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Maternal provision of non-sex-specific *transformer* messenger RNA in sex determination of the wasp *Asobara tabida*

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Abstract

In many insect species maternal provision of sex-specifically spliced messenger RNA (mRNA) of sex determination genes is an essential component of the sex determination mechanism. In haplodiploid Hymenoptera, maternal provision in combination with genomic imprinting has been shown for the parasitoid *Nasonia vitripennis*, known as maternal effect genomic imprinting sex determination (MEGISD). Here, we characterize the sex determination cascade of *Asobara tabida*, another hymenopteran parasitoid. We show the presence of the conserved sex determination genes *doublesex* (*dsx*), *transformer* (*tra*) and *transformer-2* (*tra2*) orthologues in *As. tabida*. Of these, *At-dsx* and *At-tra* are sex-specifically spliced, indicating a conserved function in sex determination. *At-tra* and *At-tra2* mRNA is maternally provided to embryos but, in contrast to most studied insects, *As. tabida* females transmit a non-sex-specific splice form of *At-tra* mRNA to the eggs. In this respect, *As. tabida* sex determination differs from the MEGISD mechanism. How the paternal genome can induce female development in the absence of maternal provision of sex-specifically spliced mRNA remains an open question. Our study reports a hitherto unknown variant of maternal effect

sex determination and accentuates the diversity of insect sex determination mechanisms.

Keywords: maternal provision, hymenoptera, sex determination, transformer, doublesex, transformer-2.

Introduction

The genetic basis of developmental pathways is presumed to be well conserved owing to their functional necessity. One of these necessary functions is sex determination, a developmental process in almost all eukaryotes that leads to sexual differentiation of female and male traits. Despite its universality within the eukaryotic domain, sex determination comprises a wide variety of fast-evolving mechanisms. Sex determination pathways consist of a primary signal that starts a cascade of interacting genes. The signal is passed on through downstream genetic components towards the bottom switch that regulates sexual differentiation genes (Herpin & Schartl, 2015). The genes at the level of the bottom switch appear more conserved than the upstream signals, in line with Wilkins' hypothesis that the cascade evolves from the bottom up (Wilkins, 1995). In insects, *doublesex* (*dsx*) has been identified in a range of insect species at the bottom of the sex determination cascade (Shukla & Nagaraju, 2010; Verhulst & van de Zande, 2015). It belongs to a group of DNA-binding motif (DM) encoding genes that are present amongst Metazoa and appear to play a role in sex determination of both invertebrates and vertebrates (Matson & Zarkower, 2012). *Dsx* is spliced into sex-specific variants that translate into male- or female-specific DSX proteins.

Sex-specific splicing of sex determination genes is a hallmark of insect sex determination. The gene upstream of *dsx*, controlling its splicing, is *transformer/feminizer* (*tra/fem*). It is not functionally conserved outside the insect class, and even within insects it is absent in Lepidoptera and basal lineages of Diptera (Geuverink

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& Beukeboom, 2014; Kiuchi *et al.*, 2014). Sex-specific *tra* messenger RNA (mRNA) is produced by alternative splicing. TRA belongs to a class of SR-type proteins that are rich in serine (S) and arginine (R) residues. For most insects an order-specific *tra* domain has been described, eg the hymenopteran (HYM) domain in Hymenoptera and the dipteran domain in Diptera (Verhulst *et al.*, 2010b). The *Ceratitis-Apis-Musca* (CAM) domain is present in all female-specific splice variants of *tra*, excluding drosophilids, but absent from male-specific splice variants (Hediger *et al.*, 2010). Only the female-specific splice form yields a functional protein. In addition to regulating *dsx* splicing, it governs splicing of premature *tra* mRNA into the female-specific form, thereby creating an auto-regulatory loop. The functional female TRA protein forms a complex with the Transformer-2 protein (TRA2) to direct female-specific splicing of *dsx* mRNA (Amrein *et al.*, 1990; Hedley & Maniatis, 1991; Inoue *et al.*, 1992). The structure of TRA2 is highly conserved and contains a RNA binding domain (RBD) flanked by two arginine/serine rich regions in all insects investigated thus far. Studies in various dipterans, the coleopteran *Tribolium castaneum* and the hymenopteran *Apis mellifera* have shown that TRA2 is necessary for female-specific *tra* splicing (Burghardt *et al.*, 2005; Concha & Scott, 2009; Salvemini *et al.*, 2009; Martín *et al.*, 2011; Sarno *et al.*, 2011; Nissen *et al.*, 2012; Shukla & Palli, 2013). The activation/deactivation of *tra* and the *tra-tra2-dsx* signal-relaying pathway appears largely conserved in insects, but is governed by a large diversity in primary signals (Bopp *et al.*, 2014).

Haplodiploid insects, comprising all Hymenoptera, Thysanoptera and several branches of Coleoptera and Hemiptera, are of special interest for sex determination research because they lack differentiated sex chromosomes. Males and females share all chromosomes but differ in ploidy level (males are haploid, females are diploid). Genetic studies of haplodiploid sex determination have focused on hymenopterans. Model organisms are the honey bee *Ap. mellifera* (Apoidea) and the parasitic wasp *Nasonia vitripennis* (Chalcidoidea). Information obtained from these species provides a framework for studies in other haplodiploid systems. *Ap. mellifera* has complementary sex determination (CSD) in which the allelic state of the *complementary sex determiner* (*csd*) locus, a paralogue of *tra* serves as the primary signal (Beye *et al.*, 2003; Hasselmann *et al.*, 2008). An individual that is homozygous or hemizygous at *csd* becomes male, whereas heterozygous individuals develop into females. A CSD-like mechanism has been inferred for over 60 species of Hymenoptera (van Wilgenburg *et al.*, 2006; Heimpel & de Boer, 2008). The molecular details of CSD have only been elucidated in *Ap. mellifera* (Beye *et al.*, 2003; Hasselmann *et al.*, 2008; Gempe *et al.*,

2009). *N. vitripennis* has no CSD, but sex determination is governed by maternal effects and genomic imprinting (MEGISD; Beukeboom & Kamping, 2006; Verhulst *et al.*, 2010a). Female-specific *Nvtra* mRNA is maternally provided to initiate female-specific splicing of zygotic *Nvtra* transcripts, necessary for female development (Verhulst *et al.*, 2010a). This constitutes the maternal effect element of the MEGISD mechanism. Zygotic transcription of *Nvtra* is hypothesized to be under the control of an activator gene, named *womanizer* (*wom*), that is silenced on the maternal complement, but active on the paternal complement in fertilized eggs (Verhulst *et al.*, 2013). This constitutes the (maternal) genomic imprinting element of MEGISD. The identity of *wom* has not been elucidated yet, but presumably non-CSD sex determination in haplodiploids depends on a difference between the paternal and maternal genome set of which the genomic imprinting element of MEGISD is an example. Thus far, MEGISD has only been demonstrated for *N. vitripennis* and its phylogenetic distribution remains unclear.

Parasitoids of the *Asobara* genus (Braconidae) are a group of well-studied ichneumonoid wasps. They occur worldwide and use *Drosophila* larvae as hosts (Carton *et al.*, 1986). CSD has been reported for numerous braconid species, yet there are also species in this group that lack CSD (van Wilgenburg *et al.*, 2006; Asplen *et al.*, 2009). *Asobara tabida* has tested negative for single-locus and multi-locus CSD through inbreeding crosses (Beukeboom *et al.*, 2000; Ma *et al.*, 2013). Sex determination in *As. tabida* could be similar to the MEGISD mechanism of *N. vitripennis*. A first step towards testing *As. tabida* for MEGISD is to elucidate its sex determination pathway in terms of genes and their regulation. Here, we investigate the presence of the sex determination genes *dsx*, *tra2* and *tra*, and examine their role in *As. tabida* sexual development.

Results

Identification of key sex determination genes

Key sex determination genes were identified using translated BLAST (Altschul *et al.*, 1997) against an *As. tabida* genomic assembly (Geuverink *et al.*; unpubl. data). Single homologues of *tra*, *tra2* and *dsx* are present, but no paralogues (ie duplications) of *tra* were detected (Table 1).

Sex-specific splice forms of At-tra

Identification of *tra* splice variants in adult *As. tabida* by rapid amplification of cDNA ends PCR (RACE-PCR) and subsequent reverse-transcription PCRs (RT-PCRs) revealed a characteristic female-specific form (Fig. 1 and Supporting Information Fig. S1A). This splice variant (*At-tra^F*) translates into a peptide containing all known

Table 1. Identified homologues of sex determination genes in *Asobara tabida*

| Gene | GenBank accession no. | Conservation (E-value compared to <i>Nasonia vitripennis</i> peptide sequence) |
|----------------------|-----------------------|--|
| <i>transformer</i> | MF074329 | 2e-34 |
| <i>doublesex</i> | MF074327 | 2e-37 |
| <i>transformer-2</i> | MF074326 | 4e-78 |

conserved TRA domains: the HYM domain, the CAM domain, an arginine-serine domain (RS domain) and a proline-rich region. It is diverged from Aculeata species (ants and bees) and *N. vitripennis* (Fig. S1B). Three male-specific isoforms were found (*At-tra*^{M1}, *At-tra*^{M2} and *At-tra*^{M3}), which contain exons that lead to different open reading frames (ORFs), all resulting in a truncated protein that only contains the HYM domain near the C-terminal end. The inclusion of male-specific exons is characteristic of the sex-specific splicing of *tra* and the CAM domain is typically absent in males (Sánchez, 2008; Verhulst *et al.*, 2010b).

Two abundantly present non-sex-specific *At-tra* splice variants are referred to as *At-tra*^{NSS1} and *At-tra*^{NSS2} (Fig. 1). These two splice variants are similar, except for the last intron, resulting in a slightly longer ORF, retained in *At-tra*^{NSS2}. Both splice variants contain a putative alternative CAM domain, followed by a shortened RS-rich region compared to the one present in *At-tra*^F

(Fig. 1). The proline-rich region associated with TRA is not found in the *AT-TRA*^{NSS} forms; however, seven prolines are detected in the C-terminal region of *AT-TRA*^{NSS1} and 12 in *AT-TRA*^{NSS2}. The two CAM regions (75 bp each) of *At-tra*^F and *At-tra*^{NSS} show high similarity at the amino acid level (Fig. 2).

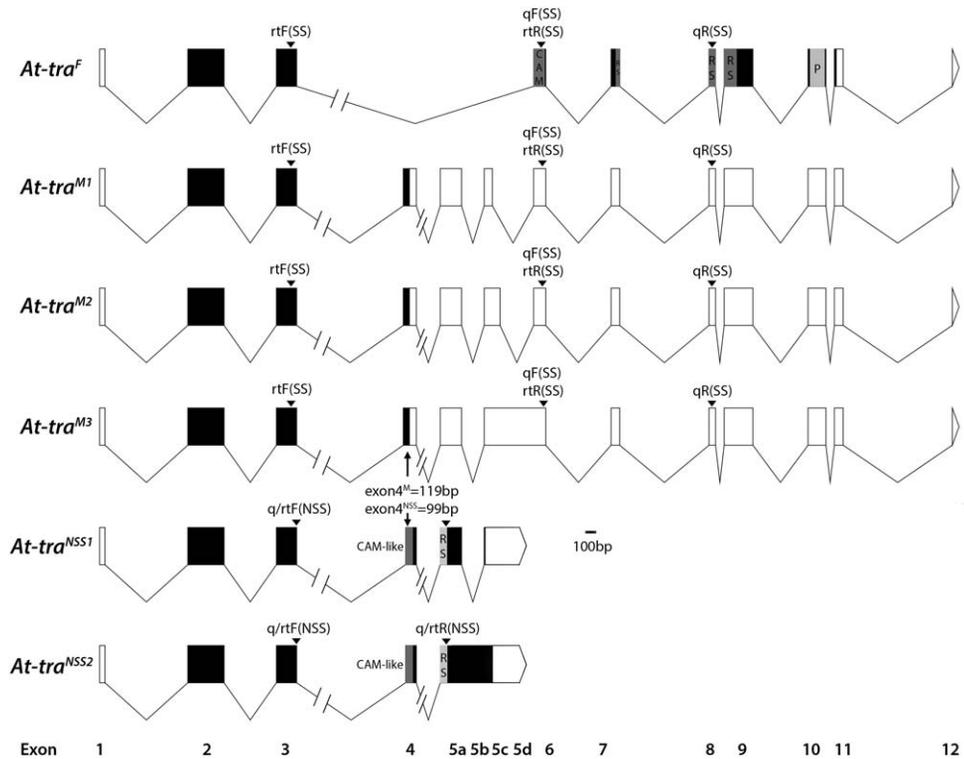
No alternative splicing of *At-tra2*

A single splice form of *tra2* was identified in *As. tabida* (Fig. 3). *AT-TRA2* is highly conserved in its amino acid sequence and contains the characteristic RBD with a large number of flanking arginine and serine residues (Fig. S2A, B). Alternative splice forms were not detected in either sex or at different stages of development. Many insect species only transcribe a single splice form of *tra2* (Burghardt *et al.*, 2005; Concha & Scott, 2009; Salvemini *et al.*, 2009; Sarno *et al.*, 2010; Schetelig *et al.*, 2012; Liu *et al.*, 2015). *Ap. mellifera* (Nissen *et al.*, 2012) and *N. vitripennis* (Geuverink *et al.*, 2017) do possess alternative splice variants of *tra2*, but these are present in both sexes and across all life stages.

Sex-specific splicing of *At-dsx*

The structure of DSX in *As. tabida* (*AT-DSX*), including the DM domain and the second oligomerization domain (OD2) domain, is conserved (Fig. S3A). *AT-DSX* clusters phylogenetically with other hymenopteran DSX orthologues (Fig. S3B). *At-dsx* is sex-specifically spliced into

Figure 1. Exon-intron structure of the female-specific (F), male-specific (M) and non-sex-specific (NSS) splice variants of *transformer (tra)* in *Asobara tabida*. All splice variants are transcribed from one locus. White boxes represent the 5' and 3' untranslated regions, black boxes the coding sequence. Primer positions for quantitative real-time PCR are labelled qF (forward) and qR (reverse). Primers used to detect splice variation by reverse-transcription PCR are labelled rtF (forward) and rtR (reverse). Two different primer sets were used to test for sex-specific (SS) and NSS splicing. Incomplete intron lengths are marked by breaks in the line. CAM, *Ceratitis-Apis-Musca*.



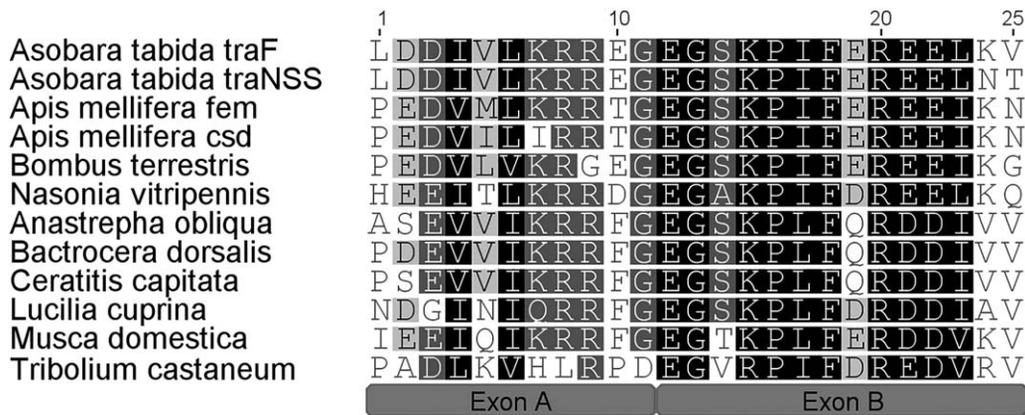


Figure 2. Alignment of the putative *Ceratitis-Apis-Musca* domain of the *Asobara tabida* transformer female-specific (*At-tra^F*) and non-sex-specific (*At-tra^{NSS}*) splice variants against hymenopteran, dipteran and coleopteran sequences. Amino acid sequences are shown with their relative conservation in greyscale (darker tones indicating higher conservation). The 11th amino acid is coded by an exon-spanning triplet and is indicative of the exclusion of male exons spliced out between exons A and B. The specific structure and number of exons A and B differ between species. In the gene structure of *As. tabida* the first 11 identical amino acids from *At-tra^F* and *At-tra^{NSS}* are translated from the same exon 3; from the 12th amino acid onwards the *At-tra^F* sequence continues on exon 7 and the *At-tra^{NSS}* sequence on exon 4.

one female-specific and one male-specific splice form (Fig. 4). The female-specific splice variant includes exon 4, which, upon translation, differs from the male-specific splice variant in yielding a shorter peptide containing a female-specific OD2 domain. The male-specific splice variant does not contain exon 4 and yields a male-specific OD2 domain in the resulting protein. The presence of sex-specific *dsx* splicing suggests functional conservation of the bottom of the sex determination cascade where *dsx* regulates sexual differentiation.

Sex-specific *At-tra* mRNA is not maternally provided

Developing embryos from both unfertilized (haploid) and fertilized (diploid) eggs, ranging from 0 to 144 h after oviposition, were collected to assess the expression of *At-tra*, *At-tra2* and *At-dsx*. RT-PCRs were conducted on the samples to portray the pattern of *At-tra* and *At-dsx* splice variants at these time points. Mated females will produce a mixture of diploid fertilized eggs and haploid unfertilized eggs; thus, a certain number of haploid males (ratio of 0.38 overall) will be present in each 'fertilized eggs' labelled sample. *At-tra^F* and *At-tra^M* mRNA are absent in 0–2-h-old embryos as measured by quantitative real-time PCR (qPCR) amplification (Fig. 5A, measured together as *At-tra^{SS}*), indicating that the mother does not provide sex-specific *tra* (*At-tra^{SS}*)

mRNA to her offspring. At 12–14 h of development *At-tra^M* expression appeared in haploid embryos (Figs 5A, 6A), indicating a zygotic origin. At this point, no splice variant can be detected by RT-PCR in diploid embryos from fertilized eggs (Fig. 6), and expression levels are equal to that of haploid embryos (Fig. 5). The RT-PCR splicing results vary in 12–14-h-old diploid embryos, where amplification covers a mixture of all four *At-tra^{SS}* splice variants (*At-tra^F*, *At-tra^{M1}*, *At-tra^{M2}* and *At-tra^{M3}*). In contrast, the qPCR assay that amplifies the common region of the *At-tra^{SS}* splice variants is more sensitive. After the start of zygotic *At-tra^{SS}* transcription higher levels of *At-tra^{SS}* mRNA are present across developing diploid embryos compared to developing haploid embryos ($Z = 2.48$, $P = 0.013$).

At 24–26 h *At-tra^F* splicing is evident in diploid embryos whereas *At-tra^F* is absent at all points in the development of haploid embryos (Fig. 6). The relative mRNA levels in haploid embryos consist solely of *At-tra^M* transcripts (Fig. 5A). The *At-tra^{SS}* expression (amplifying both *At-tra^F* and *At-tra^M*) in diploid embryos shows a similar pattern (Fig. 5A), but here the transcripts are composed mostly, but not exclusively, of *At-tra^F* mRNA. In both haploid and diploid embryos sex-specific zygotic *tra* expression peaks 48–52 h after oviposition (Fig. 5A).

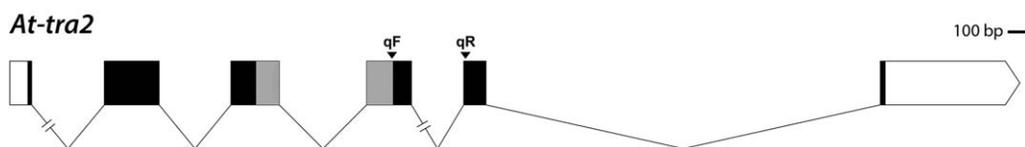


Figure 3. Exon-intron structure of *transformer-2* (*tra2*) in *Asobara tabida*. White boxes represent the 5' and 3' untranslated regions, the black boxes the coding sequence. The RNA binding domain is plotted as grey boxes on the exons. Primer positions for quantitative real-time PCR are labelled qF (forward) and qR (reverse). Incomplete intron lengths are marked by breaks in the line.

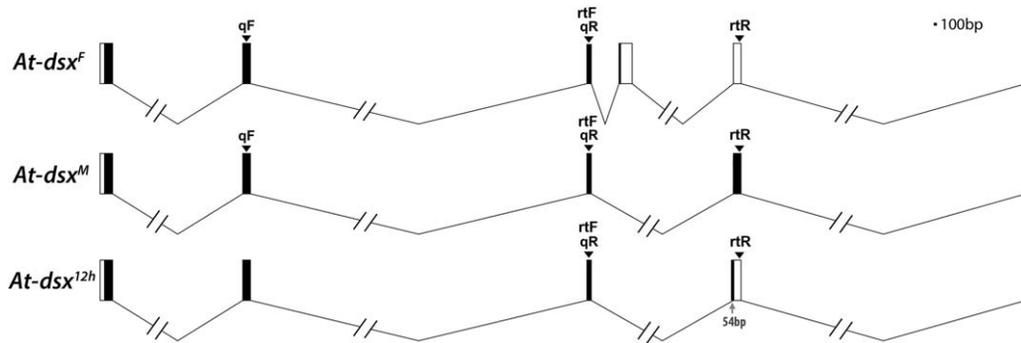


Figure 4. Exon-intron structure of the female-specific (F) and male-specific (M) splice variants of *doublesex* (*dsx*) in *Asobara tabida*. White boxes represent the 5' and 3' untranslated regions, black boxes the coding sequence. Primer positions for quantitative real-time PCR are labelled qF (forward) and qR (reverse). Primers used to detect splice variation by reverse-transcription PCR are labelled rtF (forward) and rtR (reverse). The alternative splice variant present in 12–14 h embryos is noted by the grey arrow demarking 54bp and bar in front of exon 5. Incomplete intron lengths are marked by breaks in the line.

Expression of *At-tra*^{NSS} variants does not only occur in adults but is also observed in embryos of all developmental stages and in both sexes (Figs 5B, 6). These variants are present in embryos less than 2 h of age, indicating that the mother provides these mRNAs to her eggs. The expression of the combined *At-tra*^{NSS} variants follows a pattern similar to the *At-tra*^{SS} expression in the later stages of development (Fig. 5B). The relative levels of *At-tra*^{NSS} mRNA do not differ between female and male embryonic development [$F_{(1, 57)} = 1.2975$, $P = 0.26$].

Maternal provision of *At-tra2*

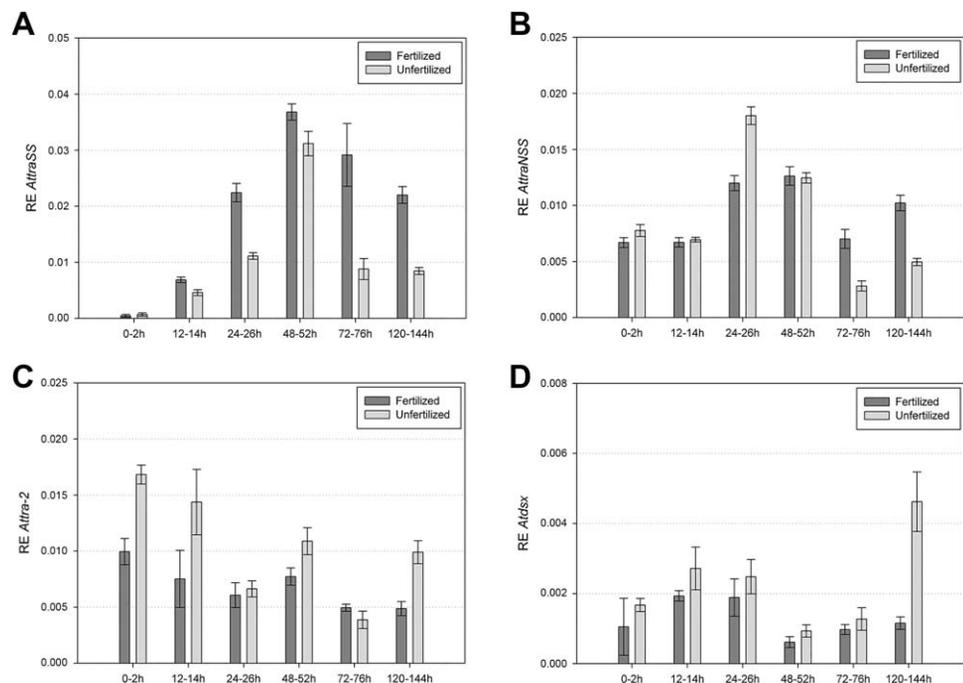
At-tra2 mRNA is maternally provided to embryos as detected with qPCRs (Fig. 5C). It remains present in all stages of both male and female development and shows

no peak of mRNA levels. *At-tra2* expression fluctuates more in males and overall has a higher level during male than female development [$F_{(1, 52)} = 19.592$, $P < 0.005$].

Expression of *At-dsx* during development

Low *At-dsx* mRNA levels measured by qPCR are visible in the earliest stages of development (Fig. 5D), indicating maternal provision. The third splice variant of *At-dsx* has a slight alteration compared to *At-dsx*^M (Fig. 4) and is only observed in some samples of both haploid and diploid embryos at the 12–14 h time point. This *At-dsx*^{12h} transcript has a small 54-bp addition at the 5' end of exon 5 causing a shortened ORF. At 12–14 h the *At-dsx*^F splice variant is not yet present in diploid embryos. Only after sufficient *At-tra*^F is present, *At-dsx*

Figure 5. Relative expression (RE) of sex determination genes during development of diploid female (fertilized eggs) and haploid male (unfertilized eggs) offspring. Note that the RNA pool of diploid embryos also contains haploids as mated females lay a mixture of fertilized and unfertilized eggs. This weakens the signal of any transcript expressed at higher levels during female development, as the reference gene is expressed similarly in both sexes. Relative mRNA levels of (A) combined *Asobara tabida* transformer sex-specific (*At-tra*^{SS}) female and male splice variants, (B) combined non-sex-specific (*At-tra*^{NSS}) splice variants, (C) transformer-2 (*At-tra2*) and (D) *doublesex* (*At-dsx*). Error bars in all figures display the SE per category.



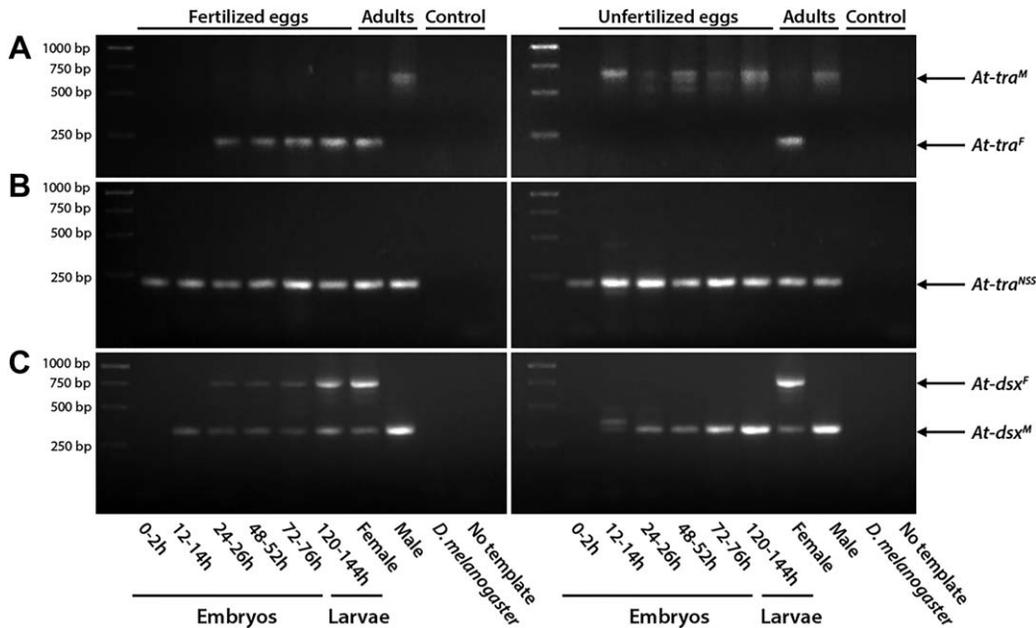


Figure 6. Splice variants of *Asobara tabida transformer* (*At-tra*) and *doublesex* (*At-dsx*) during development. Splicing of *At-tra* is depicted for diploid female (fertilized eggs) and haploid male (unfertilized eggs) offspring for female- and male-specific (*At-tra^F/At-tra^M*) (A) and non-sex-specific (*At-tra^{NS}*) (B) splice variants. Splicing of *At-dsx* in diploid female (fertilized eggs) and haploid male (unfertilized eggs) offspring is shown in (C). Adults of *As. tabida* are included for female and male splicing patterns. *Drosophila melanogaster* larvae and no template reactions are included as negative controls. Samples collected 0–2, 12–14, 24–26, 48–52 and 72–76 h after parasitization contain embryos and those collected at 120–144 h after parasitization contain larvae.

is spliced into the female mode at 24–26 h of development (Fig. 6).

After 12–14 h the haploid embryos exclusively display the male-specific splice variant of *At-dsx* (Fig. 6) and this splicing pattern continues in adult samples. In contrast, in diploid embryos and in adult females, a mixture of *At-dsx^F* and *At-dsx^M* is present in all stages after 12–14 h. This pattern of *dsx^M* leakage in females matches that observed in *Ap. mellifera* and *N. vitripennis*, where the females have both splice variants (Verhulst *et al.*, 2010a; Nissen *et al.*, 2012). This is consistent with male development as the default state of the sex determination cascade.

Discussion

Conservation of the sex determination cascade in *As. tabida*

Orthologues of the key insect sex determination genes *tra*, *tra2* and *dsx* are present in *As. tabida*. The sequence of *At-tra* is strongly diverged compared to other Hymenoptera, but its role in the sex determination cascade is evident from its sex-specific splicing patterns appearing at 12–14 h of development, followed by the activation of sex-specific splicing of *At-dsx* at 24–26 h. AT-TRA^F possesses all known TRA domains, including the HYM domain. This suggests that the HYM domain, previously documented in the Aculeata and *N.*

vitripennis (Verhulst *et al.*, 2010b; Fig. S1A), is conserved in all apocritan Hymenoptera. TRA2 distinctively lacks sex-specific isoforms and shows strong conservation of the RBD with flanking RS regions. The structure of DSX is similarly conserved with the presence of the OD2 domain. This suggests that the conservation of the transducing elements *tra* and *dsx* forms the start of the sexual differentiation process in the *As. tabida* sex determination cascade, even though *At-tra* regulation appears to deviate from the known insect mechanisms.

Regulation of female-specific *tra* and *dsx* splicing

From the 24 h developmental stage onwards, a peak of female-specific *At-tra* mRNA levels appears in diploid embryos and *At-tra* is spliced into the female mode. In haploid embryos from unfertilized eggs, *At-tra^{SS}* expression peaks around 48 h (Fig. 5A) but the male splice variant is already detectable from 12 h onwards (Fig. 6). It has to be noted that expression patterns in diploid embryo samples are not restricted to one sex, because of the experimentally unavoidable inclusion of haploid male embryos. Mated females under our experimental set-up produce mixed broods of 38% haploid male and 62% diploid female embryos. Downstream of *At-tra*, *At-dsx* is spliced into sex-specific variants, consistent with its conserved role in insect sex determination. Initially, *At-dsx* mRNA seems to be spliced into the male mode in both diploid and haploid embryos at 12–14 h of development.

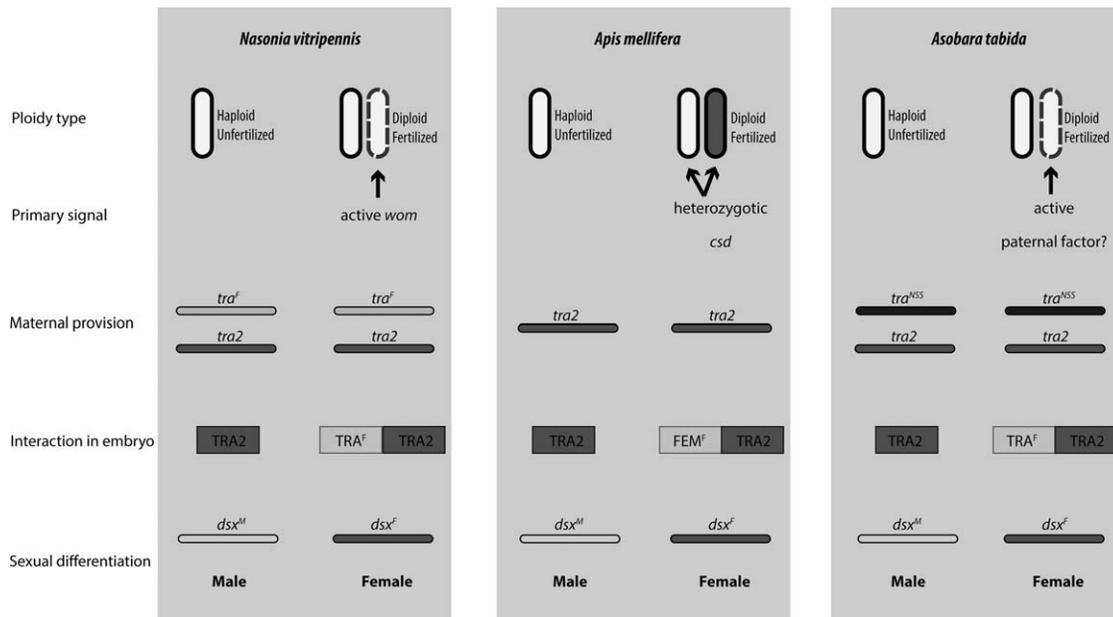


Figure 7. Characteristics of the sex determination mechanism in *Nasonia vitripennis*, *Apis mellifera* and *Asobara tabida*. Male haploid development is plotted on the left of each box and female diploid development on the right side. *csd*, complementary sex determiner; *dsx*, doublesex; FEM, feminizer; *tra*, transformer; *tra2*, transformer-2; *wom*, womanizer. Superscript F, M and non-sex-specific (NSS) indicate female-specific, male-specific and NSS splice variants, respectively.

At this time point both *At-tra*^{NSS} and *At-tra2* mRNA are already present as a result of the maternal provision, but apparently not capable of regulating *At-dsx*^F splicing in the absence of *At-tra*^F. Female *At-tra* splicing is clearly present at 24–26 h, which coincides with *At-dsx* switching to female-specific splicing in diploid embryos. As *At-dsx* is not spliced into the female-specific variant before *At-tra*^F is present, it appears that, as in all other studied insects, AT-TRA regulates *At-dsx* splicing.

tra2 mRNA is present in early embryos, similar to its early expression in *Ap. mellifera* (Nissen *et al.*, 2012) and *N. vitripennis* (Geuverink *et al.*, 2017). A potential complex with the female-specific TRA protein, as demonstrated in *Drosophila melanogaster* (Amrein *et al.*, 1994), can however only be formed at a later stage in development, when *At-tra*^F is actively transcribed in the zygote. Non-sex-specific *At-tra*^{NSS} is present prior to zygotic transcription and is most likely maternally provided, but it is not yet known whether AT-TRA^{NSS} products form a complex with AT-TRA2, or if only AT-TRA^F is involved in this interaction in *As. tabida*.

Functionality of alternative *tra* splice variants

The alternative CAM domain and the arginine-serine rich region of AT-TRA^{NSS} are shorter than those of AT-TRA^F and there is no proline-rich region. In *Drosophila melanogaster*, the proline-rich region appears not to be directly interacting with the spliceosome (Sciabica & Hertel, 2006), suggesting that AT-TRA^{NSS} may share

some aspects of TRA^F function. It is unknown whether *At-tra*^{NSS} splice variants in *As. tabida* have a similar function in starting auto-regulation as female-specific *tra* in other species. Additionally, non-sex-specific patterning and high expression in each developmental stage may indicate a role in overall development of the embryo.

Possibility of MEGISD in *As. tabida*

The absence of maternally provided female-specific *At-tra*^F would be the key difference between the MEGISD system of *N. vitripennis* and a possible MEGISD-like system in *As. tabida*. The function of the abundant maternal provision of non-sex-specific *tra* mRNA and the putative duplicate CAM domain in this splice variant are currently unclear, and further study is required to determine their role in the functioning of sex determination in *As. tabida*. Functional studies of the *At-tra*^{NSS} splice variant may provide the next important clue regarding the underlying sex determination mechanism, which deviates from thus far identified mechanisms (Fig. 7).

The default zygotic state of *tra* in every hymenopteran species is 'OFF', leading to male development (Fig. 7, left pane of each box). Maternal products are supplied to the egg, whether fertilized or not. Consequently, it is impossible that these maternal products are the sole feminizing elements as this would also direct the development of unfertilized haploid eggs into the female mode. A paternal factor, such as the paternal genome or other paternally provided epigenetic marks (eg

microRNAs), is a necessary element in the feminization of the fertilized egg, in combination with the supplied maternal products. The mechanisms of this interaction appear different for each hymenopteran species tested thus far. Therefore, early differential splicing of haploid and diploid zygotic *At-tra* transcripts depends on a paternal factor. As CSD has been refuted as the primary signal in *As. tabida* it is tempting to speculate that a paternal factor, only available in diploid zygotes, would enable female-specific splicing of zygotic *At-tra* transcripts by non-sex-specific AT-TRA. This model (Fig. 7) resembles both the start of the *Ap. mellifera* sex determination cascade where *csd* initiates the female-specific splicing of *fem* (*tra*), and the start of *N. vitripennis* sex determination, where the paternal *wom* gene initiates zygotic *Nvtra* expression. *As. tabida* does not determine sex by a CSD mechanism and the absence of a *tra* paralogue is consistent with a non-CSD system. The mechanism of *As. tabida* could be similar to *N. vitripennis*, but without maternal provision of female-specific *tra* mRNA. These conclusions are consistent with the fast evolution of sex determination mechanisms and their underlying cascade of genes, even within insect orders.

Experimental procedures

Insect culturing

The highly inbred TMS strain (Ma *et al.*, 2013), which originates from strains SOS (Sospel, France) and Italy (Pisa, Italy), was used as genomic source material and in subsequent experiments. The wasps were cultured on second-instar *D. melanogaster* host larvae (72 h of development) at 20 °C under constant light.

Orthologue identification

Orthologues of sex determination genes were identified using translated BLAST (Altschul *et al.*, 1997) against an *As. tabida* genomic assembly (Geuverink *et al.*; unpubl. data). DSX and TRA protein sequences of *Ap. mellifera* (ABW99105, NP_001128300) and *N. vitripennis* (ACJ65507, NP_001128299) and the TRA2 sequence of *Ap. mellifera* (NP_001252514) were used as queries. Prior to availability of the genomic assembly, a small fragment of *transformer* was detected in an *As. tabida* expressed sequence tag data set (Kremer *et al.*, 2012) using translated BLAST. This fragment was used for initial primer development.

RNA extraction, cDNA synthesis and splice-variant detection

Adult females and males were individually collected 24–48 h after their emergence from the *D. melanogaster* pupae. RNA extraction was performed according to the manufacturer's protocol with TriZol (Invitrogen, Carlsbad, CA, USA). RACE-PCRs were notably only performed on adult individuals and alternative transcription start sites could be present in early stages of development. For 3'RACE, reverse transcription of RNA was

conducted with a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) using 25 µM 3'RACE adapter (5'-GCGAGCACAGAATTAATACGACTCACTATAGGT12VN-3') from a FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA). For 5'RACE, RNA was processed according to the manufacturer's instructions (FirstChoice RLM-RACE kit), whereas reverse transcription was conducted with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). To assess the *At-tra* splice variants present in adult males and females 5'RACE-PCR was performed with outer primer Attra5RACEout (5'-CCATTCTGAAGTCGATCTGC-3') and inner primer Attra5RACEin (5'-CTTCGTGGACTTGATTCTCCT-3') under PCR conditions of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, concluded by a final extension of 10 min at 72 °C. Outer primer Attra3RACEout (5'-CAGGAAGGCTCGAAACCT-3') and inner primer Attra3RACEin (5'-GCGAAGAGCTGAATACTAACGA-3') were used in 3'RACE-PCR at an annealing temperature of 55 °C and otherwise identical conditions. 5'RACE-PCR of *At-dsx* was performed with outer primer Atdsx5RACEout (5'-ATCACTTTCTGTCTATCCGT-3') and inner primer Atdsx5RACEin (5'-CGGTATTTGCAATATCTCTTGTC-3') under PCR conditions of 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s, concluded by a final extension of 7 min at 72 °C. Outer primer Atdsx3RACEout (5'-GGGACAAGAGATATTGCA-3') and inner primer Atdsx3RACEin (5'-CATTCTTACGACGGATAGAC-3') were used in 3'RACE-PCR with an extension time of 2 min and otherwise identical conditions. 5'RACE-PCR of *At-tra2* did not yield any fragments after various attempts and was eliminated from the tests. RNAseq isotigs and the genomic contig yielded a putative *tra2* homologue including the 5' untranslated region (5'UTR; Table 1). 3'RACE-PCR of *At-tra2* was performed with outer primer Attra23RACEout (5'-AGGAGCAGGTCTTATTCTAGGT-3') and inner primers Attra23RACEin1/Attra23RACEin2 (5'-TAGGAGTCCAATGTCATCAAGAAGG-3'/5'-TCAATGATGCAAAGACTGGGAG-3') under PCR conditions of 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s, concluded by a final extension of 7 min at 72 °C.

RT-PCRs to confirm the splicing variation of each gene were performed with primers AttraF (5'-AAACAGTGGAGAATAGAGCA-3') and AttraSSR (5'-CTGTATGGAGGTCTGAAACGA-3') for *At-tra*, primers AtdsxfrontF (5'-CTCCACCCGTTACAAGTGAT-3') and AtdsxbackR (5'-GTAGAGCTCAGCCTCTGAC-3') for *At-dsx* and primers Attra2FrontF (5'-CCCAACGAGAAGTCCGCA-3') and Attra2exon4R (5'-CCTTCAAACCTCCCTCTATCATCC-3') for *At-tra2*. All RACE-PCR and RT-PCR products were purified using a GeneJET Gel Extraction Kit (Fermentas) and subsequently ligated into a pGEM-T vector (Promega, Madison, WI, USA). Ligation reactions were used to transform competent JM-109 *Escherichia coli* (Promega). Colony-PCR was conducted with pGEM-T primers (5'-GTA AACGACGCGCCAGT-3' and 5'-GGAAACAGCTATGCCA TG-3') under PCR conditions of 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, concluded by a final extension of 7 min at 72 °C. Both strands were sequenced on an ABI 3730XL capillary sequencer (Applied Biosystems, Foster City, CA, USA) and fragments were aligned to one another and to the assembled genomic contigs to inspect the splicing variation. The exon-intron structure of the genes was visualized with EXON-INTRON GRAPHIC MAKER (<http://wormweb.org/exonintron>). Alignments of TRA,

TRA-CAM, TRA-2 and DSX were produced with Geneious8 (Bio-matters Ltd, Auckland, New Zealand) and gene trees were constructed using a Maximum Likelihood algorithm in MEGA7 (Kumar *et al.*, 2016) based on Jones-Taylor-Thornton (JTT) (Jones *et al.*, 1992) and Le & Gascuel (2008) models. Transcript sequences were deposited in GenBank (accession numbers: MF074326–MF074334).

Embryo collection for *At-tra* expression and splice variation

Hosts containing TMS strain wasps in the pupal stage were placed individually in tubes to prevent females from mating. A batch of mated females was collected from mass culture bottles. Groups of three virgin or mated females were allowed to parasitize hosts for 2 h to train host detection. Following this pretreatment they were kept for 2 days at 12 °C and provided with honey for feeding. Next, each group of wasps was provided with 30 hosts for 2 h every third day, alternated with a period of 2 days with honey-feeding instead of hosts. This allowed for collection of embryos of life stages up to 24–26 h. As differences in development and chances of encapsulation by the host increase in later developmental stages of the hosts, the wasps were allowed more time to parasitize during the collection points of 48–52 h (4 h), 72–76 h (4 h) and 120–144 h (24 h). Petri dishes containing parasitized hosts were kept under constant light at 20 °C. After the allotted development time the Petri dishes were rinsed with water and the host larvae collected and stored crushed in TriZol at –80 °C. A subset of larvae from each group and time point was left to develop into adults. This set was used to verify the virgin state of the unmated females, as they only produce male offspring, and to measure the progeny sex ratio of the mated female. The sex ratio was on average 0.38 (proportion male). Of each group and time point, six tubes with 10 parasitized larvae each were used for RNA extraction, as described for adult tissue above. All total RNA was used for cDNA synthesis, as only a small proportion of the total RNA extracted was of parasitoid wasp origin. Reverse-transcription was performed with a mixture of 1:6 random oligo-dT : random hexamers from the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). Controls of unparasitized *D. melanogaster* larvae of similar age groups were included.

qPCR

For qPCR both embryological and adult samples were diluted 1:50. 5 µl diluted cDNA was combined with 10 µl PerfeCTa SYBR Green Fast Mix (Quanta BioSciences, Gaithersburg, Maryland, USA) and 200 nM of primers. Two different primer sets were used to differentiate between the *At-tra* splice variants: AttraSSqF (5'-GAACGTGAAGAACTGAAGGTTGAG-3') plus AttraSSqR (5'-CTTGACCTCCCCTCATGCCT-3'); and AttraNSSqF (5'-GAAGAGAAGGAGAAGGCTCG-3') plus AttraNSSqR (5'-GAAGGGTGGGATGAAATAAGG-3'). The first set measures the expression of both female-specific and male-specific splice variants, combined noted as SS (sex-specific). The latter set amplifies the non-sex-specific (NSS) splice variants of *transformer* in *As. tabida*, which were measured combined as their amino acid sequence shows very strong similarity. The primer set for *At-dsx*, consisting of AtdsxqF (5'-TTCAGCAATGTACCAATCGGTG-3') and

AtdsxqR (5'-TACCAGAATTGCTCCAGAAGTTTGAC-3'), amplified all splice variants. Primers Attra2qF (5'-TAACGCAACGAGCACATACAC-3') and Attra2qR (5'-GAGCTTCTCTACGCCTG-3') were used to amplify the single mRNA transcript of *At-tra2*. *Elongation factor 1 alpha (EF1α)* was used as the reference gene with primers EF1αF (5'-TCACCGCTCAGTTATTGTC-3') and EF1αR (5'-GGCACAAGATTGACGATAGCTG-3'). All primer sets were used on an Applied Biosystems 7300 Real Time PCR System under PCR conditions of 95 °C for 3 min, 45 cycles of 95 °C for 15 s, 56 °C (*At-dsx* and *At-tra2*) or 58 °C (*EF1α*, *At-tra*^{SS} and *At-tra*^{NSS}) for 30 s and 72 °C for 30 s. Dissociation curves were produced to check for nonspecific amplification. An amplified product of each primer set was cloned, according to the protocol above, to confirm the sequence identity of the amplicon. Negative control samples of unparasitized *D. melanogaster* larvae were tested under the same qPCR conditions and did not show any amplification after 45 cycles.

Raw data were base-line corrected using LINREGPCR 11.0 (Ramakers *et al.*, 2003). Relative mRNA levels were determined by dividing *At-tra2*, *At-tra*^{SS}, *At-tra*^{NSS} and *At-dsx* N0 (starting concentration) values by *At-EF1α* N0. As *EF1α* has stable expression in adults, assessing expression in early embryo stages is not possible. Thus, different time points were not compared and raw expression levels of each gene closely monitored to avoid biases due to reference gene use. *At-tra2*, *At-tra*^{NSS} and *At-dsx* were tested in a general linear model with categorical factors fertilization and time point. A Kruskal–Wallis test was used to compare the relative mRNA levels of *At-tra*^{SS} between fertilized and unfertilized eggs sorted by time point. Relative mRNA levels of *At-tra*^{SS} across development differences between females and males were compared with a Mann–Whitney *U*-test in STATISTICA 7 (StatSoft Inc, Tulsa, OK, USA).

Splice variant presence during development

RT-PCRs with primers AttraRTF (5'-TCTTCGTCGACTATCAATATCC-3') and AttraRTR (5'-TTCTCAACCTTCAGTTCTTCAC-3') were performed on embryo and adult cDNA samples under PCR conditions of 94 °C for 3 min, 45 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 2 min, concluded by a final extension of 7 min at 72 °C. The non-sex-specific *At-tra* transcripts were amplified with the same primers used in the qPCR: AttraNSSF (5'-GAAGAGAAGGAGAAGGCTCG-3') and AttraNSSR (5'-GAAGGGTGGGATGAAATAAGG-3'). Reactions were performed under PCR conditions of 94 °C for 3 min, 45 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, concluded by a final extension of 7 min at 72 °C. *At-dsx* splicing was amplified using AtdsxRTF (5'-GGAGCAATTCTGGTATT-CATGG-3') and AtdsxRTR (5'-CCTGGTGGATTGATACTT-TAGTG-3') under PCR conditions of 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, concluded by a final extension of 7 min at 72 °C. Products were run on a 1.5% agarose gel with ethidium bromide.

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References

- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.
- Amrein, H., Maniatis, T. and Nöthiger, R. (1990) Alternatively spliced transcripts of the sex-determining gene *tra-2* of *Drosophila* encode functional proteins of different size. *Embo J* **9**: 3619–3629.
- Amrein, H., Hedley, M.L. and Maniatis, T. (1994) The role of specific protein-RNA and protein-protein interactions in positive and negative control of pre-mRNA splicing by Transformer 2. *Cell* **76**: 735–746.
- Asplen, M.K., Whitfield, J.B., de Boer, J.G. and Heimpel, G.E. (2009) Ancestral state reconstruction analysis of hymenopteran sex determination mechanisms. *J Evol Biol* **22**: 1762–1769.
- Beukeboom, L.W. and Kamping, A. (2006) No patrigenes required for femaleness in the haplodiploid wasp *Nasonia vitripennis*. *Genetics* **172**: 981–989.
- Beukeboom, L.W., Eilers, J. and Van Alphen, J.J. (2000) Absence of single-locus complementary sex determination in the braconid wasps *Asobara tabida* and *Alysia manducator*. *Heredity* **84**: 29–36.
- Beye, M., Hasselmann, M., Fondrk, M.K., Page, R.E. and Omholt, S.W. (2003) The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. *Cell* **114**: 419–429.
- Bopp, D., Saccone, G. and Beye, M. (2014) Sex determination in insects: variations on a common theme. *Sex Dev* **8**: 20–28.
- Burghardt, G., Hediger, M., Siegenthaler, C., Moser, M., Dübendorfer, A. and Bopp, D. (2005) The *transformer2* gene in *Musca domestica* is required for selecting and maintaining the female pathway of development. *Dev Genes Evol* **215**: 165–176.
- Carton, Y., Bouletreau, M., Van Alphen, J. and Van Lenteren, J. (1986) The *Drosophila* parasitic wasps. In *The Genetics and Biology of Drosophila* (Ashburner, M., Carson, H. and Thompson, J., eds), pp. 347–394. Academic Press, London.
- Concha, C. and Scott, M.J. (2009) Sexual development in *Lucilia cuprina* (Diptera, Calliphoridae) is controlled by the *transformer* gene. *Genetics* **182**: 785–798.
- Gempe, T., Hasselmann, M., Schiøtt, M., Hause, G., Otte, M. and Beye, M. (2009) Sex determination in honeybees: two separate mechanisms induce and maintain the female pathway. *PLoS Biol* **7**: e1000222.
- Geuverink, E. and Beukeboom, L.W. (2014) Phylogenetic distribution and evolutionary dynamics of the sex determination genes *doublesex* and *transformer* in insects. *Sex Dev* **8**: 38–49.
- Geuverink, E., Rensink, A.H., Rondeel, I., Beukeboom, L.W., van de Zande, L. and Verhulst, E.C. (2017) Maternal provision of *transformer-2* is required for female development and embryo viability in the wasp *Nasonia vitripennis*. *Insect Biochem Mol Biol* **90**: 23–33.
- Hasselmann, M., Gempe, T., Schiøtt, M., Nunes-Silva, C.G., Otte, M. and Beye, M. (2008) Evidence for the evolutionary nascence of a novel sex determination pathway in honeybees. *Nature* **454**: 519–522.
- Hediger, M., Henggeler, C., Meier, N., Perez, R., Saccone, G. and 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.
- Hedley, M.L. and Maniatis, T. (1991) Sex-specific splicing and polyadenylation of *dsx* pre-mRNA requires a sequence that binds specifically to tra-2 protein in vitro. *Cell* **65**: 579–586.
- Heimpel, G.E. and de Boer, J.G. (2008) Sex determination in the Hymenoptera. *Annu Rev Entomol* **53**: 209–230.
- Herpin, A. and Schartl, M. (2015) Plasticity of gene-regulatory networks controlling sex determination: of masters, slaves, usual suspects, newcomers, and usurpaters. *EMBO Rep* **16**: 1260–1274.
- Inoue, K., Hoshijima, K., Higuchi, I., Sakamoto, H. and 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 USA* **89**: 8092–8096.
- Jones, D.T., Taylor, W.R. and Thornton, J.M. (1992) The rapid generation of mutation data matrices from protein sequences. *Bioinformatics* **8**: 275–282.
- Kiuchi, T., Koga, H., Kawamoto, M., Shoji, K., Sakai, H., Arai, Y. *et al.* (2014) A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature* **509**: 633–636.
- Kremer, N., Charif, D., Henri, H., Gavory, F., Wincker, P., Mavingui, P. *et al.* (2012) Influence of *Wolbachia* on host gene expression in an obligatory symbiosis. *BMC Microbiol* **12**: S7.
- Kumar, S., Stecher, G. and Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* **33**: 1870–1874.
- Le, S.Q. and Gascuel, O. (2008) An improved general amino acid replacement matrix. *Mol Biol Evol* **25**: 1307–1320.
- Liu, G., Wu, Q., Li, J., Zhang, G. and Wan, F. (2015) RNAi-mediated knock-down of *transformer* and *transformer 2* to generate male-only progeny in the oriental fruit fly, *Bactrocera dorsalis* (Hendel). *PLoS ONE* **10**: e0128892.
- Ma, W.J., Kuijper, B., de Boer, J.G., van de Zande, L., Beukeboom, L.W., Wertheim, B. *et al.* (2013) Absence of complementary sex determination in the parasitoid wasp genus *Asobara* (Hymenoptera: Braconidae). *PLoS One* **8**: e60459.
- Martín, I., Ruiz, M.F. and Sánchez, L. (2011) The gene *transformer-2* of *Sciara* (Diptera, Nematocera) and its effect on *Drosophila* sexual development. *BMC Dev Biol* **11**: 19.
- Matson, C.K. and Zarkower, D. (2012) Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. *Nat Rev Genet* **13**: 163–174.
- Nissen, I., Müller, M. and Beye, M. (2012) The *Am-tra2* gene is an essential regulator of female splice regulation at two levels of the sex determination hierarchy of the honeybee. *Genetics* **192**: 1015–1026.
- Ramakers, C., Ruijter, J.M., Deprez, R.H.L. and Moorman, A.F.M. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* **339**: 62–66.
- Salvemini, M., Robertson, M., Aronson, B., Atkinson, P., Polito, L.C. and Saccone, G. (2009) *Ceratitis capitata transformer-2* gene is required to establish and maintain the autoregulation

- of *Cctra*, the master gene for female sex determination. *Int J Dev Biol* **53**: 109–120.
- Sánchez, L. (2008) Sex-determining mechanisms in insects. *Int J Dev Biol* **52**: 837–856.
- Sarno, F., Ruiz, M.F., Eirín-López, J.M., Perondini, A.L.P., Selivon, D. and Sánchez, L. (2010) The gene *transformer-2* of *Anastrepha* fruit flies (Diptera, Tephritidae) and its evolution in insects. *BMC Evol Biol* **10**: 140.
- Sarno, F., Ruiz, M.F. and Sánchez, L. (2011) Effect of the *transformer-2* gene of *Anastrepha* on the somatic sexual development of *Drosophila*. *Int J Dev Biol* **55**: 975–979.
- Schetelig, M.F., Milano, A., Saccone, G. and Handler, A.M. (2012) Male only progeny in *Anastrepha suspensa* by RNAi-induced sex reversion of chromosomal females. *Insect Biochem Mol Biol* **42**: 51–57.
- Sciabica, K.S. and Hertel, K.J. (2006) The splicing regulators Tra and Tra2 are unusually potent activators of pre-mRNA splicing. *Nucleic Acids Res* **34**: 6612–6620.
- Shukla, J.N. and Nagaraju, J. (2010) *Doublesex*: a conserved downstream gene controlled by diverse upstream regulators. *J Genet* **89**: 341–356.
- Shukla, J.N. and 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.
- Verhulst, E.C., Beukeboom, L.W. and van de Zande, L. (2010a) Maternal control of haplodiploid sex determination in the wasp *Nasonia*. *Science* **328**: 620–623.
- Verhulst, E.C., van de Zande, L. and Beukeboom, L.W. (2010b) Insect sex determination: it all evolves around *transformer*. *Curr Opin Genet Dev* **20**: 376–383.
- Verhulst, E.C., Lynch, J.A., Bopp, D., Beukeboom, L.W. and van de Zande, L. (2013) A new component of the *Nasonia* sex determining cascade is maternally silenced and regulates transformer expression. *PLoS ONE* **8**: e63618.
- Verhulst, E.C. and van de Zande, L. (2015) Double nexus-*Doublesex* is the connecting element in sex determination. *Brief Funct Genomics* **14**: 396–406.
- van Wilgenburg, E., Driessen, G. and Beukeboom, L.W. (2006) Single locus complementary sex determination in Hymenoptera: an “unintelligent” design? *Front Zool* **3**: 1.
- Wilkins, A.S. (1995) Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. *Bioessays* **17**: 71–77.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. (A) Alignment of transformer (TRA) female-specific amino acid sequences. Conservation of sites is shown in greyscale with darker tones indicating higher conservation. (B) Gene tree of female-specific TRA using the maximum likelihood method under Jones-Taylor-Thornton model with incorporation of amino acid frequencies from the data set (JTT-f) (Jones *et al.* 1992). All sites of the alignment are used to infer the gene tree. Bootstrap values (1000 replicates) are shown on the branches. The scale bar shows the number of substitutions per site.

Figure S2. (A) Alignment of transformer-2 (TRA2) amino acid sequences. Conservation of sites is shown in greyscale with darker tones indicating higher conservation. (B) Gene tree of TRA2 using the maximum likelihood method based on the Le & Gascuel (2008) model. All sites of the alignment are used to infer the gene tree. Bootstrap values (1000 replicates) are shown on the branches. The scale bar shows the number of substitutions per site.

Figure S3. (A) Alignment of doublesex (DSX) female-specific amino acid sequences. Conservation of sites is shown in grey scale with darker tones indicating higher conservation. (B) Gene tree of female-specific DSX using the maximum likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992). All sites of the alignment are used to infer the gene tree. Bootstrap values (1000 replicates) are shown on the branches. The scale bar shows the number of substitutions per site.