The *Am-tra2* Gene Is an Essential Regulator of Female Splice Regulation at Two Levels of the Sex Determination Hierarchy of the Honeybee

Inga Nissen,[1] Miriam Müller, and Martin Beye
Department of Genetics, Heinrich Heine University Duesseldorf, 40225 Duesseldorf, Germany

ABSTRACT Heteroallelic and homo- or hemiallelic Complementary sex determiner (Csd) proteins determine sexual fate in the honeybee (*Apis mellifera*) by controlling the alternative splicing of the downstream gene *fem* (*feminizer*). Thus far, we have little understanding of how heteroallelic Csd proteins mediate the splicing of female *fem* messenger RNAs (mRNAs) or how Fem proteins direct the splicing of honeybee *dsx* (*Am-dsx*) pre-mRNAs. Here, we report that *Am-tra2*, which is an ortholog of *Drosophila melanogaster tra2*, is an essential component of female splicing of the *fem* and *Am-dsx* transcripts in the honeybee. The *Am-tra2* transcripts are alternatively (but non-sex-specifically) spliced, and they are translated into six protein isoforms that all share the basic RNA-binding domain/RS (arginine/serine) domain structure. Knockdown studies showed that the *Am-tra2* gene is required to splice *fem* mRNAs into the productive female and nonproductive male forms. We suggest that the *Am*-Tra2 proteins are essential regulators of *fem* pre-mRNA splicing that, together with heteroallelic Csd proteins and/or Fem proteins, implement the female pathway. In males, the *Am*-Tra2 proteins may enhance the switch of *fem* transcripts into the nonproductive male form when heteroallelic Csd proteins are absent. This dual function of *Am*-Tra2 proteins possibly enhances and stabilizes the binary decision process of male/female splicing. Our knockdown studies also imply that the *Am*-Tra2 protein is an essential regulator for *Am-dsx* female splice regulation, suggesting an ancestral role in holometabolous insects. We also provide evidence that the *Am-tra2* gene has an essential function in honeybee embryogenesis that is unrelated to sex determination.

In contrast to the well-studied sex chromosome system in *Drosophila melanogaster* (Cline and Meyer 1996; Erickson and Quintero 2007), sex in the honeybee (*Apis mellifera*) is determined by heterozygosity at the *complementary sex determiner* (*csd*) gene (Beye *et al.* 2003). Bees that are heterozygous at the *csd* locus develop into females, whereas bees that are homozygous or hemizygous at *csd* develop into males. Queens in honeybee colonies lay unfertilized eggs to produce fertile males (drones) and fertilized eggs to produce females that differentiate into either workers or queens; queen fate is determined by specific feeding of the queen larvae with royal jelly (Kucharski *et al.* 2008; Kamakura 2011). Diploid males, homozygous for the *csd* gene, do not survive in a colony because they are eaten by worker bees shortly after they hatch from the egg (Woyke 1963). The *csd* gene translates into an SR-type protein that has at least 15 major allelic variants (Beye 2004; Hasselmann *et al.* 2008) that differ at an average of ~3% of their amino acid residues in the putative specifying domain (Hasselmann and Beye 2004; Hasselmann *et al.* 2008). Females express heteroallelic Csd proteins that direct female splicing of *feminizer* (*fem*) pre-messenger RNAs (pre-mRNAs) (Hasselmann *et al.* 2008; Gempe *et al.* 2009). These female *fem* transcripts are also translated into SR-type proteins that are required for female differentiation. The Fem proteins promote the female splicing of the *A. mellifera dsx* (*Am-dsx*) transcripts, which express a transcription factor of the DM type, a protein with a female-specific carboxy-terminal end (Dearden *et al.* 2006; Cho *et al.* 2007). In addition, Fem proteins direct splicing of their own pre-mRNAs into the productive female form, which generates an autoregulatory feedback loop that maintains the female state throughout development (Gempe *et al.* 2009). In the absence of Csd protein activity in males (homo- or hemiallelic Csd proteins), *fem* transcripts are spliced into the male form, which contains a translational

doi: 10.1534/genetics.112.143925
Manuscript received July 17, 2012; accepted for publication August 17, 2012
Available freely online through the author-supported open access option.
Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.143925/-/DC1.
[1]Corresponding author: Heinrich Heine University Duesseldorf, Institute of Genetics, Universitaetsstr. 1, 40225 Duesseldorf, Germany. E-mail: inganissen@gmx.net.

stop codon in exon 3 that causes premature translation termination (Gempe *et al.* 2009). As a consequence, *Am-dsx* pre-mRNAs are spliced into the male variant expressing a Dsx protein (Gempe *et al.* 2009), which has a male-specific carboxy-terminal end as part of the oligomerization domain 2 (Cho *et al.* 2007).

The *csd* gene is thus the primary signal of sex determination in the honeybee. The *csd* gene evolved recently in the honeybee lineage by gene duplication of an ancestral copy of the *fem* gene (Hasselmann *et al.* 2008). Although substantially diverged in sequence, the *fem* gene is the ortholog of the sex-determining gene *transformer* (*tra*) of *D. melanogaster*. Thus far, we have little understanding of how the heteroallelic Csd proteins mediate *fem* splicing or how Fem proteins direct *Am-dsx* pre-mRNA splicing (Gempe and Beye 2011). Both proteins lack an RNA-binding domain (RBD), which is suggestive of a cofactor that can directly interact with the respective RNA sequence.

In this study, we explored the role of the *tra2* gene of the honeybee [*Am-tra2* (Dearden *et al.* 2006)] in regulating the sex-specific splicing of the *fem* and *Am-dsx* transcripts. In *D. melanogaster*, the RNA-binding protein Tra2 acts together with the Tra protein to promote the female splicing of *dsx* pre-mRNAs (Burtis and Baker 1989; Amrein *et al.* 1990; Hedley and Maniatis 1991; Inoue *et al.* 1992; Lynch and Maniatis, 1996, 1995; Sciabica and Hertel 2006). *D. melanogaster* females express in somatic tissues two major protein isoforms of the Tra2 proteins ($Tra2^{264}$ and $Tra2^{226}$) that, together with the Tra proteins, activate a weak 3′ splice acceptor site in *dsx* pre-mRNAs by binding to the six repeats of a 13-nucleotide exonic splicing enhancer (ESE) sequence and a single purine-rich element. This activation leads to the inclusion of the female exon 4 in female *dsx* mRNAs (Burtis and Baker 1989; Hedley and Maniatis 1991; Inoue *et al.* 1992; Lynch and Maniatis 1996, 1995; Sciabica and Hertel 2006). Splicing of honeybee *Am-dsx* has putatively evolved in that respect compared to *D. melanogaster*, as *Am-dsx* pre-mRNAs lack the canonical binding sites of Tra/Tra2 proteins.

The *tra2* gene has also been characterized in other dipteran species, including *Musca domestica*, *Anastrepha obliqua*, *Ceratitis capitata*, *Lucilia cuprina*, and *Sciara ocellaris* (Burghardt *et al.* 2005; Concha and Scott 2009; Salvemini *et al.* 2009; Sarno *et al.* 2010; Martín *et al.* 2011), as well as in the lepidopteran insect *Bombyx mori* (Niu *et al.* 2005). All Tra2 proteins share the same domain structure of a single RBD that is flanked by two arginine-/serine-rich (RS-rich) regions. The RBD consists of 80–90 amino acids that form a βαββαβ barrel-like topology. One side of the β-sheet surface (β1 and β3) of the RBD exposes two sequence elements, RNP-1 and RNP-2, which are directly involved in RNA recognition (Dreyfuss *et al.* 1988; Merrill *et al.* 1988; Nagai *et al.* 1990; Amrein *et al.* 1994).

In the dipteran insects *M. domestica*, *C. capitata*, *Anastrepha suspensa*, and *A. obliqua*, RNA interference (RNAi) knockdown studies of the *tra2* gene showed that Tra2 proteins are also involved in female splicing of *tra* mRNAs (Burghardt *et al.* 2005; Concha and Scott 2009; Salvemini *et al.* 2009; Sarno *et al.* 2010; Martín *et al.* 2011). Due to the presence of the canonical 13-nucleotide Tra/Tra2-binding motif in *transformer* of *M. domestica*, *A. suspensa*, and *C. capitata*, the authors suggest that Tra2 proteins act as cofactors in the autoregulatory splicing loop in which Tra/Tra2 proteins direct the female splicing of *tra* transcripts and thus the expression of Tra protein (Salvemini *et al.* 2009; Hediger *et al.* 2010; Schetelig *et al.* 2012). In the lepidopteran insect *B. mori*, the function of Tra2 proteins in sexual regulation of the *Bm-dsx* transcripts is not known. In this species, male splicing of *Bm-dsx* transcripts requires the splicing inhibitor (*Bm*-PSI) and the male-specific IMP (*Bm*-IMP) proteins. The activation of the female exon splicing is repressed in males by the binding of the *Bm*-PSI and the male-specific *Bm*-IMP proteins to the 20-nucleotide CE1 motif of the female exon (Suzuki *et al.* 2001, 2008, 2010).

In the male germline of *D. melanogaster*, the $Tra2^{226}$ protein isoform has an additional function in spermatogenesis in controlling the splicing of the *exuperantia* (*exu*) and *alternative-testes-transcript* (*att*) transcripts (Hazelrigg and Tu 1994; Madigan *et al.* 1996; Mattox *et al.* 1996). In the testes, $Tra2^{226}$ proteins negatively affect their own expression by promoting the splicing of $tra2^{179}$ mRNAs, which produce no functional protein (Mattox and Baker 1991; Mattox *et al.* 1996; McGuffin *et al.* 1998). This negative feedback loop controls the level of Tra2 expression, which is critical for proper spermatogenesis.

In this study, we report the cloning and functional analysis of the *A. mellifera Am-tra2* gene. Our study showed that the *Am-tra2* gene serves as a regulator in female-specific splicing of *fem* and *Am-dsx* transcripts. Furthermore, we show that *Am-tra2* has a vital function in embryogenesis that differs from its reported functions in other species.

Materials and Methods

Bee sources

Diploid female embryos were derived from the progeny of queens inseminated by semen from a single drone having a different sex allele than that of the queen. Haploid male embryos were collected from colonies that were headed by a virgin queen. These non-mated queens laid unfertilized male eggs that we induced by repeated CO_2 treatments of virgin queens.

RNA extraction, cDNA synthesis, and PCR

Total RNA was extracted using the TRIzol protocol (GIBCO BRL Life Technologies, Darmstadt, Germany). The first-strand complementary DNA (cDNA) from mRNA was generated by reverse transcription using an oligo(dT) primer following the protocol of the supplier (Fermentas, St. Leon-Rot, Germany). We quantified the amount of cDNA in our samples in a NanoDrop ND-1000 spectral photometer and

Table 1 Development of *Am-tra2*-dsRNA-treated female embryos

Treatment/amount of injected dsRNA per embryo	Embryos injected	No. of embryos showing normal development after ~70 hr		Hatched L1 larvae	
	N	*N*	%	*N*	%
Nontreated	683	594	86.9	365	53.4
ddH_20	406	182	44.8	97	23.9
Am-tra2 dsRNA-1					
200 pg	92	0	0	0	0
100 pg	25	0	0	0	0
75 pg	60	0	0	0	0
40 pg	20	0	0	0	0
20 pg	53	0	0	0	0
4 pg	40	5	12.5	2	5.0
Am-tra2 dsRNA-2					
224 pg	27	0	0	0	0
96 pg	100	15	15	0	0
67 pg	104	38	36.5	0	0
56 pg	105	14	13.3	4	3.8
33 pg	146	31	21.2	6	4.1
4 pg	155	29	18.7	9	5.8

The number of individuals (*N*) and the relative proportions (%) with respect to the total number of initially injected embryos are shown.

adjusted the amount of cDNA prior to PCR amplification. PCR was performed using GoTaq Flexi DNA Polymerase (RNAi experiments) according to the protocol of the supplier (Promega, Mannheim, Germany) and Taq polymerase (RACE and transcriptional analysis of *Am-tra2* throughout development). All RT-PCR fragments were resolved by agarose gel electrophoresis and stained with ethidium bromide. The identity of the *fem*, *Am-dsx*, *csd*, and *Am-tra2* amplicons was verified by sequencing. We amplified cDNA fragments of the *ef-1α* gene using oligonucleotides #EM033 and #EM034 for the semiquantitative studies across samples (Supporting Information, Table S1).

Characterization of Am-tra2 gene

To determine the entire sequences of the *Am-tra2* transcripts, we performed 5′ and 3′ RACE experiments following the manufacturer's instructions (FirstChoice RLM-RACE kit; Ambion). The cDNAs were generated from male and female RNA samples of honeybee embryos. Gene-specific primers for RACE reactions were designed from the sequence of the *Am-tra2* gene model at the NCBI web site (NCBI Reference Sequence: XM_001121070.2) (Table S1). All RACE products were cloned into the pGEM-T vector (Promega), and both strands were sequenced. We translated the mRNA sequences into the amino acid sequence, and we predicted the protein domains by the similarity to domains in the PROSITE database (http://www.expasy.org/prosite/). The GenBank accession numbers are JQ518311 (*Am-tra2*^{285}), JQ518314 (*Am-tra2*^{284}), JQ518312 (*Am-tra2*^{253}), JQ518314 (*Am-tra2*^{252}), JQ518313 (*Am-tra2*^{234}), and JQ518316 (*Am-tra2*^{233}).

Transcriptional studies of the Am-tra2 gene throughout development

Total RNA was extracted from male and female eggs (at 0–6, 9–24, 33–48, and 72 hr), larvae (L1 and L4 instar), pupae (3 days before hatching from comb), adult heads, and germline tissue (testes of L4 larvae and ovaries of virgin queens). We amplified cDNA fragments using oligonucleotides #359 and #421 (Table S1) that span the complete open reading frame (ORF) of all six *Am-tra2* splice variants. The identity of the amplicons of the male and female L1 larvae and pupae were verified by sequencing.

Functional studies of the Am-tra2 gene

RNAi knockdown was induced in early embryogenesis at the syncytial stage (0–4 hr after egg deposition) in females and males (Beye *et al.* 2003, 2002). *Am-tra2* double-stranded RNA-1 (dsRNA-1), encompassing the region from 322 to 767 bp (446 bp long), was generated using oligonucleotides #22M and #23M (Table S1) from cloned cDNAs of the *Am-tra2*^{285} transcript following the protocol previously described (Beye *et al.* 2003, 2002; Hasselmann *et al.* 2008). *Am-tra2* dsRNA-2, encompassing the region from 108 to 499 bp (392 bp long), was generated using oligonucleotides #591 and #592 (Table S1 and Figure S1) from cloned cDNAs of transcript *Am-tra2*^{285}. The dsRNAs were dissolved in ddH_2O and injected at a concentration of 4–200 pg/embryo (Tables 1 and 2). In the control samples, we injected only ddH_2O (Roth, Karlsruhe, Germany).

We counted the number of embryos showing normal development ~70 hr after egg deposition and the number of hatched L1 larvae 77–80 hr after egg deposition. All embryos that were malformed, showed necrotic tissue, or lacked the segmentation pattern 70 hr after egg deposition were classified as aberrant.

To study the effect of *Am-tra2* knockdown on the splice patterns of *fem* and *Am-dsx* transcripts, we collected embryos and L1 larvae from dsRNA-treated and nontreated samples 48 or 77–80 hr after egg deposition. Treated embryos and controls were reared in the incubator at 35° until an age of 48 or 77–80 hr or until reaching the L1 larval stage. The samples were directly frozen in liquid nitrogen.

Table 2 Development of *Am-tra2*-dsRNA-treated male embryos

Treatment/amount of injected dsRNA per embryo	Embryos injected	No. of embryos showing normal development after ~70 hr		Hatched L1 larvae	
	N	*N*	%	*N*	%
Nontreated	55	36	65.5	36	65.5
ddH_20	78	30	38.5	26	33.3
Am-tra2 dsRNA-2					
96 pg	73	8	11.0	0	0

The number of individuals (*N*) and the relative proportions (%) with respect to the total number of initially injected embryos are shown.

Fragments corresponding to the female *fem* mRNAs were amplified using oligonucleotides #412 and #523 (Table S1), which are composed of a part of exon 3 and exon 6 (size: 177 bp). We amplified fragments (size: 458 bp; exons 3–4 and part of exon 5) corresponding to the male *fem* mRNAs using oligonucleotides #410 and #566 (Table S1). Because the predetermined state in the early embryos is male splicing of *fem* transcripts (Gempe *et al.* 2009), the presence of female-specific fragments indicates that female splicing is induced. We used oligonucleotides #417 and #418 (Table S1) to amplify exons 4–5–6 (size: 1.2 kb) and exons 4–6 (size: 392 bp), which correspond to the female and male *Am-dsx* transcripts, respectively. The female *Am-dsx* transcripts were also specifically amplified by oligonucleotides #417 and #419 (Table S1) (size: 188 bp), which encompass exon 4–5. Fragments corresponding to the *csd* mRNAs were amplified using oligonucleotides #CS-1 and #CS-2 (Table S1), which are composed of a part of exon 6, exons 7 and 8, and a part of exon 9. As exons 7 and 8 include the hypervariable region of the *csd* gene, the length of the amplified fragments can vary substantially between the alleles.

Phylogenetic and molecular evolutionary sequence analyses

We utilized nine *tra-2* sequences from different insect species to compare the phylogenetic and molecular relationships: *Tribolium castaneum* (GenBank:XP_968550.2), *Acromyrmex echinatior* (GenBank: EGI70155.1), *D. melanogaster* (NCBI Reference Sequence: NP_476764.1), *B. mori* (GenBank: NP_001119705.1), *Nasonia vitripennis* (GenBank: XP_001601106.1), *S. ocellaris* (GenBank: CBX45935.1), *C. capitata* (GenBank: ACC68674.1), *M. domestica* (GenBank: AAW34233.1) and *Anastrepha obliqua* (GenBank: CBJ17280.1).

Results

Genomic organization of the tra2 gene in the honeybee

The existence of the *tra2* gene in the honeybee genome was predicted by the similarity of its RBD to those of other insects (Dearden *et al.* 2006). We isolated *Am-tra2* transcripts that included the 5′ and 3′ untranslated regions (UTRs) and three different polyadenylation sites (Figure 1) using RACE experiments with cDNA preparations from both male and female embryos. Using cDNA preparations from embryos and pupae of males and females, we performed RT-PCRs to amplify the entire ORF of *Am-tra2* with oligonucleotide primers that bound the 5′ and the 3′ ends of the ORF. As a result, we detected up to six splice variants (Figure 1) that were not sex-specific in embryos or pupae. The six splice variants in embryos were *Am-tra2*^{285}, *Am-tra2*^{284}, *Am-tra2*^{253}, *Am-tra2*^{252}, *Am-tra2*^{234}, and *Am-tra2*^{233}, and the four transcript variants in pupae were *Am-tra2*^{285}, *Am-tra2*^{284}, *Am-tra2*^{253}, and *Am-tra2*^{252} (Figure 1). Three splice variants showed major sequence differences in exon 2. The other three are minor variants of the other three major variants that differ in three nucleotides in exon 4. All transcripts express essentially the same protein that harbors a RBD flanked by two RS-rich domains but differ in the length in the first RS domain (RS1) (Figure 1, A–C). The RBD amino acid sequence has the strongest similarity (61–85% sequence identity) to the Tra2 proteins of a variety of insects (*N. vitripennis*, *T. castaneum*, *A. echinatior*, *B. mori*, *S. ocellaris*, *D. melanogaster*, *M. domestica*, *C. capitata* and *A. obliqua*) (Amrein *et al.* 1990; Mattox *et al.* 1996; Burghardt *et al.* 2005; Niu *et al.* 2005; Tribolium Genome Sequencing Consortium 2008; Salvemini *et al.* 2009; Sarno *et al.* 2010; Nygaard *et al.* 2011), supporting our notion that we have identified the Tra2 ortholog of the honeybee (Figure 2).

The largest transcript, *Am-tra2*^{285}, consists of 1401 nt and five exons and harbors an ORF coding for 285 amino acids (Figure 1A). The other two major transcript variants (*Am-tra2*^{253} and *Am-tra2*^{234}) consist of six exons in which different parts of exon 2 are spliced out. These two variants are produced by sharing the same splice donor but using two alternative splice acceptors. They are 1248 and 1305 nt long and are putatively translated into 253- and 234-amino-acid proteins, respectively (Figure 1, B and C). All three major splice variants have minor splice variants that utilize an alternative splice acceptor site at exon 4, producing a single-serine amino acid deletion in the RS2 domain of the putative protein (Figure 1D).

Am-Tra2 has a typical Tra2 RBD that has evolved in the RNP-1 element

We compared our deduced amino acid sequence with those of dipteran (*D. melanogaster*, *M. domestica*, *S. ocellaris*, and *A. obliqua*), coleopteran (*T. castaneum*), lepidopteran (*B. mori*), and other hymenopteran [*N. vitripennis* (wasp)

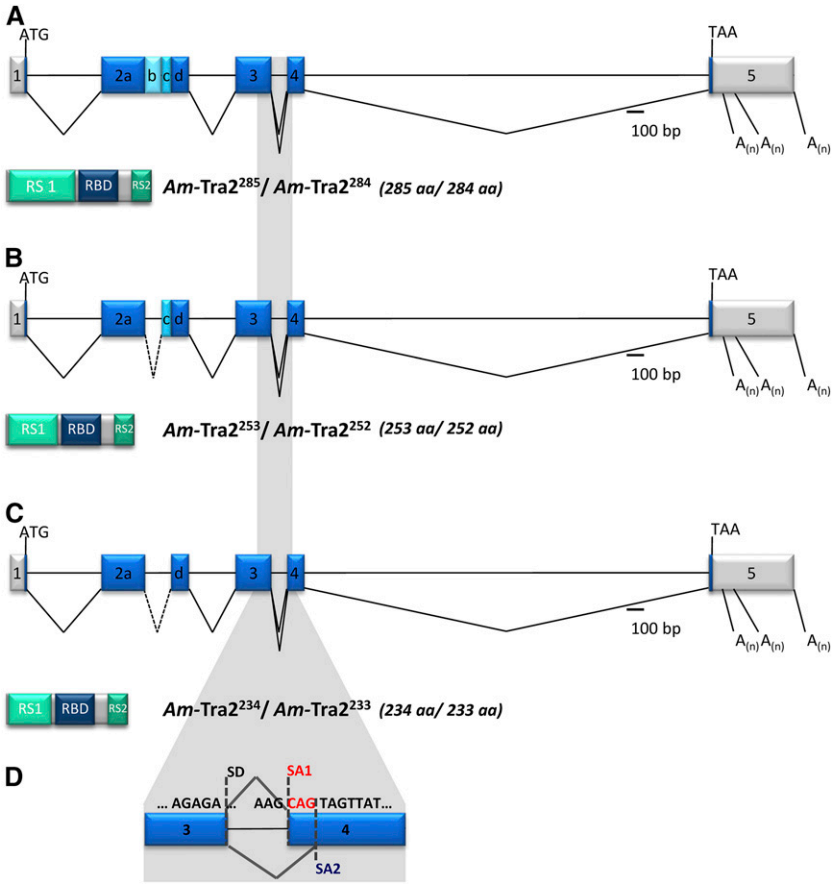

Figure 1 Genomic organization of the *Am-tra2* gene and the alternatively spliced mRNAs. Schematic representation of the intron and exon organization (presented as lines and boxes, respectively). The alternatively spliced transcripts are indicated by the connecting lines between exons. The three alternative polyadenylation sites are labeled "$A_{(n)}$." The scale denotes the relative size of the introns and exons. The 5′- and 3′-UTRs are presented in gray and the ORF in blue boxes. Below the genomic organization, the domain structure and relative size of the predicted *Am*-Tra2 proteins are shown (RS, arginine/serine-rich domain; RBD, RNA-binding domain). Superscript of *Am*-Tra2 proteins denotes the number of amino acids in that particular protein isoform. (A) *Am*-Tra2^{285} and *Am*-Tra2^{284}. (B) *Am*-Tra2^{253} and *Am*-Tra2^{252}. (C) *Am*-Tra2^{234} and *Am*-Tra2^{233}. (D) Alternatively spliced variants in exon 4 producing trinucleotide and single-amino-acid differences. This splicing affected all three transcripts shown in A–C that are denoted as the *Am-tra2*^{284}, *Am-tra2*^{252}, and *Am-tra2*^{233} transcripts. SA_1 and SA_2 label the alternatively splice acceptors in exon 4.

and *A. echinatior* (ant)] insects to identify shared structural features of Tra2 proteins in holometabolous insects. We were able to unambiguously align the amino acid sequences only for the RBD and neighboring regions, but not for other parts of the protein (Figure S2), suggesting that the RBD is evolutionarily constrained. All Tra2 proteins in the different organisms share the two RS domains, but the arginine- and serine-rich sequence is highly diverged (Figure S2), suggesting that these domains are faster-evolving and evolutionarily less constrained than the RBD.

In *D. melanogaster*, the RBD domain of Tra2 protein binds to ESEs, which are composed of six nearly identical 13-nucleotide-long sequences, the *dsx* repeat elements (RE). Similar motifs have also been detected in the *dsx* gene sequences of other dipteran insects, but are lacking in the *Am-dsx* pre-mRNAs of the honeybee (Crampton *et al.* 1998; Hediger *et al.* 2004; Lagos *et al.* 2005; Ruiz *et al.* 2005, 2007; Cho *et al.* 2007; Saccone *et al.* 2008; Concha *et al.* 2010; Permpoon *et al.* 2011). We next studied the sequence similarities within the RBDs of Tra2 proteins of different holometabolous insects (Figure 2). The RBD amino acid sequence diverges in relation to phylogenetic distance. The RBD domains of the hymenopteran species honeybees, *N. vitripennis*, and *A. echinatior* show a pairwise sequence identity of 82–85%, whereas RBDs of the honeybee and dipteran species have pairwise sequence identity of 61–68%. Within

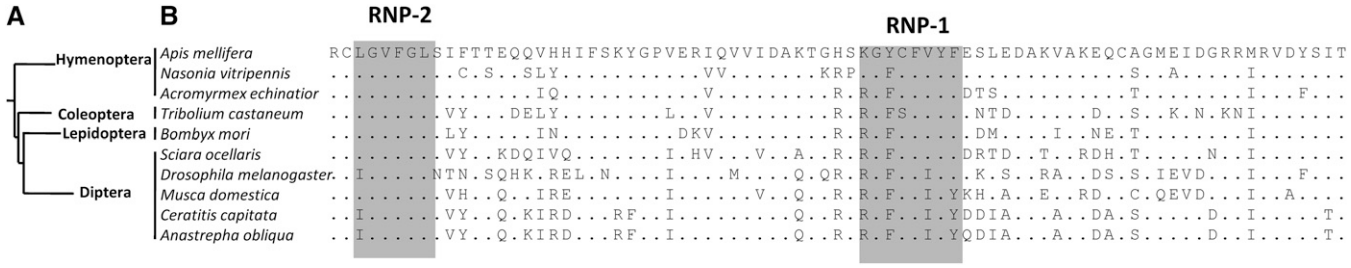

Figure 2 The RBD domain of the honeybee *Am*-Tra2 protein and its relation to the Tra2 RBD domain of other holometabolous insects. (A) The phylogenetic relationship of the members of the different insect orders used in this comparison (Savard *et al.* 2006). (B) Amino acid sequence alignment of Tra2 RBDs of *Apis mellifera*, *N. vitripennis*, *A. echinatior*, *T. castaneum*, *B. mori*, *S. ocellaris*, *D. melanogaster*, *M. domestica*, *C. capitata*, and *A. obliqua*. Dots indicate amino acids identical to the predicted *Am*-Tra2 RBD of the honeybee. Shaded areas denote the RNP sequence elements.

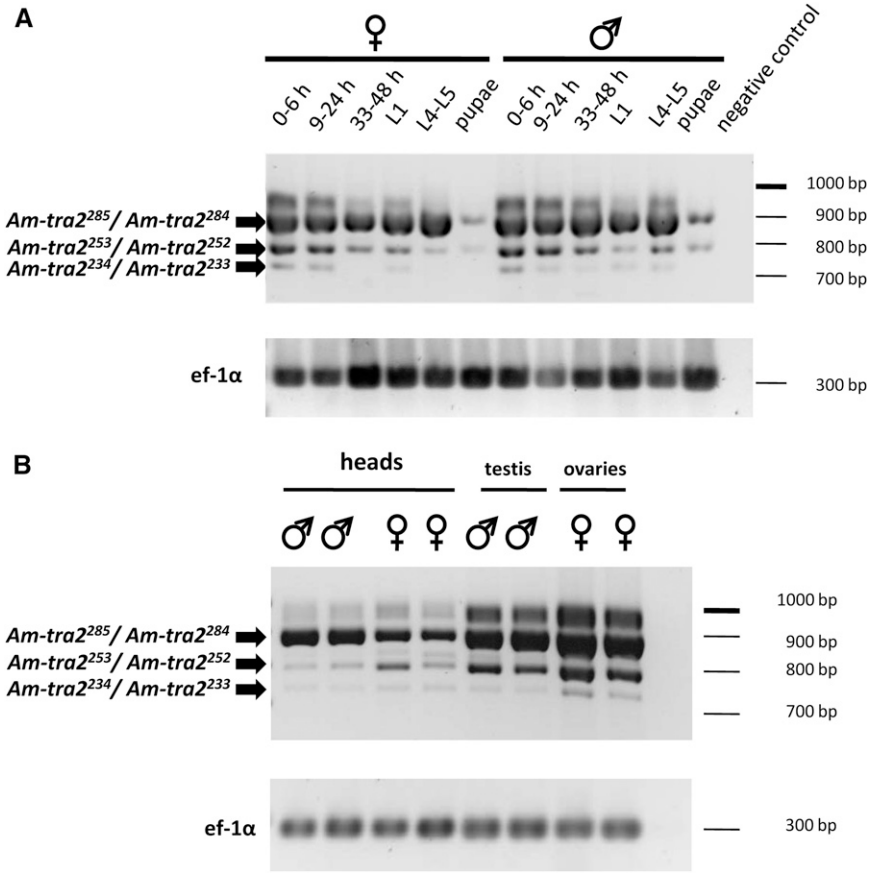

Figure 3 Semiquantitative transcriptional profile of *Am-tra2* transcripts throughout male and female development (A) and in somatic and gonadal tissue (B). Fragments of *Am-tra2* transcripts spanning the entire ORF were amplified by RT-PCR, resolved by agarose gel electrophoresis, and stained with ethidium bromide. We used amplification of the cDNAs of the gene *ef-1α* as a relative control to semiquantify *Am-tra2* transcripts across samples (Beye *et al.* 2003). The fragments in 0- to 6-hr-old embryos and pupae were cloned and sequenced. Ovaries were dissected from virgin queens as were testes from L4 larvae; heads are from adult bees. L1, larval stage 1; L4–L5, larval stages 4 and 5. For the embryonic stages, the hours (h) after egg deposition are indicated.

the RBD of the *Am*-Tra2 protein, there are two putative ribonucleoprotein consensus peptide (RNP) elements (Figure 2). These elements can directly interact with the *dsx* pre-mRNAs in *D. melanogaster* (Dreyfuss *et al.* 1988; Merrill *et al.* 1988; Nagai *et al.* 1990; Amrein *et al.* 1994). In the eight-amino-acid-long honeybee RNP-1 sequence element, we detected three amino acid differences compared with that of *D. melanogaster*. A mutation in the first amino acid of RNP-1 has been shown in *D. melanogaster* to be essential for female *dsx* splicing (Amrein *et al.* 1994). Interestingly, this functionally important first amino acid has especially evolved from amino acid R to K within the honeybee and the *Nasonia* lineage (Figure 2). We propose that the target RNA sequences of the RNP-1 element in the *Am*-Tra2 protein may have correspondingly evolved and thus differ from those found in *D. melanogaster*.

Am-tra2 transcript variants are not sex-specifically regulated and are transcribed throughout development

In *D. melanogaster*, the *tra2* gene produces three transcript variants that translate into three Tra2 protein isoforms (Tra-2^{264}, Tra-2^{226}, and Tra-2^{179}), which differ in the length of the first RS domain (Amrein *et al.* 1990; Mattox *et al.* 1996). Tra-2^{264} and Tra-2^{226} are expressed in somatic tissues in both sexes and are involved in *dsx* regulation. To identify sex-specific transcripts and the relative abundance of transcripts at different stages of development and sex determination in the honeybee, we simultaneously amplified all *Am-tra2* transcript variants in 0- to 6-, 9- to 24-, and 33-to 48-hr-old embryos; L1 and L4–L5 larvae; and pupae by semiquantitative RT-PCR (Figure 3A). At the stage of 0- to 6-h-old embryos, the primary signal *csd* does not yet determines the sexual fate. The *csd* gene is not transcribed at this syncytial embryonic stage (Beye *et al.* 2003; Gempe *et al.* 2009). The transcripts are most abundant in the embryonic stages before and after the primary signal is active, but also at the larval stages and in both sexes. However, the level of expression substantially decreases at the pupal stage. The *Am-tra2*^{285} and *Am-tra2*^{284} transcripts, which are translated into the largest RS domain-containing proteins of all the transcripts, have the highest level of expression (Figure 3A). The *Am-tra2*^{253}/*Am-tra2*^{252} transcripts are present in sizable amounts in early embryos, whereas the *Am-tra2*^{234}/*Am-tra2*^{233} transcripts are apparently minor splice products.

In *D. melanogaster*, there is a male-specific *tra2* transcript, *tra-2*^{179}, whose splicing is mediated by Tra-2^{226} proteins derived from the general *tra-2*^{226} transcript. This negative feedback loop at the level of splicing regulates proper Tra2 protein expression in the germline, which is essential for correct sperm formation (McGuffin *et al.* 1998). To identify a similar role of the *Am-tra2* gene in sperm formation of the honeybee, we specifically searched for sex-specific *Am-tra2* transcripts in the female (ovary tissue) and male (testis tissue) germlines (Figure 3B). We detected no sex-specific

Table 3 Production of female and male *fem* and *Am-dsx* transcripts in 72- to 80-hr-old individuals in response to embryonic *Am-tra2* dsRNA-2 treatment

		No. of individuals with transcripts					
		fem			*Am-dsx*		
Treatment	No. of embryos	Solely male	Solely female	Male and female	Solely male	Solely female	Male and female
Nontreated controls							
Males	10	9	0	0	10	0	0
Females	14	1	8	5	1	12	1
Treated females							
ddH_20	15	0	8	15	1	13	1
dsRNA-2 (33 pg)	12	6	0	1	12	0	0
dsRNA-2 (4 pg)	11	0	4	7	1	1	9

transcripts, suggesting that *Am-tra2* mRNAs are not sexually spliced in male germ cells.

Taken together, these results indicate that *Am-tra2* transcripts are not sex-specifically spliced and present before the primary decision of sexual fate is made by the *csd* gene. The *Am-tra2*^{285} and *Am-tra2*^{284} are the dominant transcripts, and the relative amount of *Am-tra2* decreases at the pupal stage.

Knockdown of the Am-tra2 gene affects embryonic viability and female splicing of the fem and Am-dsx transcripts

In the honeybee, the primary signal *csd* mediates, in the heteroallelic condition, the female splicing of *fem* transcripts. The downstream target of *csd*, the *fem* gene, is required to direct splicing of *Am-dsx* pre-mRNAs and its own *fem* transcripts (Gempe *et al.* 2009), the latter of which establishes a positive feedback loop of self-regulated *fem* female splicing (Pane *et al.* 2002; Gempe *et al.* 2009). Although substantially diverged in sequence, the *fem* gene and the *tra* gene of *D. melanogaster* are orthologs, whereas the *csd* gene was derived by gene duplication of an ancestral copy of the *fem* gene (Hasselmann *et al.* 2008). As Csd and Fem proteins harbor no RBD but have similar sex-determining and splice regulation functions as the Tra protein in *D. melanogaster* (Gempe *et al.* 2009), we proposed that *Am*-Tra2 is the RNA-binding cofactor that is essential for *fem* and *Am-dsx* splicing. To study the role of the *Am-tra2* gene in female splicing, we induced *Am-tra2* knockdown by RNAi in 0- to 3-hr-old female embryos. We injected two dsRNAs in which the first (dsRNA-1) targets the region expressing the RS1 and the RBD domains and the second (dsRNA-2) targets the entire RS1 domain (Figure S1). Both dsRNAs overlap in a small segment with no stretches of sequence identity over the small interfering RNA length of 20–22 bp to other genes in the honeybee genome.

No female embryo reached larval stage L1 when we injected ~200 pg of dsRNA-1 or dsRNA-2 per embryo (Table 1). This is an amount that is substantially below the ~900 pg of dsRNA per embryo that we repeatedly used in previous studies, in which we observed no lethal effect (Beye *et al.* 2003; Hasselmann *et al.* 2008). Phenotype data from null mutants suggest that the *tra2* gene in *D. melanogaster* is not essential for viability (Watanabe 1975; Fujihara *et al.* 1978). We further reduced the amount of *Am-tra2*-dsRNAs until we observed fully developed L1 female larvae (Table 1). At concentrations of 56 pg of dsRNA-2 and 4 pg of dsRNA-1 per embryo, we obtained the first viable L1 female larvae, but at a very low frequency (5% compared to 24% in our ddH_2O-treated controls). When we further reduced the dsRNA-2 concentration from 56 to 4 pg per embryo, the hatching rate still did not substantially improve. We studied whether the lethality is sex-specific and injected 96 pg of dsRNA-2 into the male embryos for comparison (Table 2). Also, no male embryos reached larval stage L1, suggesting that knockdown of *Am-tra2* caused some non-sex-specific lethality during embryogenesis. Because none of the hatched L1 female larvae reached the L4 stage (data not shown), we were not able to further study the role of *Am-tra2* in morphological sexual differentiation. Taken together, our knockdown results suggest that the *Am-tra2* gene is essential for embryogenesis in the honeybee.

We proposed that, in addition to a vital role for *Am-tra2* in embryogenesis, *Am-tra2* possibly has another function in sex determination, specifically in promoting female-specific splicing of the *fem* and *Am-dsx* transcripts. The knockdown of the *fem* and the *csd* genes, which regulate female splicing and sex determination, had no general effects on lethality, suggesting that the putative role of *Am-tra2* in activation of the female pathway did not cause the embryonic lethality (Beye *et al.* 2003; Gempe *et al.* 2009). To study the sex-determining role of the *Am-tra2* gene, we injected 4 or 33 pg of dsRNA-2 in embryos and after 77–80 hr studied the splice patterns, irrespective of whether the larvae hatched. If *Am-tra2* promotes female splicing, we expected that knockdown of this gene would induce male-like splice patterns in these females.

The injection of 4 pg of dsRNA-2 (Table 3) induced male splicing of *Am-dsx* mRNAs in females (Figure 4A, lanes 1–10), which is entirely absent in the control embryos (Figure 4A, lanes 21–30), which produce only the *Am-dsx* female splice product. This result suggests that the *Am-tra2* gene is essential to promote female splicing of *Am-dsx* transcripts. The female splicing of *fem* mRNAs is obviously not sizably

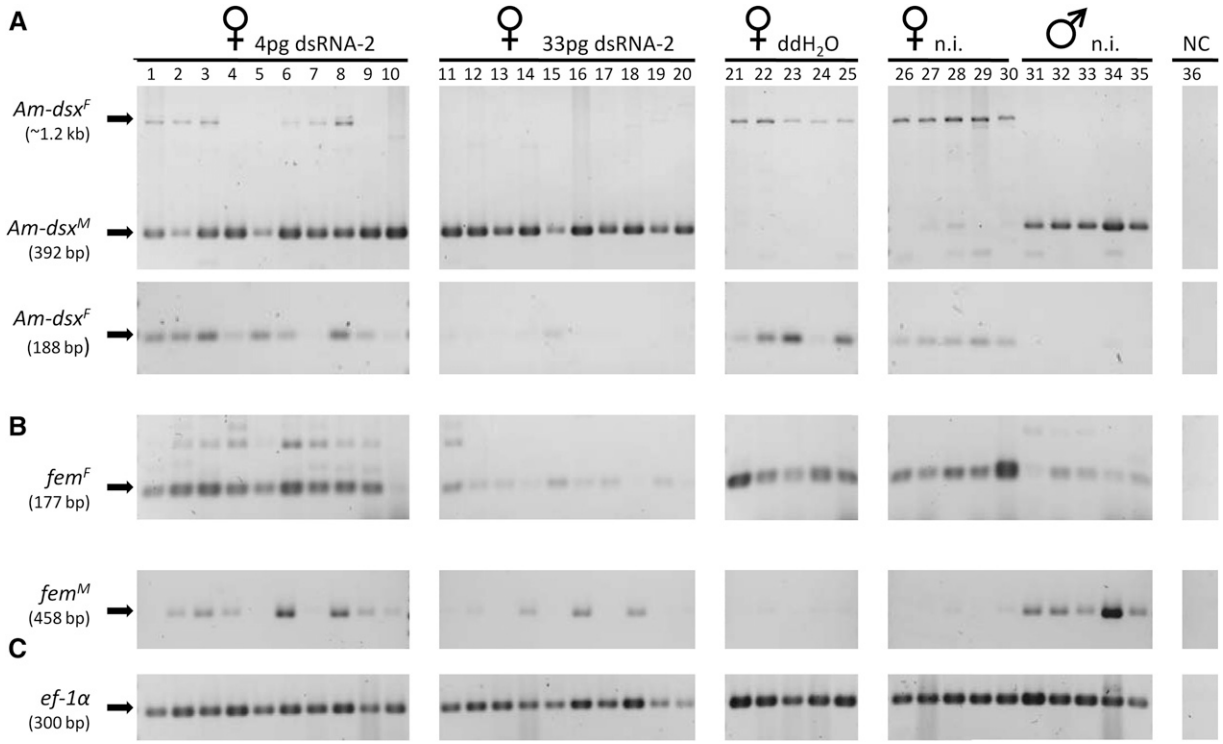

Figure 4 Sex-specific splicing of the *fem* and *Am-dsx* transcripts in *Am-tra2* dsRNA-2-treated embryos. The male and female *Am-dsx* (A) and *fem* (B) mRNAs of individuals 77–80 hr after egg laying were studied using semiquantitative RT-PCR. Early embryos were injected with 4 pg of *Am-tra2* dsRNA-2 (lanes 1–10), 33 pg of *Am-tra*2 dsRNA-2 (lanes 11–20), or ddH_20 (lanes 21–25). The untreated (labeled as "n.i.") female and male controls are shown in lanes 26–30 and 31–35, respectively. "NC" denotes our control PCR in which no cDNA was added (lane 36). Fragments corresponding to the *fem* female (size of 177 bp) and male (size of 458 bp) mRNAs and the *Am-dsx* female (size of 1.2 kb and 188 bp) and male (size of 392 bp) mRNAs were resolved by agarose gel electrophoresis and stained with ethidium bromide. We used amplification of the cDNAs of the gene *ef-1α* (C) as a relative control to semiquantify *Am-tra2* transcripts across embryonic samples.

affected as we observed comparable amounts of the corresponding female *fem* fragments in treated and nontreated female embryos in our semiquantitative PCR analysis (Figure 4B, lanes 1–10 and lanes 21–30).

The treatment of female embryos with the higher concentration of 33 pg of dsRNA-2 per embryo compromised female splicing of the *fem* mRNAs (Figure 4B, lanes 11–20) compared to our control embryos (Figure 4B, lanes 26–35; Table 3), indicating that *Am*-Tra2 protein is also required for *fem* female transcript splicing. To provide further evidence that the effect on splicing is specific for *dsx* and *fem* transcripts, we also assayed the splice products of our control gene *ef-1α* (Figure 4C) and that of the *csd* gene (Figure S3) in the dsRNA-treated and nontreated embryos. We detected in embryos that were treated with 4 or 33 pg *Am-tra2* dsRNA-2 the *csd* and the *ef-1α* exon spanning fragments of cDNA, suggesting that our treatment or the knockdown of *Am-tra2* has not generally affected the splice process or degradation of embryonic mRNAs. This latter result supports our notion that the *Am*-Tra2 protein is specifically involved in the sex-specific splice regulation of *Am-dsx* and *fem* transcripts.

In contrary to our expectation, the knockdown of the *Am-tra2* gene in females did not produce the alternative, male splice form of the *fem* transcripts. We consistently observed in the 33-pg-treated females the absence of the male *fem* transcript (Figure 4B, lanes 11–20). We thus studied in male (haploid) embryos that only produce the male splice variant the influence of the *Am-tra2* gene on male *fem* splicing. The injection of 33 and 67 pg dsRNA-2 repeatedly produced males that lacked the male *fem* transcript in 48-hr-old embryos (Figure S4, lanes 1–19) whereas the transcript of our control gene *ef-1α* was present. This result suggests a role of *Am*-Tra2 protein in splicing the *fem* pre-mRNAs into the male form.

Taken together, these results suggest that *Am*-Tra2 promotes female splicing of the productive female *fem* mRNAs and also of the nonproductive male *fem* mRNAs.

Discussion

Heteroallelic Csd proteins determine honeybee femaleness and set the downstream regulator of the sex determination cascade, *fem*, into the female mode by alternative splicing (Beye *et al.* 2003; Hasselmann *et al.* 2008). The Fem proteins in females maintain the female-determined state by promoting female splicing of the *fem* mRNAs (positive autoregulation) and direct female splicing of the *Am-dsx*

transcripts (Hasselmann *et al.* 2008; Gempe *et al.* 2009). In this study, we showed that the *Am-tra2* gene, an ortholog of the *tra2* gene of *D. melanogaster*, is a component of the honeybee sex determination hierarchy. The *Am-tra2* proteins are required to regulate female and male splicing of *fem* mRNAs and female splicing of the *Am-dsx* mRNAs. In addition, we showed that the *Am-tra2* gene has an essential role in embryogenesis that is not related to sex determination.

We characterized the *Am-tra2* gene in the honeybee and showed that the deduced *Am*-Tra2 proteins share the same domain structure as other Tra2 orthologs described thus far (Amrein *et al.* 1988; Bandziulis *et al.* 1989; Goralski *et al.* 1989; Mattox *et al.* 1996; Burghardt *et al.* 2005; Salvemini *et al.* 2009; Sarno *et al.* 2010). *Am*-Tra2 protein contains a RBD that is supposed to directly interact with the pre-mRNA and two flanking RS-rich domains that provide a potential surface for an interaction with other proteins, such as Tra proteins (Amrein *et al.* 1988; Hoshijima *et al.* 1991; Graveley 2000; Sciabica and Hertel 2006). We identified six splice variants of *Am-tra2* mRNAs that translate into proteins that differ in the length of the first RS domain and in the absence/presence of one amino acid (serine) in the second RS domain. The six splice variants are not sex-specifically regulated throughout development, suggesting that *Am*-Tra2 proteins are constitutively expressed. The *tra2* transcripts are present in early embryos before the primary signal *csd* is transcribed and thus before the primary decision of sexual fate is made, which is after blastoderm formation (Beye *et al.* 2003; Gempe *et al.* 2009). However, we observed that the level of *Am-tra2* transcription substantially decreases at the pupal stage, possibly at a stage when sexual signals of the primary sex determination cascade are less important. We also showed that the *Am-tra2* gene is not sex-specifically spliced in the gonadal tissues (Figure 3B). This finding is in contrast to the germline-specific control of *tra2* transcripts in males of *D. melanogaster*. Here, the Tra-2^{226} protein directs splicing of the *tra-*2^{179} transcript in the fruit fly germline, thereby regulating the level of Tra-2^{226} protein expression that is critical for proper sperm formation (McGuffin *et al.* 1998; Mattox *et al.* 1990).

When we repressed the *Am-tra2* gene by injecting 4-pg dsRNAs into early embryos, we observed that female splicing of *Am-dsx* switched into male splicing. This *Am-tra2* knockdown had no sizable effect on female splicing of *fem* transcripts, suggesting that the *Am-dsx* switch of splicing was not caused by affecting splice regulation of the upstream regulator *fem*. We also showed that the knockdown did not affect the splicing of control genes, the *csd*, and the *ef-1α* gene, suggesting that embryonic lethality has not compromised our testing. Taken together, these results suggest that the *Am-tra2* gene plays a role in the regulation of female *Am-dsx* mRNA splicing (Figure 5). Our result suggests a conserved role of the *Am*-Tra2 protein in *Am-dsx* regulation, although the canonical Tra/Tra2-binding motifs that have been reported in different dipteran insects are absent in the *Am-dsx* gene. This finding suggests that the Tra2

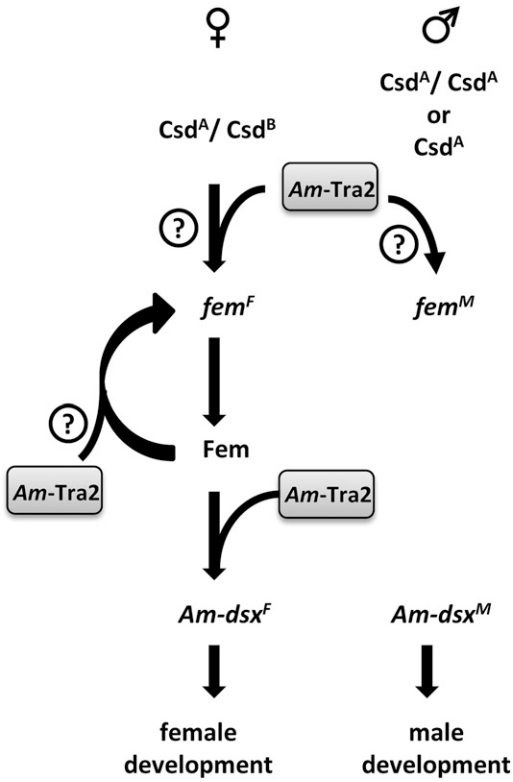

Figure 5 Model of the role of the *Am*-Tra2 protein in honeybee sex determination. *Am*-Tra2 protein is necessary for splicing of the productive female and *fem* and *Am-dsx* mRNAs. It is also required to splice the nonproductive male *fem* splice form. In females, the *Am*-Tra2 proteins either act together with heterozygous Csd proteins (Csd^A/Csd^B) and/or together with Fem proteins (*fem^F*-positive autoregulatory loop) to promote female processing of the *fem* pre-mRNAs (*fem^F*). *Am*-Tra2, together with with Fem proteins, directs female-specific splicing of the *Am-dsx* pre-mRNA (*Am-dsx^F*). In males, *Am*-Tra2 protein directs splicing of male *fem* mRNAs in the presence of inactive Csd proteins (that are homoallelic Csd^A/Csd^A or Csd^A proteins) and Fem proteins.

protein-binding sites have evolved. In *D. melanogaster*, Tra2, together with the Tra proteins, binds to six repeats of a 13-nucleotide motif [TC(T/A)(A/T)C(A/G)ATCAACA] on the *dsx* pre-mRNA and promotes the activation of the weak female splice acceptor that directs the production of the female *dsx* transcripts. In other dipteran species (*M. domestica*, *C. capitata*, *Bactrocera oleae*, *Bactrocera dorsalis*, *Bactrocera correcta*, *Bactrocera tyroni*, and different *Anastrepha* species), the canonical Tra/Tra2-binding motifs are consistently present in the *dsx* genes (Crampton *et al.* 1998; Hediger *et al.* 2004; Lagos *et al.* 2005; Ruiz *et al.* 2005, 2007; Saccone *et al.* 2008; Concha *et al.* 2010; Permpoon *et al.* 2011) and are proposed to be utilized in promoting female splicing (Burghardt *et al.* 2005; Salvemini *et al.* 2009; Sarno *et al.* 2010). We propose that the *Am*-Tra2 protein, like its ortholog in *D. melanogaster*, is an essential, constitutively expressed cofactor that, together with the female-specific Fem protein, directs the female processing of the *Am-dsx* transcript. The honeybees are a member of the hymenopteran insects and are at the base of the

phylogeny of holometabolous insects (Savard *et al.* 2006). The shared function in *dsx* regulation across the different insect orders thus suggests that the role of Tra2 proteins in regulating female *dsx* splicing is the ancestral state in holometabolous insects.

Consistent with an evolved binding site of the Fem/*Am*-Tra2 proteins, we identified several amino acid replacements in the RBD that affect the designated binding nucleotide sequence of the RNA. We found three amino acid sites in the RNP-1 sequence element that diverged with respect to the *D. melanogaster* sequence. The RNP-1 and RNP-2 sequence elements are part of the RBD, which has a βαββαβ barrel-like structure. The RNP sequence elements have exposed positions at the surface of the β-sheets β1 and β3 and are used to bind directly to the ribonucleotide sequence (Dreyfuss *et al.* 1988; Merrill *et al.* 1988; Nagai *et al.* 1990; Amrein *et al.* 1994). Mutation of the first amino acid arginine of the RNP-1 sequence element in *D. melanogaster* abolishes the female processing of *dsx* pre-mRNAs (Amrein *et al.* 1994). This critical arginine amino acid residue in the RNP-1 sequence element is replaced in the honeybee by a lysine. These findings support our conclusion that the corresponding Fem/*Am*-Tra2 protein-binding sites diverge from that of the fruit fly.

When we repressed the *Am-tra2* gene by injecting higher amounts of dsRNAs (33 pg) into female embryos, we also observed a reduction of the productive female and the nonproductive male *fem* splice variants. This amount of dsRNA did not affect splicing of the paralogous gene *csd* and the *ef-1α* gene, suggesting that embryonic lethality has not compromised our testing of splicing. We also confirmed that the *Am-tra2* gene is essential for the male splicing of *fem* mRNAs by knockdown of the *Am-tra2* gene in males. These results together indicate a function of the *Am-tra2* gene also on the level of splicing of the *fem* gene. We propose that the *Am*-Tra2 protein is a required cofactor of heteroallelic Csd proteins in females that mediates the binding and splicing of female *fem* pre-mRNAs (Figure 5). *Am*-Tra2 proteins may together with Fem proteins direct female splicing of the *fem* transcripts, which are maintaining the female determined state through development by a positive feedback loop (Figure 5). In males, where the active, heteroallelic Csd proteins and Fem proteins are absent, the *Am*-Tra2 proteins may enhance the switch of the *fem* transcripts into the nonproductive male form. In the absence of the *Am-tra2*-dependent splicing, the *fem* RNAs may undergo RNA decay machinery and are removed. We speculate that the dual function of the *Am-tra2* gene as a regulator in male and female splicing may enhance the binary decision when the female-specific proteins (Fem, heteroallelic Csd) are present or absent. The proper male and female regulation of *fem* splicing is important to implement and maintain the sexual fate as the analysis of gynandromorphs indicates that they were induced by knockdowns of the *fem* gene (Gempe *et al.* 2009).

The *fem* gene is an ortholog of the *tra* gene of dipteran insects. In *D. melanogaster*, Tra2 proteins are not deployed in regulating female splicing of *tra* transcripts. Female *tra* mRNA processing in this species is regulated by the Sxl proteins (Bell *et al.* 1988; Sosnowski *et al.* 1989; Bell *et al.* 1991; Valcárcel *et al.* 1993). In contrast, Tra2 proteins in the dipteran insects *M. domestica* and *C. capitata* are, as in the honeybee, required to splice *tra* transcripts into the female form (Burghardt *et al.* 2005; Salvemini *et al.* 2009; Hediger *et al.* 2010). In these dipteran species, Tra2 proteins are presumably regulators of an autoregulatory loop in females in which the maternally provided Tra proteins mediate female *tra* mRNAs. The presence of a male-determining factor, M, apparently impairs this *tra*-positive regulatory loop, resulting in male *tra* pre-mRNA splicing and male differentiation (Pane *et al.* 2002; Hediger *et al.* 2010). The role of *Am-tra2* gene in controlling the male splicing of the *fem* gene has not been reported in other insects so far.

Our knockdown studies in early embryos also suggest that the *Am-tra2* gene is essential and has a vital role in embryogenesis. We suggest that this role is independent of the sex determination process as no sex-specific lethality has been observed and the other components (*fem* and *csd*) regulating sex determination produce no lethal phenotype (Beye *et al.* 2003; Hasselmann *et al.* 2008; Gempe *et al.* 2009). We suggest that this lethal effect during embryogenesis is not caused by unspecific effects due to our dsRNA method as (i) different regions of the transcript with our dsRNAs were targeted, (ii) lethal effects with dsRNA concentrations were observed that were substantially below that of previous experiments (4–40 times) that showed no lethal embryonic effects (Beye *et al.* 2003; Hasselmann *et al.* 2008), and (iii) the viability of embryos did not further increase above ∼5% when we further decreased the dsRNA concentration by 1/10th (Tables 1 and 2). This additional role in embryogenesis is absent in other dipteran insects (Watanabe 1975; Fujihara *et al.* 1978; Burghardt *et al.* 2005; Salvemini *et al.* 2009), suggesting that this role evolved in insects.

Taken together, our results suggest that the *Am-tra2* gene is a non-sex-specifically expressed regulator that is essential for generating the productive female and nonproductive male *fem* transcripts. We propose that the *Am*-Tra2 protein acts together with heteroallelic Csd proteins and/or Fem proteins to mediate female *fem* splicing by binding to *fem* pre-mRNAs in accordance with the function of its ortholog in *D. melanogaster*. This predicted role, however, needs to be further tested in splice assays and protein-binding studies. The *Am-tra2* gene thus may have a central role in initiating the primary signal *csd* in females and in maintaining the female determined state by the positive regulatory loop at the level of the *fem* gene (Figure 5). In males with hemi- or homoallelic Csd proteins, *Am*-Tra2 may enhance the switch of *fem* transcripts into the nonproductive male form. The use of the *Am*-Tra2 proteins in male and female splicing may enhance and stabilize the male and female splicing state at the level of the *fem* gene. We also provided evidence that the *Am*-Tra2 protein is an essential regulator of female *Am*-

dsx splice regulation, a feature that is shared with other dipteran insects, suggesting an ancestral role in *dsx* splice regulation in holometabolous insects. In addition, the *Am-tra2* gene has an essential function in honeybee embryogenesis that is unrelated to sex determination and has thus far not been reported in other insects.

Acknowledgments

We thank two anonymous reviewers for helpful comments on the manuscript; Eva-Maria Theilenberg, Marion Mueller-Borg, Isabel Röös, and Jessica Langer for technical support; Dalibor Titera for providing bee crosses; and Michael Griese for beekeeping support. This work was supported by grants from the Deutsche Forschungsgemeinschaft.

Literature Cited

Amrein, H., M. Gorman, and R. Nöthiger, 1988 The sex-determining gene *tra-2* of *Drosophila* encodes a putative RNA binding protein. Cell 55: 1025–1035.

Amrein, H., T. Maniatis, and R. Nöthiger, 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., M. L. Hedley, and T. Maniatis, 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.

Bandziulis, R. J., M. S. Swanson, and G. Dreyfuss, 1989 RNA-binding proteins as developmental regulators. Genes Dev. 3: 431–437.

Bell, L. R., E. M. Maine, P. Schedl, and T. W. Cline, 1988 *Sex-lethal*, a *Drosophila* sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins. Cell 55: 1037–1046.

Bell, L. R., J. I. Horabin, P. Schedl, and T. W. Cline, 1991 Positive autoregulation of sex-lethal by alternative splicing maintains the female determined state in *Drosophila*. Cell 65: 229–239.

Beye, M., 2004 The dice of fate: the *csd* gene and how its allelic composition regulates sexual development in the honey bee, *Apis mellifera*. Bioessays 26: 1131–1139.

Beye, M., S. Härtel, A. Hagen, M. Hasselmann, and S. W. Omholt, 2002 Specific developmental gene silencing in the honey bee using a homeobox motif. Insect Mol. Biol. 11: 527–532.

Beye, M., M. Hasselmann, M. K. Fondrk, R. E. Page, and S. W. Omholt, 2003 The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. Cell 114: 419–429.

Burghardt, G., M. Hediger, C. Siegenthaler, M. Moser, A. Dübendorfer *et al.*, 2005 The *transformer2* gene in *Musca domestica* is required for selecting and maintaining the female pathway of development. Dev. Genes Evol. 215: 165–176.

Burtis, K. C., and B. S. Baker, 1989 *Drosophila doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell 56: 997–1010.

Cho, S., Z. Y. Huang, and J. Zhang, 2007 Sex-specific splicing of the honeybee *doublesex* gene reveals 300 million years of evolution at the bottom of the insect sex-determination pathway. Genetics 177: 1733–1741.

Cline, T. W., and B. J. Meyer, 1996 Vive la différence: males *vs.* females in flies *vs.* worms. Annu. Rev. Genet. 30: 637–702.

Concha, C., and M. J. Scott, 2009 Sexual development in *Lucilia cuprina* (Diptera, Calliphoridae) is controlled by the *transformer* gene. Genetics 182: 785–798.

Concha, C., F. Li, and M. J. Scott, 2010 Conservation and sex-specific splicing of the *doublesex* gene in the economically important pest species *Lucilia cuprina*. J. Genet. 89: 279–285.

Crampton, J. M., A. A. James, D. C. A. Shearman, and M. Frommer, 1998 The *Bactrocera tyroni* homologue of the *Drosophila melanogaster* sex-determination gene *doublesex*. Insect Mol. Biol. 7: 355–366.

Dearden, P. K., M. J. Wilson, L. Sablan, P. W. Osborne, M. Havler *et al.*, 2006 Patterns of conservation and change in honey bee developmental genes. Genome Res. 16: 1376–1384.

Dreyfuss, G., M. S. Swanson, and S. Pinol-Roma, 1988 Heterogeneous nuclear ribonucleoprotein particles and the pathway of mRNA formation. Trends Biochem. Sci. 13: 86–91.

Erickson, J. W., and J. J. Quintero, 2007 Indirect effects of ploidy suggest X chromosome dose, not the X:A ratio, signals sex in *Drosophila*. PLoS Biol. 5: e332.

Fujihara, T., M. Kawabe, and K. Oishi, 1978 A sex-transformation gene in *Drosophila*. J. Hered. 69: 229–236.

Gempe, T., and M. Beye, 2011 Function and evolution of sex determination mechanisms, genes and pathways in insects. Bioessays 33: 52–60.

Gempe, T., M. Hasselmann, M. Schiøtt, G. Hause, M. Otte *et al.*, 2009 Sex determination in honeybees: two separate mechanisms induce and maintain the female pathway. PLoS Biol. 7: e1000222.

Goralski, T. J., J.-E. Edstrom, and B. S. Baker, 1989 The sex determination locus *transformer-2* of *Drosophila* encodes a polypeptide with similarity to RNA binding proteins. Cell 56: 1011–1018.

Graveley, B. R., 2000 Sorting out the complexity of SR protein functions. RNA 6: 1197–1211.

Hasselmann, M., and M. Beye, 2004 Signatures of selection among sex-determining alleles of the honey bee. Proc. Natl. Acad. Sci. USA 101: 4888–4893.

Hasselmann, M., T. Gempe, M. Schiøtt, C. G. Nunes-Silva, M. Otte *et al.*, 2008 Evidence for the evolutionary nascence of a novel sex determination pathway in honeybees. Nature 454: 519–522.

Hazelrigg, T., and C. Tu, 1994 Sex-specific processing of the *Drosophila exuperantia* transcript is regulated in male germ cells by the *tra-2* gene. Proc. Natl. Acad. Sci. USA 91: 10752–10756.

Hediger, M., G. Burghardt, C. Siegenthaler, N. Buser, D. Hilfiker-Kleiner *et al.*, 2004 Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator *doublesex*. Dev. Genes Evol. 214: 29–42.

Hediger, M., C. Henggeler, N. Meier, R. Perez, G. Saccone *et al.*, 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 T. Maniatis, 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.

Hoshijima, K., K. Inoue, I. Higuchi, H. Sakamoto, and Y. Shimura, 1991 Control of *Doublesex* alternative splicing by transformer and transformer-2 in *Drosophila*. Science 252: 833–836.

Inoue, K., K. Hoshijima, I. Higuchi, H. Sakamoto, and Y. Shimura, 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.

Kamakura, M., 2011 Royalactin induces queen differentiation in honeybees. Nature 473: 478–483.

Kucharski, R., J. Maleszka, S. Foret, and R. Maleszka, 2008 Nutritional control of reproductive status in honeybees via DNA methylation. Science 319: 1827–1830.

Lagos, D., M. F. Ruiz, L. Sánchez, and K. Komitopoulou, 2005 Isolation and characterization of the *Bactrocera oleae* genes orthologous to the sex determining *Sex-lethal* and *doublesex* genes of *Drosophila melanogaster*. Gene 348: 111–121.

Lynch, K. W., and T. Maniatis, 1995 Synergistic interactions between two distinct elements of a regulated splicing enhancer. Genes Dev. 9: 284–293.

Lynch, K. W., and T. Maniatis, 1996 Assembly of specific SR protein complexes on distinct regulatory elements of the *Drosophila doublesex* splicing enhancer. Genes Dev. 10: 2089–2101.

Madigan, S. J., P. Edeen, J. Esnayra, and M. McKeown, 1996 *att*, a target for regulation by tra2 in the testes of *Drosophila melanogaster*, encodes alternative RNAs and alternative proteins. Mol. Cell. Biol. 16: 4222–4230.

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

Mattox, W., and B. S. Baker, 1991 Autoregulation of the splicing of transcripts from the *transformer-2* gene of *Drosophila*. Genes Dev. 5: 786–796.

Mattox, W., M. J. Palmer, and B. S. Baker, 1990 Alternative splicing of the sex determination gene *transformer-2* is sex-specific in the germ line but not in the soma. Genes Dev. 4: 789–805.

Mattox, W., M. E. McGuffin, and B. S. Baker, 1996 A negative feedback mechanism revealed by functional analysis of the alternative isoforms of the *Drosophila* splicing regulator *transformer-2*. Genetics 143: 303–314.

McGuffin, M. E., D. Chandler, D. Somaiya, B. Dauwalder, and W. Mattox, 1998 Autoregulation of *transformer-2* alternative splicing is necessary for normal male fertility in *Drosophila*. Genetics 149: 1477–1486.

Merrill, B. M., K. L. Stone, F. Cobianchi, S. H. Wilson, and K. R. Williams, 1988 Phenylalanines that are conserved among several RNA-binding proteins form part of a nucleic acid-binding pocket in the A1 heterogeneous nuclear ribonucleoprotein. J. Biol. Chem. 263: 3307–3313.

Nagai, K., C. Oubridge, T. H. Jessen, J. Li, and P. R. Evans, 1990 Crystal structure of the RNA-binding domain of the U1 small nuclear ribonucleoprotein A. Nature 348: 515–520.

Niu, B.-L., Z.-Q. Meng, Y.-Z. Tao, S.-L. Lu, H.-B. Weng *et al.*, 2005 Cloning and alternative splicing analysis of *Bombyx mori Transformer-2* gene using silkworm EST database. Acta Biochim. Biophys. Sin. (Shanghai) 37: 728–736.

Nygaard, S., G. Zhang, M. Schiøtt, C. Li, Y. Wurm *et al.*, 2011 The genome of the leaf-cutting ant *Acromyrmex echinatior* suggests key adaptations to advanced social life and fungus farming. Genome Res. 21: 1339–1348.

Pane, A., M. Salvemini, P. Delli Bovi, C. Polito, and G. Saccone, 2002 The *transformer* gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. Development 129: 3715–3725.

Permpoon, R., N. Aketarawong, and S. Thanaphum, 2011 Isolation and characterization of *Doublesex* homologues in the *Bactrocera* species: *B. dorsalis* (Hendel) and *B. correcta* (Bezzi) and their putative promoter regulatory regions. Genetica 139: 113–127.

Ruiz, M. F., R. N. Stefani, R. O. Mascarenhas, A. L. P. Perondini, D. Selivon *et al.*, 2005 The gene *doublesex* of the fruit fly *Anastrepha obliqua* (Diptera, Tephritidae). Genetics 171: 849–854.

Ruiz, M. F., J. M. Eirín-López, R. N. Stefani, A. L. P. Perondini, D. Selivon *et al.*, 2007 The gene *doublesex* of *Anastrepha* fruit flies (Diptera, Tephritidae) and its evolution in insects. Dev. Genes Evol. 217: 725–731.

Saccone, G., M. Salvemini, A. Pane, and L. C. Polito, 2008 Masculinization of XX *Drosophila* transgenic flies expressing the *Ceratitis capitata* DoublesexM isoform. Int. J. Dev. Biol. 52: 1051–1057.

Salvemini, M., M. Robertson, B. Aronson, P. Atkinson, L. C. Polito *et al.*, 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.

Sarno, F., M. F. Ruiz, J. M. Eirín-López, A. L. P. Perondini, D. Selivon *et al.*, 2010 The gene *transformer-2* of *Anastrepha* fruit flies (Diptera, Tephritidae) and its evolution in insects. BMC Evol. Biol. 10: 140.

Savard, J., D. Tautz, S. Richards, G. M. Weinstock, R. A. Gibbs *et al.*, 2006 Phylogenomic analysis reveals bees and wasps (Hymenoptera) at the base of the radiation of Holometabolous insects. Genome Res. 16: 1334–1338.

Schetelig, M. F., A. Milano, G. Saccone, and A. M. Handler, 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 K. J. Hertel, 2006 The splicing regulators Tra and Tra2 are unusually potent activators of pre-mRNA splicing. Nucleic Acids Res. 34: 6612–6620.

Sosnowski, B. A., J. M. Belote, and M. McKeown, 1989 Sex-specific alternative splicing of RNA from the *transformer* gene results from sequence-dependent splice site blockage. Cell 58: 449–459.

Suzuki, M. G., F. Ohbayashi, K. Mita, and T. Shimada, 2001 The mechanism of sex-specific splicing at the *doublesex* gene is different between *Drosophila melanogaster* and *Bombyx mori*. Insect Biochem. Mol. Biol. 31: 1201–1211.

Suzuki, M. G., S. Imanishi, N. Dohmae, T. Nishimura, T. Shimada *et al.*, 2008 Establishment of a novel *in vivo* sex-specific splicing assay system to identify a trans-acting factor that negatively regulates splicing of *Bombyx mori dsx* female exons. Mol. Cell. Biol. 28: 333–343.

Suzuki, M. G., S. Imanishi, N. Dohmae, M. Asanuma, and S. Matsumoto, 2010 Identification of a male-specific RNA binding protein that regulates sex-specific splicing of *Bmdsx* by increasing RNA binding activity of *Bm*PSI. Mol. Cell. Biol. 30: 5776–5786.

Tribolium Genome Sequencing Consortium, 2008 The genome of the model beetle and pest *Tribolium castaneum*. Nature 452: 949–955.

Valcárcel, J., R. Singh, P. D. Zamore, and M. R. Green, 1993 The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of *transformer* pre-mRNA. Nature 362: 171–175.

Watanabe, T. K., 1975 A new sex-transforming gene on the second chromosome of *Drosophila melanogaster*. Jpn. J. Genet. 50(3): 269–271.

Woyke, J., 1963 Drone larvae from fertilized eggs of the honeybee. J. Apic. Res. 2: 19–24.

Communicating editor: T. Schupbach

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.112.143925/-/DC1

The *Am-tra2* Gene Is an Essential Regulator of Female Splice Regulation at Two Levels of the Sex Determination Hierarchy of the Honeybee

Inga Nissen, Miriam Müller, and Martin Beye

DOI: 10.1534/genetics.112.143925

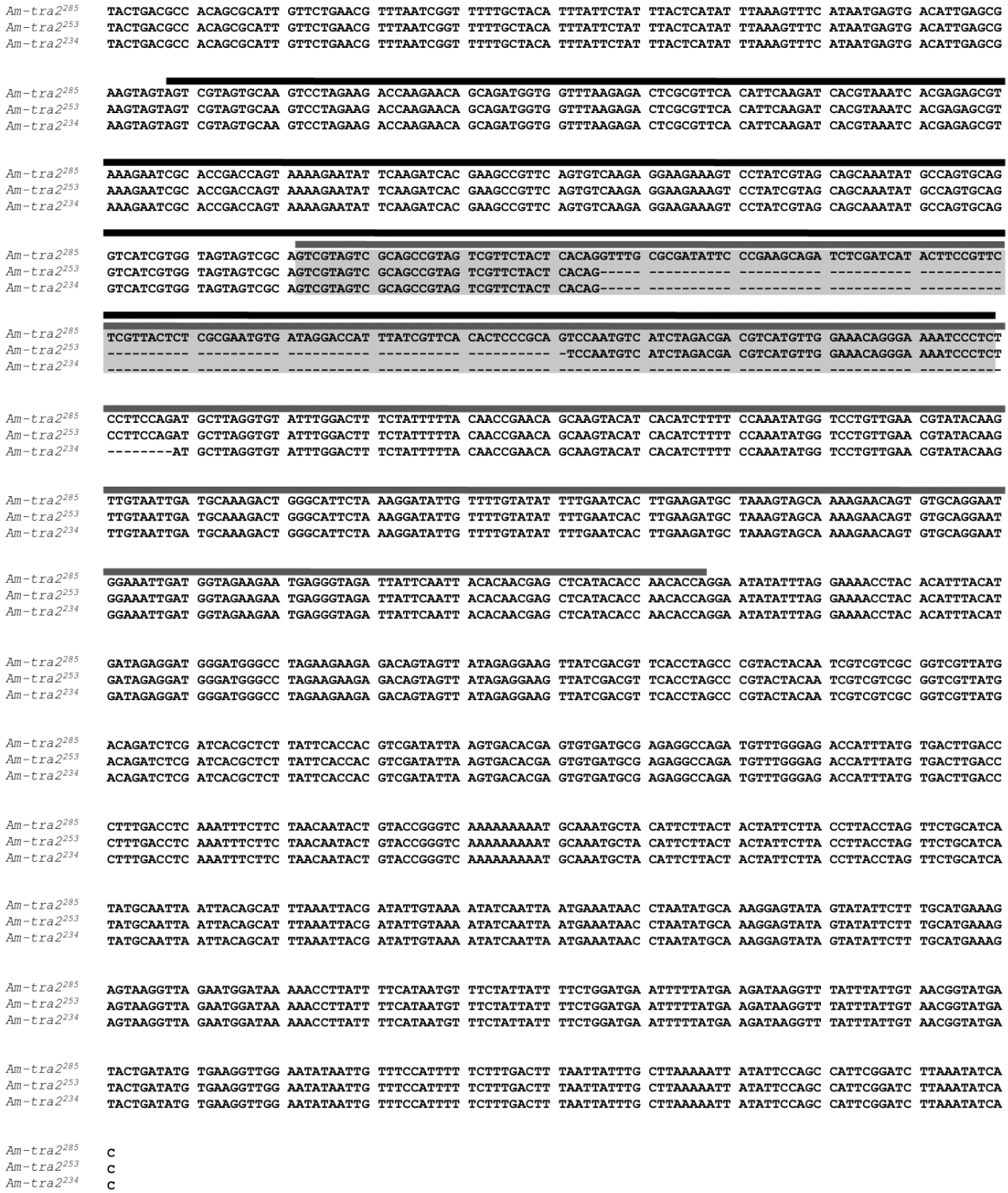

Figure S1 Multiple Nucleotide Sequence Alignment of the Am-tra2 cDNAs *Am-tra2*^{285}, *Am-tra2*^{253}, *Am-tra2*^{234}. Black (dsRNA-2) and grey (dsRNA-1) boxes mark the region that we used to produce our dsRNAs.

Figure S2 Multiple Sequence Alignment of the Tra2 proteins of *Apis mellifera (A.m.), Drosophila melanogaster (D.m.)* and *Bombyx mori (B.m)*. The RNA binding domain (RBD) is highlighted in black, and the linker region is highlighted in dark grey. The arginine-serine rich motifs (RS1 and RS2) are shown in light grey. The RNP-1 and RNP-2 sequence elements are marked by black bars.

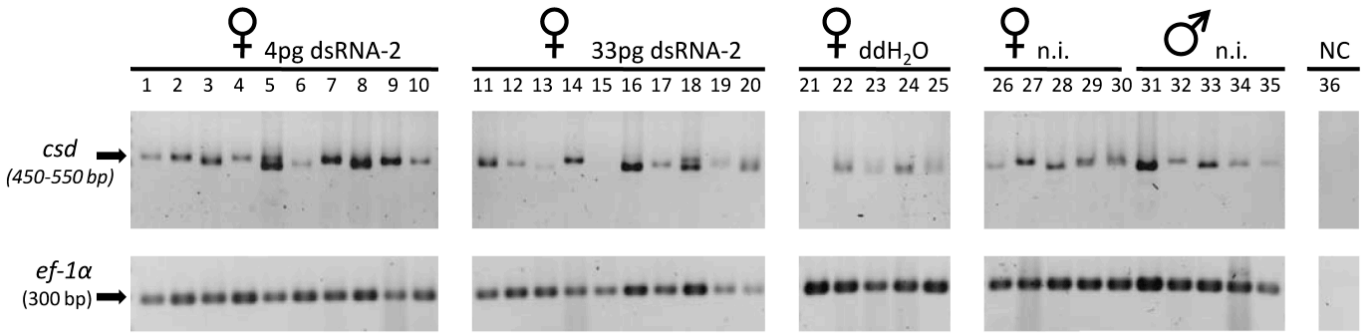

Figure S3 Constitutive splicing of *csd* transcripts in *Am-tra2* dsRNA-2 treated embryos. The *csd* mRNAs of individuals 77-80 hours after egg-laying were studied using semiquantitative RT-PCR. Early embryos were injected with 4 pg of *Am-tra2* dsRNA-2 (lanes 1-10), 33 pg of *Am-tra*2 dsRNA-2 (lanes 11-20) or ddH_20 (lanes 21-25). The untreated female and male controls (labeled as n.i.) are shown in lanes 26-30 and 31-35, respectively. NC denotes our control PCR in which no cDNA was added (lane 36). Fragments corresponding to the *csd* transcripts including the hypervariable region (size of ~450-550 bp) were resolved by agarose gel electrophoresis and stained with ethidium bromide. The size of *csd* fragments varies due to length differences of the hypervariable that substantially varies between *csd* alleles. Because *csd* alleles can vary substantially in the nucleotide sequence (10-15%) we cannot amplify to the same extend all the *csd* alleles. We amplified cDNAs of the gene *elongation factor 1α* (ef-1α) as a relative control to semiquantify *csd* transcripts across embryonic samples.

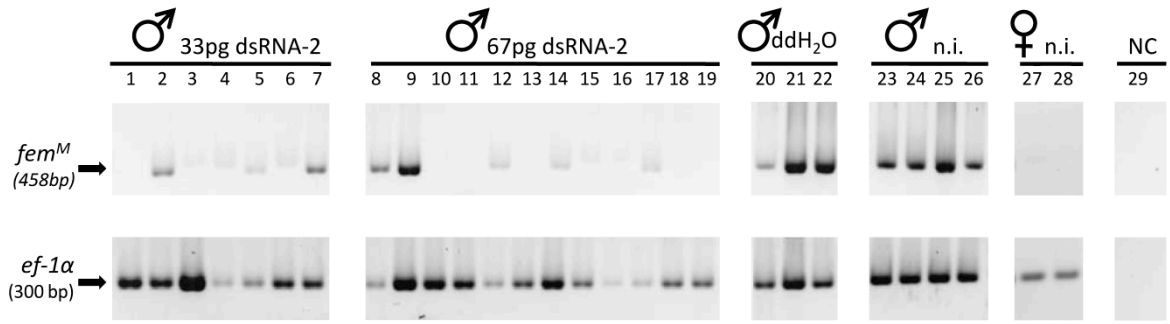

Figure S4 *fem* transcripts in male embryos treated with *Am-tra2* dsRNA-2. The male *fem* mRNAs of individuals 48 hours after egg-laying were studied using semiquantitative RT-PCR. Early embryos were injected with 33 pg of *Am-tra2* dsRNA-2 (lanes 1-7), 67 pg of *Am-tra*2 dsRNA-2 (lanes 8-19) or ddH_20 (lanes 20-22). The untreated male and female controls (labeled as n.i.) are shown in lanes 23-26 and 27-28, respectively. NC denotes our control PCR in which no cDNA was added (lane 29). Fragments were resolved by agarose gel electrophoresis and stained with ethidium bromide. The corresponding fragment of the male *fem* transcripts has a size of 458 bp. We used amplification of the cDNAs of the gene *elongation factor 1α* (ef-1α) as a relative control to semiquantify *csd* transcripts across embryonic samples.

Table S1 Sequences of oligonucleotides that were used

to synthesize dsRNA	
#22M: tra-2_ds_FOR	TAATACGACTCACTATAGGGCGAAGTCGTAGTCGCAGCCGTAGTCGTT
#23M: tra-2_ds_REV	TAATACGACTCACTATAGGGCGACTGGTGTTGGTGTATGAGCTCGTTG
#591	TAATACGACTCACTATAGGGAGTCGTAGTGCAAGTCCTAGAAGACC
#592	TAATACGACTCACTATAGGGATTTTCCCTGTTTCCAACATGAC
to analyze *Am-dsx* splicing	
#417	CTATTGGAGCACAGTAGCAAACTTG
#418	GGCTACGTATGTTTAGGAGGACC
#419	GAAACAATTTTGTTCAAAATAGAATTCC
to analyze *fem* splicing	
#412	CTGATTTTTCAATATTTACAGCTAAAACTGTAC
#523	CAACATCTGATGAACTTAAACGG
#410	TGAAGTTAATAACATATTTTTAATTCATCAATGAAG
#566	TGTACCATCTGAAGATTCTAATTTTTCG
to amplify *elongation factor-1α*	
#EM033	CGTTCGTACCGATCTCCGGATG
#EM034	GCTGCTGGAGCGAATGTTAC
in 5'RACE experiments	
5'RACE OLLI:	TGAACGGCTTCGTG
in 3'RACE experiments	
#33M (3'RACE J1 OUTER)	ACT CTC GCG AAT GTG ATA GGA CCA T
#34M (3'RACE J2 INNER)	TCA CAC TCC CGC AGT CCA ATG TCA T
3'RACE OLLI	AGAACAGTGTGCAG
to clone full ORF of *Am-tra2*	
#359	GATCGGATCCATGAGTGACATTGAGCGAAGTAGTAG
#421	TGACACGCGTTTAATATCGACGTGGTGAATAAGAGC
to amplify *csd* transcripts	
#CS-1	ATGAAAAAGAAAAACTTTTAGAAGAAAGAAC
#CS-2	TAAAATTTTTATAGTTTTCATTGATGCGTAG

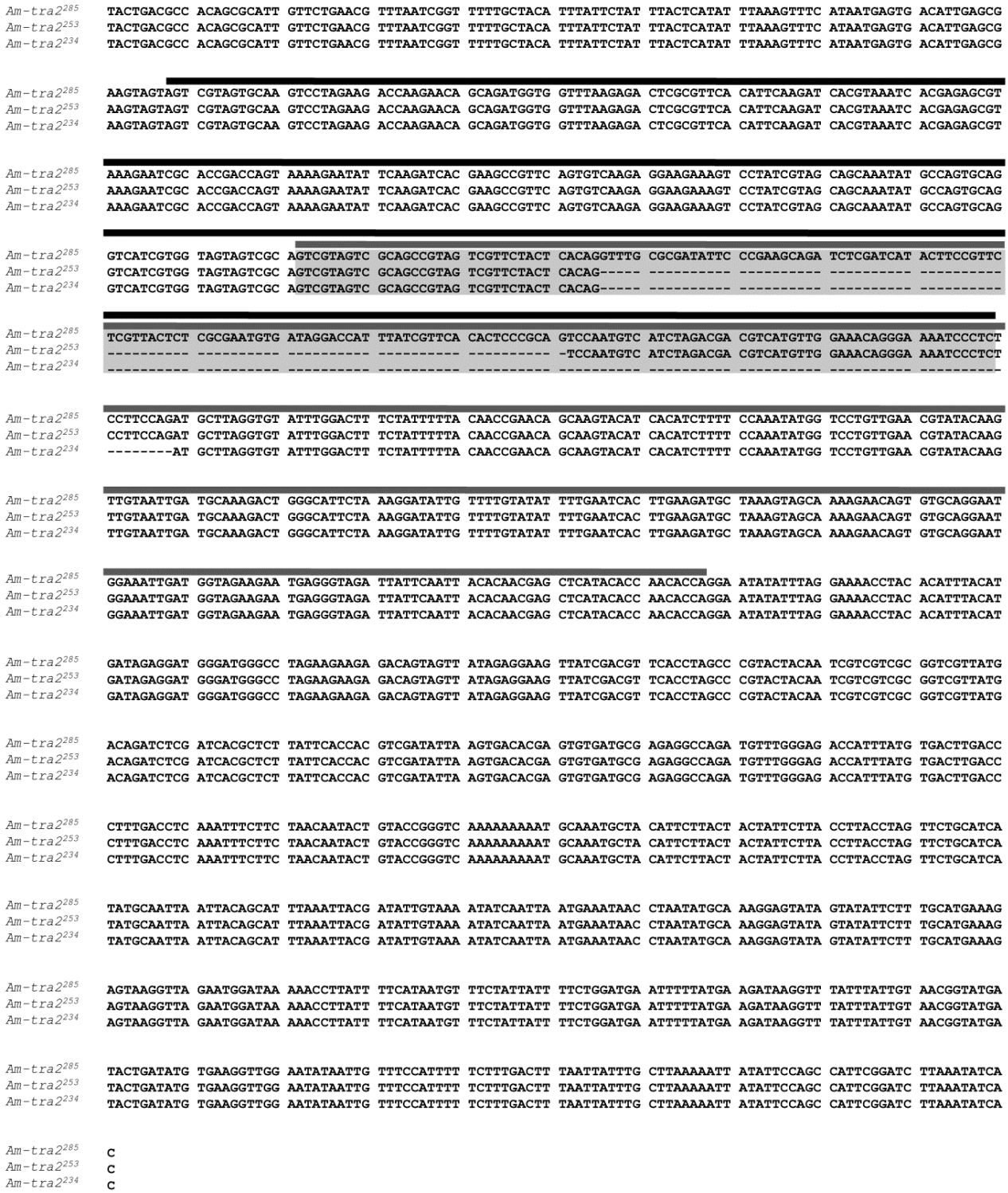

Figure S1 Multiple Nucleotide Sequence Alignment of the Am-tra2 cDNAs *Am-tra2*^{285}, *Am-tra2*^{253}, *Am-tra2*^{234}. Black (dsRNA-2) and grey (dsRNA-1) boxes mark the region that we used to produce our dsRNAs.

Figure S2 Multiple Sequence Alignment of the Tra2 proteins of *Apis mellifera (A.m.), Drosophila melanogaster (D.m.)* and *Bombyx mori (B.m)*. The RNA binding domain (RBD) is highlighted in black, and the linker region is highlighted in dark grey. The arginine-serine rich motifs (RS1 and RS2) are shown in light grey. The RNP-1 and RNP-2 sequence elements are marked by black bars.

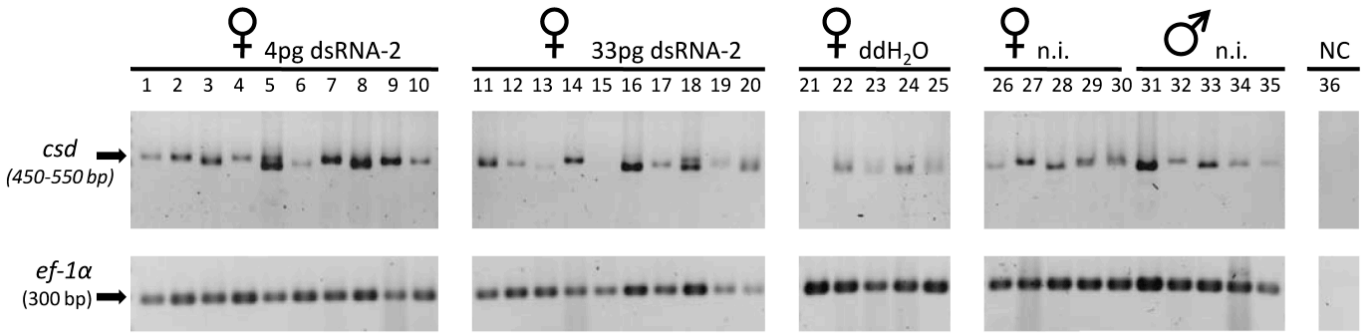

Figure S3 Constitutive splicing of *csd* transcripts in *Am-tra2* dsRNA-2 treated embryos. The *csd* mRNAs of individuals 77-80 hours after egg-laying were studied using semiquantitative RT-PCR. Early embryos were injected with 4 pg of *Am-tra2* dsRNA-2 (lanes 1-10), 33 pg of *Am-tra*2 dsRNA-2 (lanes 11-20) or ddH_20 (lanes 21-25). The untreated female and male controls (labeled as n.i.) are shown in lanes 26-30 and 31-35, respectively. NC denotes our control PCR in which no cDNA was added (lane 36). Fragments corresponding to the *csd* transcripts including the hypervariable region (size of ~450-550 bp) were resolved by agarose gel electrophoresis and stained with ethidium bromide. The size of *csd* fragments varies due to length differences of the hypervariable that substantially varies between *csd* alleles. Because *csd* alleles can vary substantially in the nucleotide sequence (10-15%) we cannot amplify to the same extend all the *csd* alleles. We amplified cDNAs of the gene *elongation factor 1α* (ef-1α) as a relative control to semiquantify *csd* transcripts across embryonic samples.

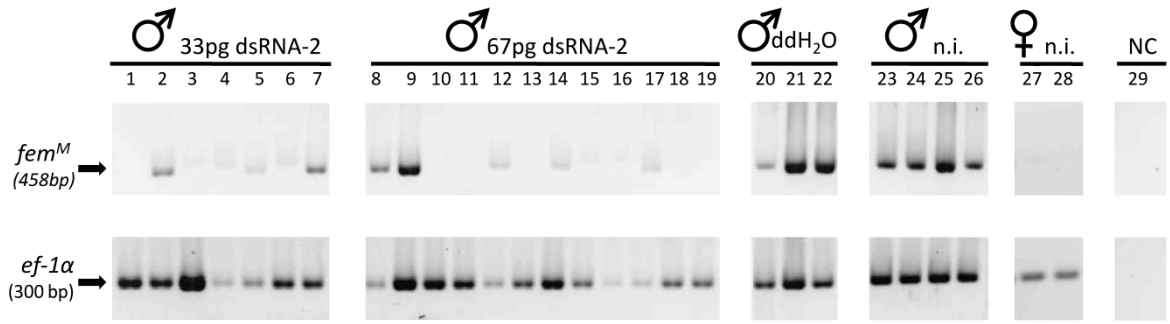

Figure S4 *fem* transcripts in male embryos treated with *Am-tra2* dsRNA-2. The male *fem* mRNAs of individuals 48 hours after egg-laying were studied using semiquantitative RT-PCR. Early embryos were injected with 33 pg of *Am-tra2* dsRNA-2 (lanes 1-7), 67 pg of *Am-tra*2 dsRNA-2 (lanes 8-19) or ddH_20 (lanes 20-22). The untreated male and female controls (labeled as n.i.) are shown in lanes 23-26 and 27-28, respectively. NC denotes our control PCR in which no cDNA was added (lane 29). Fragments were resolved by agarose gel electrophoresis and stained with ethidium bromide. The corresponding fragment of the male *fem* transcripts has a size of 458 bp. We used amplification of the cDNAs of the gene *elongation factor 1α* (ef-1α) as a relative control to semiquantify *csd* transcripts across embryonic samples.

Table S1 Sequences of oligonucleotides that were used

to synthesize dsRNA	
#22M: tra-2_ds_FOR	TAATACGACTCACTATAGGGCGAAGTCGTAGTCGCAGCCGTAGTCGTT
#23M: tra-2_ds_REV	TAATACGACTCACTATAGGGCGACTGGTGTTGGTGTATGAGCTCGTTG
#591	TAATACGACTCACTATAGGGAGTCGTAGTGCAAGTCCTAGAAGACC
#592	TAATACGACTCACTATAGGGATTTTCCCTGTTTCCAACATGAC
to analyze *Am-dsx* splicing	
#417	CTATTGGAGCACAGTAGCAAACTTG
#418	GGCTACGTATGTTTAGGAGGACC
#419	GAAACAATTTTGTTCAAAATAGAATTCC
to analyze *fem* splicing	
#412	CTGATTTTTCAATATTTACAGCTAAAACTGTAC
#523	CAACATCTGATGAACTTAAACGG
#410	TGAAGTTAATAACATATTTTTAATTCATCAATGAAG
#566	TGTACCATCTGAAGATTCTAATTTTTCG
to amplify *elongation factor-1α*	
#EM033	CGTTCGTACCGATCTCCGGATG
#EM034	GCTGCTGGAGCGAATGTTAC
in 5'RACE experiments	
5'RACE OLLI:	TGAACGGCTTCGTG
in 3'RACE experiments	
#33M (3'RACE J1 OUTER)	ACT CTC GCG AAT GTG ATA GGA CCA T
#34M (3'RACE J2 INNER)	TCA CAC TCC CGC AGT CCA ATG TCA T
3'RACE OLLI	AGAACAGTGTGCAG
to clone full ORF of *Am-tra2*	
#359	GATCGGATCCATGAGTGACATTGAGCGAAGTAGTAG
#421	TGACACGCGTTTAATATCGACGTGGTGAATAAGAGC
to amplify *csd* transcripts	
#CS-1	ATGAAAAAGAAAAACTTTTAGAAGAAAGAAC
#CS-2	TAAAATTTTTATAGTTTTCATTGATGCGTAG