The *Am-tra2* gene is an essential regulator of female splice regulation at two levels of the sex determination hierarchy of the honeybee.
Running Title:

The function of tra2 in *honeybees*

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**Abstract**

Heteroallelic and homo- or hemiallelic Csd (Complementary sex determiner) 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* 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 RBD (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 non-productive 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 non-productive 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 suggest 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.
Introduction

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 either differentiate into 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) which differ at an average of approximately 6% of their amino acid residues in the putative specifying domain (PSD) (Hasselmann et al., 2008; Hasselmann and Beye, 2004). Females express heteroallelic Csd proteins that direct female splicing of *feminizer* (*fem*) 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 *Apis mellifera dsex* (*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 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 (OD2) (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; Inoue et al., 1992; Hedley and Maniatis, 1991; Lynch and Maniatis, 1996, 1995; Sciabica and Hertel, 2006; Amrein et al., 1990). D. melanogaster females express in somatic tissues two major protein isoforms of the Tra2 proteins (Tra2264 and Tra2226) 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 (PRE). This activation leads to the inclusion of the female exon 4 in female dsx mRNAs (Burtis and Baker, 1989; Inoue et al., 1992; Hedley and Maniatis, 1991; 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; Salvemini et al., 2009; Sarno et al., 2010; Concha and Scott, 2009; 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 βαβαβαβ 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, that are directly involved in RNA recognition (Amrein et al., 1994; Nagai et al., 1990; Dreyfuss et al., 1988).

In the dipteran insects M. domestica, C. capitata, A. suspensa and A. obliqua, RNAi knockdown studies of the tra2 gene showed that Tra2 proteins are also involved in female splicing of tra mRNAs (Burghardt et al., 2005; Salvemini et al., 2009; Sarno et al., 2010; Concha and Scott, 2009; 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 (Hediger et al., 2010; Salvemini et al., 2009; 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., 2010, 2001, 2008).

In the male germline of D. melanogaster, the Tra2\textsuperscript{226} protein isoform has an additional function in spermatogenesis in controlling the splicing of the exuperantia (exu) and alternative-testes-transcript (att) transcripts (Mattox et al., 1996; Hazelrigg and Tu, 1994; Madigan et al., 1996). In the testes, Tra2\textsuperscript{226} proteins negatively affect their own expression by promoting the splicing of tra\textsuperscript{2179} mRNAs, which produce no functional protein (Mattox et al., 1996; McGuffin et al., 1998; Mattox and Baker, 1991). 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 CO₂ treatments of queens.

RNA extraction, cDNA synthesis and PCR

Total RNA was extracted using the TRIzol protocol (GIBCO BRL Life Technologies, Darmstadt, Germany). The first-strand 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 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 elongation factor 1α (ef-1α) gene using oligonucleotides #EM033 and #EM034 for the semiquantitative studies across samples (supplementary data, table S1).

Characterization of Am-tra2 gene

To determine the entire sequences of the Am-tra2 transcripts, we performed 5’ and 3’ rapid amplification of cDNA ends (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) (supplementary data, 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*), JQ518314 (*Am-tra2*), JQ518312 (*Am-tra2*), JQ518314 (*Am-tra2*), JQ518313 (*Am-tra2*), JQ518316 (*Am-tra2*).

**Transcriptional studies of the *Am-tra2* gene throughout development**

Total RNA was extracted from male and female eggs (0-6 h, 9-24 h, 33-48 h, 72 h), 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 (supplementary data, 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 h after egg deposition) in females and males (Beye et al., 2003, 2002). *Am-tra2* dsRNA-1, encompassing the region from 322 to 767 bp (446 bp long), was generated using oligonucleotides #22M and #23M (supplementary data, table S1) from cloned cDNAs of the *Am-tra2* transcript.
following the protocol previously described (Hasselmann et al., 2008; Beye et al., 2003, 2002). *Am-tra2* dsRNA-2, encompassing the region from 108 to 499 bp (392 bp long), was generated using oligonucleotides #591 and #592 (supplementary data, table S1, figure S1) from cloned cDNAs of transcript *Am-tra2*. The dsRNAs were dissolved in ddH2O and injected at a concentration of 4-200 pg per embryo (Table 1). In the control samples, we only injected ddH2O (Roth, Karlsruhe, Germany).

We counted the number of embryos showing normal development approximately 70 hours after egg deposition and the number of hatched L1 larvae 77-80 hours after egg deposition. All embryos that were malformed, showed necrotic tissue or lack the segmentation pattern 70 hours 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 non-treated samples 48 or 77-80 hours after egg deposition. Treated embryos and controls were reared in the incubator at 35°C until an age of 48 or 77-80 hours or until reaching the L1 larval stage. The samples were directly frozen in liquid nitrogen.

Fragments corresponding to the female *fem* mRNAs were amplified using oligonucleotides #412 and #523 (supplementary data, table S1) that are composed of a part of exon 3 and exon 6 (size, 177 bp). We amplified fragments (size, 458 bp; exon 3-4 and part of exon 5) corresponding to the male *fem* mRNAs using oligonucleotides #410 and #566 (supplementary data, table S1). Because the predetermined state in the early embryos is male splicing of *fem* transcripts (Gempe 2009) the presence of female-specific fragments is indicative that female splicing is induced. We used oligonucleotides #417 and #418 (supplementary data, 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 & #419 (supplementary data, table S1) (size,
188 bp) that encompass exon 4-5. Fragments corresponding to the csd mRNAs were amplified using oligonucleotides #CS-1 and #CS-2 (supplementary data, table S1) that 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), Drosophila melanogaster (NCBI Reference Sequence: NP_476764.1), Bombyx mori (GenBank: NP_001119705.1), Nasonia vitripennis (GenBank: XP_001601106.1), Sciara ocellaris (GenBank: CBX45935.1), Ceratitis capitata (GenBank: ACC68674.1), Musca 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 (Fig. 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 open reading frame (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 (Fig. 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} (Fig. 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) (Fig. 1 A-C). The RBD amino acid sequence has the strongest similarity (61 to 85% sequence identity) to the Tra2 proteins of a variety of insects (*Nasonia vitripennis, Tribolium castaneum, Acromyrmex echinatior, B. mori, Sciara ocellaris, D. melanogaster, M. domestica, C. capitata* and *A. obliqua*) (Burghardt et al., 2005; Salvemini et al., 2009; Sarno et al., 2010; Amrein et al., 1990; Mattox et al., 1996; Niu et al., 2005; The Tribolium Genome Sequencing Consortium, 2008; Nygaard et al., 2011), supporting our notion that we have identified the Tra2 ortholog of the honeybee (Fig. 2).

The largest transcript, Am-tra2^{285}, consists of 1401 nt and 5 exons and harbors an ORF coding for 285 amino acids (Fig. 1A). The other two major transcript variants (Am-tra2^{253} and...
Am-tra2\textsuperscript{234}) consist of 6 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 nt and 1305 nt long and are putatively translated into 253- and 234-amino-acid proteins, respectively (Fig. 1B and C). All three major splice variants have minor splice variants that utilize an alternative splice acceptor site at exon 4, producing single-serine amino acid deletion in the RS2 domain of the putative protein (Fig. 1D).

\textit{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 (\textit{D. melanogaster}, \textit{M. domestica}, \textit{S. ocellaris} and \textit{A. obliqua}), coleopteran (\textit{T. castaneum}), lepidopteran (\textit{B. mori}) and other hymenopteran (\textit{N. vitripennis} (wasp) and \textit{A. echinatior} (ant)) insects to identify shared structural features of Tra2 proteins in holometabolous insects. We were only able to unambiguously align the amino acid sequences for the RBD and neighboring regions, but not for other parts of the protein (supplementary data, Fig. 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 (supplementary data, Fig. S2), suggesting that these domains are faster-evolving and evolutionarily less constrained than the RBD.

In \textit{D. melanogaster}, the RBD domain of Tra2 protein binds to ESEs, which is comprised of six nearly identical 13-nucleotide-long sequences, the \textit{dsx} repeat elements (RE). Similar motifs have also been detected in the \textit{dsx} gene sequences of other dipteran insects, but are lacking in the \textit{Am-dsx} pre-mRNAs of the honeybee (Hediger et al., 2004; Ruiz et al., 2005; Saccone et al., 2008; Ruiz et al., 2007; Lagos et al., 2005; Permpoon et al., 2011; Concha et al., 2010; Crampton et al., 1998; Cho et al., 2007). We next studied the sequence...
similarities within the RBDs of Tra2 proteins of different holometabolous insects (Fig. 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 to 85%, whereas RBDs of the honeybee and dipteran species have pairwise sequence identity of 61 to 68%. Within the RBD of the *Am*-Tra2 protein, there are two putative RNP (ribonucleoprotein consensus peptide) elements (Fig. 2). These elements can directly interact with the *dsx* pre-mRNAs in *D. melanogaster* (Amrein et al., 1994; Nagai et al., 1990; Dreyfuss et al., 1988; Merrill et al., 1988). In the eight-amino-acid-long honeybee RNP-1 sequence element, we detected 3 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 functional important first amino acid has especially evolved from amino acid R to K within the honeybee and the *Nasonia* lineage (Fig. 2). We propose that the target RNA sequences of 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 *tra2* transcript variants in 0-6 h, 9-24 h and 33-48 h old embryos; L1 and L4-L5 larvae; and pupae by semiquantitative RT-
PCR (Fig. 3A). At the stage of 0-6 h old embryos the primary signal \textit{csd} not yet determines the sexual fate. The \textit{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 that in both sexes.

However, the level of expression substantially decreases at the pupal stage. The \textit{Am-tra2}\textsuperscript{285} and \textit{Am-tra2}\textsuperscript{284} transcripts, which are translated into the largest RS domain-containing proteins of all the transcripts, have the highest level of expression (Fig. 3A). The \textit{Am-tra2}\textsuperscript{253}/\textit{Am-tra2}\textsuperscript{252} transcripts are present in sizeable amounts in early embryos, whereas the \textit{Am-tra2}\textsuperscript{234}/\textit{Am-tra2}\textsuperscript{233} transcripts are apparently minor splice products.

In \textit{D. melanogaster}, there is a male-specific \textit{tra2} transcript, \textit{tra-2}\textsuperscript{179}, whose splicing is mediated by \textit{Tra-2}\textsuperscript{226} proteins derived from the general \textit{tra-2}\textsuperscript{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 \textit{Am-tra2} gene in sperm formation of the honeybee, we specifically searched for sex-specific \textit{Am-tra2} transcripts in the female (ovary tissue) and male (testis tissue) germ lines (Fig. 3B). We detected no sex-specific transcripts, suggesting that \textit{Am-tra2} mRNAs are not sexually spliced in male germ cells.

Taken together, these results indicate that \textit{Am-tra2} transcripts are not sex-specifically spliced and present before the primary decision of sexual fate is made by the \textit{csd} gene. The \textit{Am-tra2}\textsuperscript{285} and \textit{Am-tra2}\textsuperscript{284} are the dominant transcripts and the relative amount of \textit{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 (Gempe et al., 2009; Pane et al., 2002). 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 0-3-hour-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 (Supplementary data, figure S1). Both dsRNAs overlap in a small segment with no stretches of sequence identity over the siRNA length of 20-22 bp to other genes in the honeybee genome.

No female embryo reached larval stage L1 when we injected approximately 200 pg of dsRNA-1 or dsRNA-2 per embryo (Table 1A). This is an amount that is substantially below the approximately 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 1A). 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 ddH2O-treated controls). When we further reduced the dsRNA-2 concentration from 56 pg to 4 pg per embryo, the hatching rate still not substantially improved. We studied whether the lethality is sex-specific and injected 96 pg of dsRNA-2 into the male embryos for comparison (Table 1B). Also no male embryos reached larval stage L1, suggesting that knockdown of \textit{Am-tra2} caused some non-sex-specific lethality during embryogenesis. Because none the hatched L1 female larvae reached the L4 stage (data not shown) we were not able to further study the role of \textit{Am-tra2} in morphological sexual differentiation. Taken together, our knockdown results suggest that the \textit{Am-tra2} gene is essential for embryogenesis in the honeybee.

We proposed that in addition to a vital role for \textit{Am-tra2} in embryogenesis, \textit{Am-tra2} possibly has another function in sex determination, specifically in promoting female-specific splicing of the \textit{fem} and \textit{Am-dsx} transcripts. The knockdown of the \textit{fem} and the \textit{csd} genes, which regulate female splicing and sex determination, had no general effects on lethality, suggesting that the putative role of \textit{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 \textit{Am-tra2} gene, we injected 4 pg or 33 pg of dsRNA-2 in embryos and studied after 77-80 hours the splice patterns, irrespective of whether the larvae hatched. If \textit{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 2) induced male splicing of \textit{Am-dsx} mRNAs in females (Fig. 4A, lanes 1-10), which is entirely absent in the control embryos (Fig. 4A, lanes 21-30), which produce only the \textit{Am-dsx} female splice product. This result suggests that the \textit{Am-tra2} gene is essential to promote female splicing of \textit{Am-dsx} transcripts. The female splicing of \textit{fem} mRNAs is obviously not sizeable affected as we observed comparable
amounts of the corresponding female \textit{fem} fragments in treated and non-treated female embryos in our semiquantitative PCR analysis (Fig. 4B, lanes 1-10, 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 \textit{fem} mRNAs (Fig. 4B, lanes 11-20) compared to our control embryos (Fig. 4B, lanes 26-35; Table 2), indicating that \textit{Am}-Tra2 protein is also required for \textit{fem} female transcript splicing. To provide further evidence that the effect on splicing is specific for \textit{dsx} and \textit{fem} transcripts we also assayed the splice products of our control gene \textit{ef-1α} (Fig. 4C) and that of the \textit{csd} gene (Supplementary Data, figure S3) in the dsRNA treated and non-treated embryos. We detected in embryos that were treated with 4 pg or 33 pg \textit{Am-tra2} dsRNA-2 the \textit{csd} and the \textit{ef-1α} exon spanning fragments of cDNA suggesting that our treatment or the knockdown of \textit{Am-tra2} has not generally affected the splice process or degradation of embryonic mRNAs. This latter result supports our notion that the \textit{Am}-Tra2 protein is specifically involved in the sex-specific splice regulation of \textit{Am-dsx} and \textit{fem} transcripts.

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

Taken together, these results suggest that \textit{Am}-Tra2 promotes female splicing of the productive female \textit{fem} mRNAs and also of the non-productive male \textit{fem} mRNAs.
Discussion

Heteroallelic Csd proteins determine honeybee femaleness and set the downstream regulator of the sex determination cascade, \textit{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 \textit{fem} mRNAs (positive autoregulation) and direct female splicing of the \textit{Am-dsx} transcripts (Hasselmann et al., 2008; Gempe et al., 2009). In this study, we showed that the \textit{Am-tra2} gene, an ortholog of the \textit{tra2} gene of \textit{D. melanogaster}, is a component of the honeybee sex-determination hierarchy. The \textit{Am-tra2} proteins are required to regulate female and male splicing of \textit{fem} mRNAs and female splicing of the \textit{Am-dsx} mRNAs. In addition, we showed that the \textit{Am-tra2} gene has an essential role in embryogenesis that is not related to sex determination.

We characterized the \textit{Am-tra2} gene in the honeybee and showed that the deduced \textit{Am-Tra2} proteins share the same domain structure as other \textit{Tra2} orthologs described thus far (Salvemini et al., 2009; Burghardt et al., 2005; Sarno et al., 2010; Mattox et al., 1996; Amrein et al., 1988; Goralski et al., 1989; Bandziulis et al., 1989). \textit{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 \textit{Tra} proteins (Sciabica and Hertel, 2006; Graveley, 2000; Hoshijima et al., 1991; Amrein et al., 1988). We identified six splice variants of \textit{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 \textit{Am-Tra2} proteins are constitutively expressed. The \textit{tra2} transcripts are present in early embryos before the primary signal \textit{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 \textit{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 (Fig. 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 germ line, 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 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 sizeable effect on female splicing of fem transcripts suggesting that the Am-dsx switch of splicing has not been 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 Am-tra2 gene plays a role in the regulation of female Am-dsx mRNA splicing (Fig. 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 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 (Hediger et al., 2004; Ruiz et al., 2005; Saccone et al., 2008; Ruiz et al., 2007; Lagos et al., 2005; Permpoon et al., 2011;
Concha et al., 2010; Crampton et al., 1998) 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 β-sheets β1 and β3 and are used to bind directly to the ribonucleotide sequence (Nagai et al., 1990; Amrein et al., 1994; Dreyfuss et al., 1988; Merrill et al., 1988). 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 diverged from this of the fruitfly.

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 non-productive 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 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 (Fig. 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
(Fig. 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 non-productive
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 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., 1991; Valcárcel
et al., 1993; Bell et al., 1988; Sosnowski et al., 1989). 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 (Hediger et al., 2010; Pane et al., 2002). 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 (Gempe et al., 2009; Hasselmann et al., 2008; Beye et al., 2003). 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 approximately 5% when we further decreased the dsRNA concentration by 1/10th (Table 1). This additional role in embryogenesis is absent in other dipteran insects (Watanabe, 1975; Fujihara et al., 1978; Salvemini et al., 2009; Burghardt et al., 2005), 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 non-productive 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. Am-tra2 gene may thus have a central role in initiating the primary signal csd in females and maintaining the female determined state by the positive regulatory loop at the level of the fem
gene (Fig. 5). In males with hemi- or homoallelic Csd proteins, Am-Tra2 may enhance the switch of fem transcripts into the non-productive 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.
Acknowledgements

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 bee keeping support. This work was supported by grants from the Deutsche Forschungsgemeinschaft DFG.

Supplementary information is linked to the website.....
Figure 1: Genomic organization of the *Am-tra2* gene and the alternative 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 grey 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}, *Am*-Tra2^{284} (B) *Am*-Tra2^{253}, *Am*-Tra2^{252} (C) *Am*-Tra2^{234} and *Am*-Tra2^{233}. (D) Alternatively spliced variants in exon 4 producing tri-nucleotide 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 alternative splice acceptors in exon 4.

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, Nasonia vitripennis, Acromyrmex echinatior, Tribolium castaneum, Bombyx mori, Sciara ocellaris, Drosophila melanogaster, Musca domestica, Ceratitis capitata* and *Anastrepha obliqua*. Dots indicate amino acids identical to the predicted *Am*-Tra2 RBD of the honeybee. Boxes denote the RNP sequence elements.
Figure 3: Semi-quantitative 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 open reading frame (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 elongation factor 1α (ef-1α) as a relative control to semiquantify Am-tra2 transcripts across samples (Beye et al., 2003). All the fragments in 0-6-h-old embryos and pupae were cloned and sequenced. Ovaries were dissected from virgin queens and 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.

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 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-tra2 dsRNA-2 (lanes 11-20) or ddH2O (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 elongation factor 1α (ef-1α) as a relative control to semiquantify Am-tra2 transcripts across embryonic samples.
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 non-productive male fem splice form. In females, the Am-Tra2 proteins either acts together with heterozygous Csd proteins (CsdA/CsdB) and/or together with Fem proteins (femF positive autoregulatory loop) to promote female processing of the fem pre-mRNAs (femF). Am-Tra2 together with with Fem proteins direct female-specific splicing of the Am-dsx pre-mRNA (Am-dsxF). In males, Am-Tra2 protein directs splicing of male fem mRNAs in the presence of inactive Csd proteins (that is homoallelic CsdA/CsdA or CsdA proteins) and Fem proteins.
Table 1: Development of *Am-tra2*-dsRNA treated (A) female and (B) male embryos.

**A**

<table>
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<th>Treatment/ amount of injected dsRNA per embryo</th>
<th>Embryos injected</th>
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<th>Hatched L1 larvae</th>
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<td></td>
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The number of individuals (N) and the relative proportions (%) with respect to the total number of initially injected embryos are shown.

**B**

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<th>Treatment/ amount of injected dsRNA per embryo</th>
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The number of individuals (N) and the relative proportions (%) with respect to the total number of initially injected embryos are shown.
**Table 2:** The production of female and male *fem* and *Am-dsx* transcripts in 72- to 80-hour-old individuals in response to embryonic *Am-tra2* dsRNA-2 treatment.

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Reference list


Figure 1:

A

$\text{Am-Tra}^{285}/\text{Am-Tra}^{284}$ (285 aa/ 284 aa)

B

$\text{Am-Tra}^{253}/\text{Am-Tra}^{252}$ (253 aa/ 252 aa)

C

$\text{Am-Tra}^{234}/\text{Am-Tra}^{233}$ (234 aa/ 233 aa)

D

... AGAGA AAG CAG TAGTTAT...
Figure 2:

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Figure 3:

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ef-1α

heads  | testis | ovaries

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ef-1α 300 bp
Figure 4:

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Figure 5:

Csd²/Csd² or Csd²/Csd²

Csd²/Csd² or Csd²/Csd²

Am-Tra2

Am-Tra2

Am-dsx²

female development

male development