecular mechanism to determine the location in the sex spectrum.

1.2 Sex-Determining Molecular Mechanism in Holometabolous Insects

This subsection first introduces a sex-determining molecular mechanism in the *D. melanogaster*, the most investigated model insect. Moreover, we also show the commonality of the sex-determining mechanism.

The primary signal for sex determination is the number of the X chromosome (Erickson and Quintero 2007). The sex of an individual with two X chromosomes, i.e., an XX individual, is finally determined as female; on the other hand, the sex of an individual with a single X chromosome (i.e., XY individual) is finally determined as male as a default state.

The initial signals from X chromosomes produce the sex-lethal (Sxl) protein only in XX early embryos (Cline 1978, 1986, 1988; Kramer et al. 1999; Sefton et al. 2000; Penalva and Sánchez 2003; Salz 2007). Sxl autoregulates and maintains the expression of another Sxl isoform (Cline 1984; Bell et al. 1988, 1991; Penalva and Sánchez 2003). The functional Sxl protein controls the RNA splicing of *tra*, and functional Tra protein is translated (Boggs et al. 1987; Bell et al. 1988; Penalva and Sánchez 2003). Then, Tra protein yields female-specific Dsx (DsxF) by regulating alternative splicing of *dsx* mRNA, which leads to female differentiation (Hoshijima et al. 1991).

On the other hand, in XY embryos, a non-functional Sxl protein is produced because of the lack of the initial signal from the X chromosome (Bell et al. 1988; Samuels et al. 1991; Keyes et al. 1992; Penalva and Sánchez 2003). Lack of functional Sxl leads to expression of non-functional Tra protein, and a male-specific Dsx (DsxM) is translated through lack of Tra-dependent splicing regulation, which leads to male differentiation (Bell et al. 1988).

Whether the sex-determining mechanism in *Drosophila* is conserved in other holometabolous insects has been recently studied by focusing on non-*Drosophila* insects. *Sxl* is a ‘master switch’ gene for sex determination in *D. melanogaster*. *Sxl* orthologues have been found in many holometabolous insects, including Diptera, Lepidoptera, Hymenoptera and Coleoptera (Traut et al. 2006). However, some dipteran species’ research revealed that *Sxl* orthologues are not responsible for sex determination (Meise et al. 1998; Saccone et al. 1998; Sievert et al. 2000). Furthermore, the *Sxl* orthologue in the silkworm *Bombyx mori* does not contribute to sex determination but regulates spermatogenesis (Niimi et al. 2006; Sakai et al. 2019). Therefore, *Sxl* is not a broadly conserved sex determination gene in holometabolous insects.

tra is an intermediate factor in the sex determination cascade of *D. melanogaster*. *Tra* orthologues are identified in holometabolous insects such as some species in Diptera, Coleoptera and Hymenoptera (O'Neil and Belote 1992; Pane et al. 2002; Kulathinal et al. 2003; Lagos et al. 2007; Ruiz et al. 2007; Hasselmann et al. 2008; Concha and Scott 2009; Schmieder et al. 2012; Shukla and Palli 2012; Geuverink and Beukeboom 2014; Morita et al. 2019). Furthermore, in some of these species, *tra* orthologues regulate female determination (Pane et al. 2002; Hasselmann et al. 2008; Concha and Scott 2009; Hediger et al. 2010; Shukla and Palli 2012; Morita et al. 2019). On the other hand, *tra* orthologues seem to have been lost in Lepidoptera and in some species in Strepsiptera and Diptera (Salvemini et al. 2013; Geuverink and Beukeboom 2014). These findings indicated that although *tra* orthologues are not found in some species, the sex-determining function of *tra* is conserved in a much more comprehensive range of holometabolous insect species than that of *Sxl*.

dsx is a bottom factor in the sex determination cascade of *D. melanogaster,* which directly regulates the transcription of a battery of genes responsible for sex differentiation. *dsx* orthologues are conserved in all of the insects investigated so far (Price et al. 2015). Furthermore, the *dsx* has sex-specific transcripts and contributes to sex determination in the various holometabolous insects such as Diptera, Lepidoptera, Hymenoptera and Coleoptera (Schütt and Nöthiger 2000; Ohbayashi et al. 2001; Cho et al. 2007; Chen et al. 2008; Oliveira et al. 2009; Shukla and Palli 2012; Ito et al. 2013; Mine et al. 2017; Taracena et al. 2019). Therefore, *dsx* is the conserved regulatory factor in the sex determination cascade among holometabolous insects.

The above comparative analysis focusing on the conserved genes associated with the insect sex determination pathway suggests that downstream genes *tra* and *dsx* are the core regulatory genes conserved among holometabolous insects. Therefore, to understand the determination mechanism of position in the sex spectrum in holometabolous insects, we focus on the role of *tra* and *dsx* in sexual traits. In the next section, we discuss the role of *tra* and *dsx* in the sex spectrum using the Japanese rhinoceros beetle, in which we can manipulate the location in the sex spectrum by controlling the expression level of sex-determining genes using RNA interference (RNAi) methods.

1.3 Sex Spectrum in *T. dichotomus* Horn Visualised by Manipulating the Sex Determination Pathway

The horn of the Japanese rhinoceros beetle *Trypoxylus dichotomus* (Coleoptera, Scarabaeoidea, Scarabaeidae) exhibits sexual dimorphism (Fig. 1.1a, *EGFP*). A male has an exaggerated long horn on the head and a short horn on the first thoracic segment (pronotum). On the other hand, a female has no horn neither on the head nor on the pronotum but has three small projections in the rostral region of the head (clypeolabral region).

As described in the previous sections, sex-specific *tra* and *dsx* isoforms are essential genes to a sex-differentiating mechanism in holometabolous insects. In *T. dichotomus*, downregulation of *dsx* expression by RNAi results in short head horns in both males and females, while thoracic horns form in neither males nor females (Fig. 1.1a) (Ito et al. 2013). *tra* RNAi males showed no morphological changes, while females developed ectopic male-like horns on both the head and pronotum (Fig. 1.1a) (Morita et al. 2019). These facts indicated that in *T. dichotomus*, Tra and Dsx regulate sexual dimorphism in a horn (Fig. 1.1) (Morita et al. 2019).

Next, Morita et al. (2019) insufficiently suppressed *tra* expression levels in females. As a result, female-like and male-like traits coexist in a single *tra* RNAi female (Fig. 1.2) because *tra* regulates sex-specific splicing of *dsx* like other holometabolous insects (Fig. 1.1b) (Morita et al. 2019), and insufficient suppression of *tra* expression in *tra* RNAi females leads to incomplete switching from *dsxF* to *dsxM*.

Detailed observation of the *tra* RNAi phenotypes in Fig. 1.2 revealed that the degree of similarity to males is different between head and pronotum regions. This suggests that the location on the sex spectrum at the tissue level is different between head and pronotum regions. For example, in the head region, *tra* RNAi individuals show female-specific traits (three small projections) and male-specific traits (head

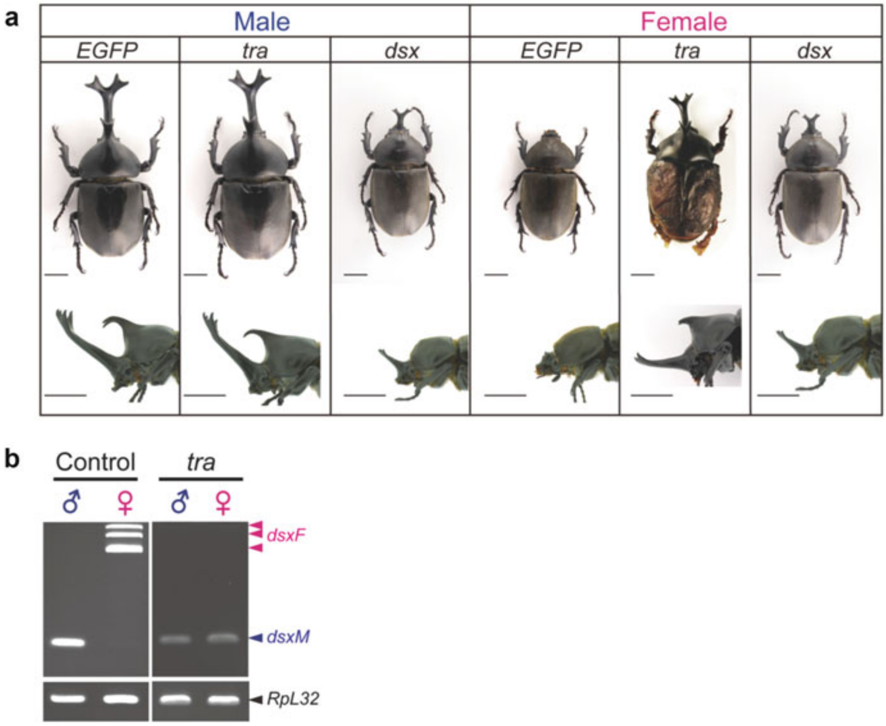

Fig. 1.1 RNAi-mediated loss of function of sex-determining genes and morphological change of horn primordia. (**a**) Representative individuals in *dsx* and *tra* RNAi treatment in males and females. *EGFP* RNAi treatment (negative control) showed no morphological defects. The upper row, the dorsal views of adults. The second row, the lateral views of adults. Scale bars are 1 cm. (**b**) Sex-specific splicing of *dsx* in RNAi treatments targeting *tra*. RpL32 was used as an internal control for RT-PCR. Blue arrowheads, male-specific splicing patterns (*dsxM*). Magenta arrowheads, female-specific splicing patterns (*dsxF*). Black arrowheads, RpL32. (Adapted from Morita et al. *PLOS Genet*., 15: e1008063, 2019)

horns) (Fig. 1.2b, c). However, in one individual (Fig. 1.2b), the ectopic head horn branched twice, forming a short stalk of head horns. On the other hand, the ectopic head horn branched only once in the other individual (Fig. 1.2c), and no stalk was formed. This suggests that the former phenotype was more similar to male-specific traits than the latter phenotype. Therefore, the locations on the sex spectrum of these *tra* RNAi phenotypes in the head region can be explained as shown in Fig. 1.3a.

Next, in the pronotum region, only the *tra* RNAi phenotype in Fig. 1.2b was that the ectopic thoracic horn was apparently like a male thoracic horn (Fig. 1.2d). The thoracic horn length is similar to that of a *tra* RNAi female injected with a sufficient amount of dsRNA (Fig. 1.2d). In contrast to the head horn in *tra* RNAi females, the incomplete formation of the thoracic horn has not been observed so far. This observation suggests that the thoracic horn phenotype is located closely at either endpoint on the sex spectrum (Fig. 1.3b).

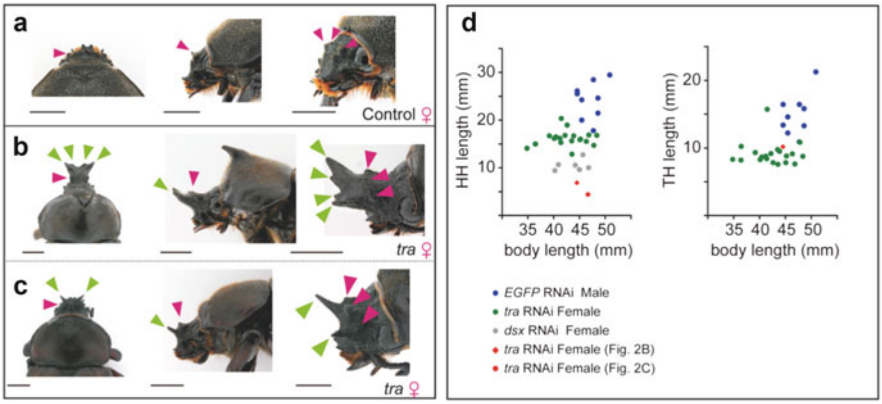

Fig. 1.2 *tra* RNAi phenotype induced by injection of small amounts of *tra* dsRNA. (**a**–**c**) Comparison of a wild-type female and ectopic intermediate sexual transformation of horns in females induced by *tra* RNAi treatments. (**a**) A wild-type female. (**b**, **c**) Small ectopic horn formation in *tra* RNAi females. Magenta arrowheads, three small protrusions formed in clypeolabrum. Green arrowheads, ectopic head horns formed in the region anterior to the three small protrusions in *tra* RNAi treatments. Scale bars are 5 mm. (**d**) Relationship between the head horn (left) and thoracic horn (right) length and body size in RNAi-treated individuals. The head and thoracic horn lengths and body sizes of control males (*EGFP*, blue dots), *dsx* RNAi-treated females (grey dots), and *tra* RNAi-treated females (green dots: **b** magenta diamond, **c** magenta hexagon) are plotted. HH, head horn; TH, thoracic horn. (Adapted from Morita et al. *PLOS Genet*., 15: e1008063, 2019)

These observations indicate that even within a single individual, the locations on the sex spectrums at the tissue level are tissue-dependent. Then, what mechanisms produce these differences? One possibility is a difference in the role of the sex-specific *dsx* isoforms among tissues. In the head region, male and female traits (the head horn and the three small projections, respectively) coexist in a single *tra* RNAi female (Fig. 1.2). In *T. dichotomus*, the *dsx* RNAi phenotype in both males and females showed the head horn was formed, albeit short (Fig. 1.1a). This head horn phenotype suggests that *dsxM* promotes the expression of the horn formation genes and *dsxF* represses (Ito et al. 2013). In Fig. 1.2, it is considered that *dsxF* and *dsxM* coexist in a single individual due to the insufficient suppression of *tra* expression. The antagonistic effects of *dsxM* and *dsxF* on the expression of horn formation genes might define the degree of similarity to males in the head region. In a region of the clypeolabrum where *dsxF* and *dsxM* coexist (Fig. 1.2b and c), *dsxM* functions to promote head horn formation, whereas *dsxF* functions to suppress head horn formation; therefore, this antagonistic effect would define the location on the sex spectrum at the tissue level in the head region. Conversely, male and female traits did not coexist in a *tra* RNAi female's thoracic horn (Fig. 1.2). In the *dsx* RNAi phenotype, the thoracic horn was not formed in either males or females. These phenotypes indicate that *dsxM* promotes thoracic horn formation, while *dsxF* does not contribute to thoracic horn formation. This fact means that thoracic horn formation is regulated depending on the only *dsxM*. In other words, in *tra* RNAi

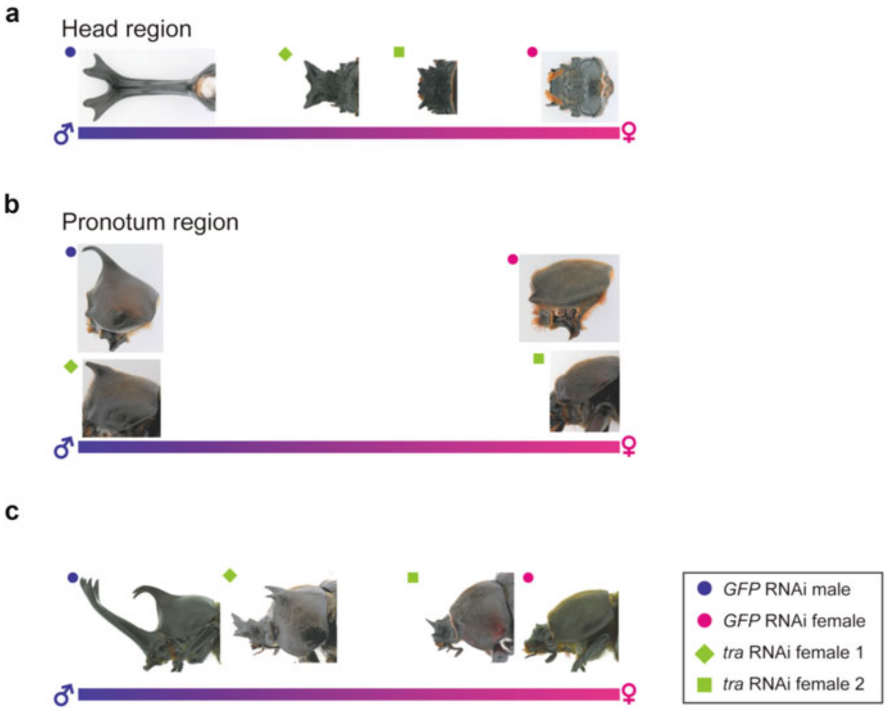

Fig. 1.3 Sex spectrum of *T. dichotomus* estimated from *tra* RNAi phenotypes. (**a**) The sex spectrum at the tissue level in the head region. (**b**) The sex spectrum at the tissue level in the pronotum region. The shorter head and thoracic horns in *tra* RNAi females than in control males may be due to differences in body size between males and females at the timing of injection. (**c**) The individual-level sex spectrum is understood from the sum of tissue-level sex spectrums. Blue dots, *GFP* RNAi male; magenta dots, *GFP* RNAi female; green diamond, *tra* RNAi female 1 (Fig. 1.2b); green square, *tra* RNAi female 2 (Fig. 1.2c). (Adapted from Morita et al. *PLOS Genet.*, 15: e1008063, 2019)

females with insufficiently suppressed *tra* expression, when the expression level of *dsxM* exceeds a certain threshold, thoracic horn formation is promoted; otherwise, the horn is not formed. Therefore, the phenotype of the thoracic horn by *tra* RNAi (Fig. 1.2) is considered to be located at either endpoint on the sex spectrum (Fig. 1.3b).

This section described how sex is interpreted as a spectrum of continuous phenotypes in *T. dichotomus*, using the horn as an indicator of sex by manipulating the expression level of a sex-determining gene *tra*. In addition, the sex spectrum at the tissue level showed different locations in the head and pronotum regions. In the concept of sex spectrum, the sum of the sex spectrum at the tissue level can be understood as the sex spectrum at the individual level (Preface). Therefore, the location of the sex spectrum at the individual level for *tra* RNAi in *T. dichotomus* could be shown as in Fig. 1.3c. From the view of the sex spectrum, rather than the traditional binary sex view, it can be uniformly explained for individuals that cannot be classified by their appearance, as in Fig. 1.2.

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