Parental and endosymbiont effects on sex determination in haplodiploid wasps
Geuverink, Elzemiek

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 6

Identification, sex-specific splicing and maternal provision of *transformer* and *transformer-2* in arrhenotokous and *Wolbachia*-infected thelytokous *Asobara japonica*

Elzemiek Geuverink
Marloes van Leussen
Wen-Juan Ma
Leo W. Beukeboom
Louis van de Zande
Alternative splicing is a hallmark of insect sex determination. In most insect species the sex-specifically spliced transformer (tra) gene with its co-factor transformer-2 (tra2) are the central gear in the sex determination cascade. Orthologs of tra and tra2 were identified in the parasitoid Asobara japonica (Hymenoptera, Braconidae). The female-specific splice form of AJ-TRA contains the characteristic Ceratitis-Apis-Musca (CAM) domain, arginine-serine region and proline-rich region. Aj-tra exhibits alternative splice variants that are composed of exons containing a variant of the putative CAM domain. Arrhenotokous females only provide these alternative splice forms to their offspring and lack maternal provision of female-specific Aj-tra mRNA, though they do transcribe female-specific Aj-tra mRNA throughout their own development. Thelytokous Wolbachia-infected females shift their maternal input to the female-specific Aj-tra splice variant. Removal of Wolbachia by antibiotic curing reverts the maternal provision to contain only the alternative splice forms, indicating active manipulation of splicing regulation by Wolbachia. Aj-tra2 has four splice variants resulting in different lengths of the arginine/serine region. The pattern of splice forms is different between males and females, without a clear male-specific splice form. The longest splice form is only detected in adult females and early embryonic samples. All splice forms of tra2 are maternally provided by thelytokous Wolbachia-infected, thelytokous Wolbachia-cured and arrhenotokous females, indicating that Wolbachia does not interfere with this gene in the sex determination cascade.
INTRODUCTION

Sex determination in insects follows a gene cascade that evolves from the bottom upwards (Wilkins, 1995). At the bottom of the cascade is the switch *doublesex (dsx)*, leading to male and female differentiation. It is controlled by *transformer (tra)* and *transformer-2 (tra2)*, which are both provided by the mother to her eggs in most insect species (Nissen et al., 2012; Shukla & Palli, 2013; Bopp et al., 2014). *Tra* splices in a female-specific pattern which switches the sex determination cascade into the female mode by maintaining an autoregulatory loop on itself. This autoregulation is suggested to act on the CAM domain (*Ceratitis-Apis-Musca* domain), which is only present on the female isoform of TRA (Hediger et al., 2010). The maternal input of *tra2* and female-specific *tra* leads to female specific splicing of *dsx* and thus female development. While *tra* and *dsx* require sex-specific splice variation for their functionality, *tra2* typically demonstrates no sex-specific splicing, except for a negative feedback loop of a *tra2* splice variant in the testis of *Drosophila melanogaster* (Mattox & Baker, 1991; Mattox et al., 1996; McGuffin et al., 1998). *Tra* is regulated by highly variable primary signals on top of the cascade, few of which are identified. In Hymenoptera these primary signals are the *csd* gene (a duplicate of *tra*) in the honeybee *Apis mellifera* (Beye et al., 2003; Hasselmann et al., 2008a; Gempe et al., 2009) and the hypothesized *womanizer (wom)* gene in the parasitoid wasp *Nasonia vitripennis* (Verhulst et al., 2013).

Molecular investigations point at a large diversity of haplodiploid sex determination mechanisms, similar to other organismal groups (Beukeboom & Perrin, 2014). In some groups, including *A. mellifera*, sex is determined by a single complementary sex determination locus (sl-CSD), heterozygotes become female and homo- and hemizygotes become male. For many other hymenopterans this complementary sex determination mechanism has been ruled out (van Wilgenburg et al., 2006; Heimpel & de Boer, 2008; Asplen et al., 2009). Little is known regarding the genetic basis of non-CSD sex determination and how conserved and widespread these molecular mechanisms are. The molecular basis of Maternal Effect Genomic Imprinting sex determination (MEGISD) has been elucidated in *N. vitripennis* (Beukeboom et al., 2007b; Verhulst et al., 2010a, 2013). It requires the maternal provision of female-specific *tra* and an active paternal copy of the maternally imprinted *wom* gene.

Many Hymenopterans are infected with endosymbionts which can manipulate reproduction in various ways (Werren et al., 2008; Ma et al., 2014b). The parasitoid *Asobara tabida* is infected with three *Wolbachia* strains of which one is necessary for oogenesis (Dedeine et al., 2001). The other two *Wolbachia* strains cause cytoplasmic incompatibility in this species (Dedeine et al., 2004). *Asobara* wasps are solitary endoparasitoids of *Drosophila* larvae and its species are distributed widely (Carton et al., 1986). Four species of the genus tested negative for CSD up to 10 loci (Beukeboom et al., 2000; Ma et al., 2013), including *Asobara japonica* which we investigate in this study. *A. tabida* contains homologs of *tra, tra2* and *dsx*, but reveals a notable absence of maternal provisioning of female-specific *tra*. A MEGISD mechanism...
(Beukeboom et al., 2007b), as demonstrated for N. vitripennis, can still be valid in Asobara wasps, albeit with modifications (Chapter 4).

In contrast to the oogenesis function in A. tabida, Asobara japonica that are infected with the endosymbiont Wolbachia reproduce thelytokously, whereas uninfected populations reproduce arrhenotokously (Kremer et al., 2009; Murata et al., 2009). We have recently found that these bacteria cause thelytokous reproduction in two steps: duplication of the haploid genome during oogenesis followed by feminization of diploid individuals (Ma et al., 2015). Interestingly, these two steps are differentially dependent on the Wolbachia titer. The additional feminization step implies that the bacteria interfere with the regulation of the host’s sex determination pathway. To test this prediction tra and tra2 regulation need to be compared between thelytokous and arrhenotokous strains. We examine which splice variants of tra and tra2 are maternally provided and how this splicing pattern changes during development. We further revert the thelytokous reproduction to arrhenotoky by curing the wasps with antibiotics to examine the direct effects of Wolbachia removal on the regulation of tra in the sex determination cascade.

MATERIAL AND METHODS

A. japonica strains

Genome drafts of arrhenotokous strain AM and thelytokous strain KG (Murata et al., 2009) were assembled (unpublished data). These strains were used for the identification of candidate sex determination genes and to collect embryonal samples for a developmental time series. As the AM strain was subsequently lost from laboratory cultures, further identification of male-specific Asobara japonica tra (Aj-traM) and non-sex-specific Aj-tra (Aj-traNSS) splicing was performed with the arrhenotokous IR strain (Murata et al., 2009). No strain differences between AM and IR were detected in splicing patterns of sex determination genes and nucleotide sequences of Aj-tra2. A small number of SNPs however distinguish the Aj-tra nucleotide sequences. All laboratory culturing and experiments took place at 25°C and L:D 24:0 conditions. Wasps were provided with second instar D. melanogaster larvae as hosts.

Ortholog identification

Orthologs of tra and tra2 were identified by translated BLAST (Altschul et al., 1997) against the two A. japonica genomic assemblies. TRA protein sequences of A. mellifera (NP_001128300) and N. vitripennis (NP_001128299), and the TRA2 sequence of A. mellifera (NP_001252514) were used as queries.

RNA extraction, cDNA synthesis and splice-variant detection

Adult females and males were individually collected after their emergence from the host pupae. RNA extraction was performed with TriZol (Invitrogen, Carlsbad, California, USA) according to
Identification, splicing and maternal provision of tra and tra2 in arrhenotokous and thelytokous A. japonica

manufacturer’s protocol. For 3’RACE (Rapid Amplification of cDNA Ends), RNA was reverse-transcribed with the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) using 25µM 3’RACE adapter (5’-GCG AGC ACA GAA TTA ATA CGA CTC ACT ATA GGT 12VN-3’) from FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA). For 5’RACE, RNA was processed according to manufacturer’s instructions (FirstChoice RLM-RACE kit, Ambion, Austin, TX, USA) and reverse-transcribed using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). To assess the Aj-tra splice variants present in adult males and females 5’RACE-PCR was performed with primer Ajtra_5RACEout (5’-GCTTCCTACCTCCTTTAATAACGG-3’). Reaction conditions were 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes, with a final extension of 7 minutes at 72°C. Outer primer Ajtra_3RACEout (5’-CTTGAAATTGAAGAAGCAGCAGG-3’) and inner primer Ajtra_3RACEin (5’-GGAAGACACATCTCTGCTTGG-3’) were used in a nested 3’RACE-PCR at 94°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 2 minutes, with a final extension of 10 minutes at 72°C. Reverse Transcription (RT-)PCRs to confirm the splicing variation of Aj-tra were performed with primer sets Ajtra exon3F1 (5’-CGTTATTTAAGGGTAGTGAAAG-3’)/Ajtra exon4R (5’-GCTGGTCTTTTGTTGTAACTC-3’), Ajtra exon3F1/Ajtra exon8R (5’-GGAATCAGCTCTTCTGAG-3’) and Ajtra exon3F2 (5’-ACATTGAAGACATTCCA’)/Ajtra exon6R1 (5’-CCCTCAATACACCACCTCC-3’) and Ajtra exon4F (5’-AAGAGTTAACAACGAAAGGACC-3’)/Ajtra exon6R2 (5’-AGTTGGTGCTCTCATCATAGTC-3’). All sets were used at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 2 minutes, with a final extension of 7 minutes at 72°C. An additional PCR was performed with primers Ajtra exon7F (5’-AGAAGCTGAATGCTTGG-3’) and Ajtra exon9R (5’-GCTCTGGAATGACGGACTTTGG-3’) to identify the full gDNA region between exon 7 and 9. The reaction conditions were 94°C for 3 minutes, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes, with a final extension of 7 minutes at 72°C.

To amplify the 5’region of Aj-tra2 5’RACE-PCR was performed with outer primer Ajtra2_5RACEout (5’-AGGTCTGAATGATGCTAAGACTG-3’) and nested inner primer Ajtra2_5RACEin (5’-ATGTCTTTAAAGAAGTGGCAG-3’) at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 2 minutes, with a final extension of 10 minutes at 72°C. 3’RACE-PCR of Aj-tra2 was performed with outer primer Ajtra2_3RACEout (5’-GTCTTTCAATAATTTTCCCTTCTGTG-3’) and nested inner primers Ajtra2_3RACEin (5’-CGACGTGGAGATAAGAAGGAC-3’) in a reaction at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 2 minutes, with a final extension of 10 minutes at 72°C. RT-PCRs to confirm the splicing variation of Aj-tra2 were performed with primers Ajtra2F1 (5’-TCTTCAACAATCTCAGCCAC-3’) and Ajtra2R1 (5’-CACTTTCTCCTCTCTCACTG-3’). The reaction conditions were 94°C for 3 minutes, 45 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 45 seconds, with a final extension of 7 minutes at 72°C.
All RACE-PCR and RT-PCR products were ligated into pGEM-T vector (Promega, Madison, WI, USA) after purification using GeneJET Gel Extraction Kit (Fermentas, Hanover, MD, USA). Ligation reactions were used to transform competent JM-109 *Escherichia coli* (Promega, Madison, WI, USA). Colony-PCR was conducted with pGEM-T primers (5’-GTAAACGACGGCCAGT-3’ and 5’-GGAACACGCTATGACCAG-3’) at 94°C for 3 minutes, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes, with a final extension of 7 minutes at 72°C. Both strands were sequenced and fragments were aligned to one another and to the genomic contigs from the assembly to inspect the splicing variation. The structure of the genes was visualized with Exon-Intron Graphic Maker (http://wormweb.org/exonintron).

**Phylogenetic trees**

Phylogenetic trees were constructed in MEGA7 (Kumar et al., 2016). Within-genera divergence of TRA, TRA2 and COI was calculated in MEGA7 by computing the within group mean distance with 1000 bootstraps. TRA amino acid sequences of the following species (Genbank accession) were used: *A. cerana* (ABV56230), *A. dorsata* (NP_001276123), *A. mellifera* (NP_001128300), *Bombus impatiens* (XP_003493796), *Bombus terrestris* (NP_001267853) and *N. vitripennis* (NP_001128299). TRA sequences of *Leptopilina* and *A. tabida* were detected in chapter 4 and 5. Amino acid sequences of *Nasonia giraulti* and *Nasonia longicornis* were predicted comparing the *N. vitripennis* TRA sequence with tblastn to the transcriptome shotgun archives and whole genome shotgun sequences of *N. giraulti* (GBEC00000000/ADAO01000000) and *N. longicornis* (ADAP01000000) (Werren et al., 2010; Hoedjes et al., 2015). TRA2 amino acid sequences used were: *A. dorsata* (XP_006623033), *A. mellifera* (AFJ15561), *B. impatiens* (XP_012249279) and *B. terrestris* (XP_012169168). Amino acid sequences of TRA2 from *N. giraulti* (ADAO01000000), *N. longicornis* (ADAP01000000), *L. boulardi* (GAJA01019094), *L. clavipes* (GAXY02014083) and *L. heterotoma* (GAJC01027588) were predicted by tblastn searches of the *N. vitripennis* amino acid sequence obtained in chapter 3 against the transcriptome shotgun archives and whole genome shotgun sequences of these species (Werren et al., 2010; Goecks et al., 2013; Misof et al., 2014). The amino acid sequence of *A. tabida* TRA2 was obtained in chapter 4. Nucleotide sequences of COI (Genbank accession) used were: *A. cerana* (DQ016088), *A. dorsata* (DQ020235), *A. mellifera* (AF250946), *B. impatiens* (JF799028), *B. terrestris* (AY181171), *N. giraulti* (EU746515), *N. longicornis* (EU746524), *N. vitripennis* (EU746534), *Leptopilina boulardi* (JQ808437), *Leptopilina clavipes* (HM999666), *Leptopilina heterotoma* (AB456712), *A. tabida* (JQ808428) and *A. japonica* (JF430429).

**Splicing patterns of Aj-tra<sup>M</sup> and Aj-tra<sup>NSS</sup> in females**

Amplification of *Aj-tra<sup>M</sup>* and *Aj-tra<sup>NSS</sup>* transcripts in adult thelytokous females, arrenhotokous females and arrenhotokous males was performed with primers Ajap_tra_exon3F (5’-CGTTATTTAAGGGTATGAAAGG-3’) and Ajap_tra_exon4R (5’-GCTGGTCTTTGTTGTAACTC-3’) at 94°C for 3 minutes, 40 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 30
Identification, splicing and maternal provision of tra and tra2 in arrhenotokous and thelytokous A. japonica

...seconds, with a final extension of 7 minutes at 72°C. Products were run and visualized on an ethidiumbromide-containing 1.5% agarose gel.

Presence and splicing of Aj-tra and Aj-tra2 during embryonic development

Offspring of arrhenotokous mated, arrhenotokous virgin, thelytokous Wolbachia-infected and thelytokous antibiotics-cured females was collected along a range of time-points to examine the presence of Aj-tra and Aj-tra2 transcripts prior to zygotic transcription and during embryonic development. Hosts containing arrhenotokous (AM strain) wasps in the pupal stage were placed individually in tubes to prevent emerging females from mating. Mated arrhenotokous females and untreated thelytokous (KG strain) females were collected from mass culture bottles. Immediately after emergence, a subset of thelytokous females was provided with 1mg/g rifampicin dissolved in honey, to cure their Wolbachia infection. Groups of three females were given hosts for 2 hours to stimulate oogenesis and to improve host detection. Following this pre-treatment the wasps were kept for 2 days at 12°C and L:D 24:0 in tubes with a layer of agar for moisture and with honey for feeding. Next, each group of wasps was provided with 30 hosts in dried yeast solution on a petri dish for 2 hours. After the allotted development time at standard culturing conditions the petri dishes were rinsed with water and 10 host larvae were collected by freezing them in liquid nitrogen and subsequent storage at -80 °C. Time-points were chosen to cover maternal provision prior to zygotic transcription (0-2h), the predicted start of zygotic sex determination gene transcription (6-8h and 12-14h) and subsequent early sexual differentiation (24-26h and 48-50h). A subset of larvae from each group and time-point was left to develop into adults. This served as a control for the parasitization itself, the virginit of the unmated arrhenotokous females, the success of rifampicin treatment, and possible spontaneous male production of the thelytokous untreated females (Reumer et al., 2012; Ma et al., 2014a).

The progenies from mated arrhenotokous females yielded information about offspring sex ratio, which was on average 0.47 (proportion males). All thelytokous females treated with rifampicin and all virgin arrhenotokous females produced only sons. Less than 5% of thelytokous untreated females produced male offspring and these samples were excluded from RNA extractions. Of each group and time-point, 3 to 6 samples with each 10 parasitized larvae were used for RNA extraction, as described for adult tissue above. Reverse transcription was performed with a mixture of 1:6 random oligo-dT:random hexamers from the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). Controls were unparasitized D. melanogaster larvae of similar age groups.

For the amplification of Aj-tra splice variants during development, primers Ajtra_RTF (5’-TTAACGAAAGAAGGTGAGG-3’) and Ajtra_RTR (5’-GAGAATCCAGCTTTCTCTGAG-3’) were used in reactions at 94°C for 3 minutes, 45 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 1 minute, with a final extension of 7 minutes at 72°C. Assessment of Aj-tra2 splicing patterns in these developmental series was performed with the same primer set used in the initial splice-variant detection. Detection of Aj-tra2α and Aj-tra2β could not be separated on gel...
as these transcripts are exactly the same size. The band was extracted from the gel and cloned according to the procedures described in the splice variant detection above. Two samples of an adult of each category and one sample of 0-2h old embryos of each category were used to clone, as described above, and 8 to 12 colonies of each cloned fragment were sequenced.

**Figure 6.1.** Exon-intron structure of the female-specific (F), male-specific (M) and non-sex-specific (NSS) splice variants of *tra* in *A. japonica*. White boxes represent the 5’ and 3’UTR’s, black boxes contain the coding sequence. The CAM domain is shown in dark grey on exon 3 and 7, the alternative 3’ CAM version of the *Aj-tra*<sup>NSS</sup> splice forms is shown in lighter grey on exon 4. The length of the first and second intron is marked with a break as the genomic contigs only contain the regions flanking the exons and do not inform on the full length.

**RESULTS**

*Identification and splicing variation of tra in A. japonica*

A single homolog of *tra* could be detected in *A. japonica* genomic assemblies of both an arrhenotokous and a thelytokous strain. The ORF was spanning multiple incomplete contigs and a large variety of splice variants was detected by amplification and sequencing of RACE-PCR and
RT-PCR fragments. One female-specific splice variant was found, indicated by with Aj-tra\(^F\) (Figure 6.1), containing a CAM domain (3’ part located on exon 6) followed by an arginine/serine domain and a proline-rich region. This pattern is typical for a female-specific splice form of tra. All hymenopteran tra homologs contain a conserved domain at the 5’part of the ORF, before the sex-specific exons. This Hymenoptera-specific HYM domain (Verhulst et al., 2010b) is found in the female-specific variant of Aj-tra and all alternative splice forms. Six alternative splice forms were detected, of which three were male-specific (Aj-tra\(^{M1}\), Aj-tra\(^{M2}\), Aj-tra\(^{M3}\)), based on the inclusion of exons before the CAM domain leading to a change in ORF and an early STOP-codon. This short ORF is consistent with male tra variants in other species. Three splice variants had a longer ORF and contained an alternative diverged CAM domain (3’ part located on exon 4) followed by a short arginine/serine rich region, alike A. tabida (Chapter 4). These were termed non-sex-specific (Aj-tra\(^{NSS1}\), Aj-tra\(^{NSS2}\), Aj-tra\(^{NSS3}\)) due to their presence in both sexes.

Figure 6.2. Gene tree of TRA/FEM inferred using the Maximum Likelihood method based on the (Jones et al., 1992) model (1000 bootstraps). The evolutionary distances are scaled in units of the number of amino acid substitutions per site. Numbers at the tree nodes indicate bootstrap support values. Amel = Apis mellifera, Ador = Apis dorsata, Acer = Apis cerana, Ajap = Asobara japonica, Atab = Asobara tabida, Bter = Bombus terrestris, Bimp = Bombus impatiens, Lhet = Leptopilina heterotoma, Lbou = Leptopilina boulardi, Lcla = Leptopilina clavipes, Ngir = Nasonia giraulti, Nlon = Nasonia longicornis and Nvit = Nasonia vitripennis.
The female-specific TRA peptides of *A. japonica* and *A. tabida* are highly divergent in comparison to orthologs within other genera, except the *Leptopilina* genus which also contains low conservation of TRA (Figure 6.2). This pattern is not reflective of the evolutionary history of the species. Within genera mean distances of the COI marker reveal a slightly higher differentiation in *Asobara* and *Leptopilina*, but the differences are markedly increased for TRA (Table 6.1). This indicates that strong divergence of the *tra* gene in *Asobara* and *Leptopilina* is not a result of a more remote speciation event.

**Table 6.1.** Average evolutionary divergence over sequence pairs within genera. Average number of amino acid (TRA/TRA2) or base (COI) substitutions per site are shown, followed by their standard error estimates. Analyses were conducted using the JTT matrix-based model (TRA/TRA2) and the Tamura 3-parameter model (COI).

<table>
<thead>
<tr>
<th>Genus</th>
<th>TRA d</th>
<th>SE</th>
<th>TRA2 d</th>
<th>SE</th>
<th>COI d</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apis</em></td>
<td>0.043</td>
<td>0.014</td>
<td>0.000</td>
<td>0.000</td>
<td>0.091</td>
<td>0.017</td>
</tr>
<tr>
<td><em>Bombus</em></td>
<td>0.007</td>
<td>0.007</td>
<td>0.000</td>
<td>0.000</td>
<td>0.134</td>
<td>0.025</td>
</tr>
<tr>
<td><em>Leptopilina</em></td>
<td>0.358</td>
<td>0.049</td>
<td>0.033</td>
<td>0.011</td>
<td>0.209</td>
<td>0.028</td>
</tr>
<tr>
<td><em>Nasonia</em></td>
<td>0.009</td>
<td>0.007</td>
<td>0.003</td>
<td>0.003</td>
<td>0.145</td>
<td>0.023</td>
</tr>
<tr>
<td><em>Asobara</em></td>
<td>0.386</td>
<td>0.063</td>
<td>0.045</td>
<td>0.014</td>
<td>0.188</td>
<td>0.032</td>
</tr>
</tbody>
</table>

**Presence of Aj-tra splice variants during adult stages**

In adult males of *A. japonica* the female specific splice form of *Aj-tra* is never expressed, regardless of their arrhenotokous or thelytokous genomic origin (Figure 6.3). Males can splice *Aj-tra* in a mixture of *Aj-tra*\(^M\) and *Aj-tra*\(^NSS\) splice forms (Figure 6.4a and 6.4b). Arrhenotokous females are not exclusively transcribing the *Aj-tra*\(^F\) variant, but contain a mixture of all splice variants (Figure 6.3 and 6.4a). Thelytokous females that have been cured from their *Wolbachia* infection do not differ in splice-form composition from arrhenotokous females, providing further evidence that the underlying sex determination mechanism and gene function is similar in thelytokous and arrhenotokous populations. *Wolbachia*-infected females, however, demonstrate a strong bias towards the *Aj-tra*\(^F\) splice form (Figure 6.3). When only exons specific to *Aj-tra*\(^NSS\) and *Aj-tra*\(^M\) are amplified, transcripts of these splice variants can be seen in *Wolbachia* infected females albeit at low abundance (Figure 6.4a and 6.4b). Notably, the level of *Aj-tra*\(^M\) mRNA appears slightly lower in thelytokous females (Figure 6.4b) while that of *Aj-tra*\(^NSS\) is similar in all types of adults (Figure 6.4a).
Identification, splicing and maternal provision of `tra` and `tra2` in arrhenotokous and thelytokous *A. japonica*.

**Figure 6.3.** Splice variants of *Aj-tra* during embryo development and in adults of thelytokous and arrhenotokous *A. japonica*. *D. melanogaster* larvae and no template reactions are included as negative controls.

**Figure 6.4.** Presence of *Aj-tra*\textsuperscript{NSS} (a) and *Aj-tra*\textsuperscript{M} (b) in adult *A. japonica*. The *Aj-tra*\textsuperscript{NSS} band in (a) represents an amplification of transcripts spanning exon 3 and 4 from *Aj-tra*\textsuperscript{NSS1}, *Aj-tra*\textsuperscript{NSS2} and *Aj-tra*\textsuperscript{NSS3} which are of the same size. The *Aj-tra*\textsuperscript{M} fragment amplified in (b) spans exons 3 and 4, where the structure of the *Aj-tra*\textsuperscript{M1} and *Aj-tra*\textsuperscript{M2} transcripts is identical.
Dynamics of Aj-tra splice variants during embryonic development

The female-specific splice variant of Aj-tra is not present in early embryos of arrhenotokous females (Figure 6.3). This implies that arrhenotokous A. japonica do not maternally provide traF mRNA to the offspring. The alternative splice forms of Aj-traNSS are provided to the embryos of arrhenotokous females (Figure 6.3), where Aj-traNSS1 and/or Aj-traNSS3 transcripts are present in all embryo samples (0-2h and 3-5h of development) before the presumed start of zygotic transcription. Embryos of thelytokous females of 0-2h and 3-5h after parasitization contain Aj-traF (Figure 6.3), indicating that thelytokous females, in contrast to arrhenotokous females, do maternally provide Aj-traF mRNA to their eggs. Thelytokous females that were treated with antibiotics to remove the thelytoky-inducing Wolbachia did not maternally provide Aj-traF mRNA to the eggs. Apparently, maternal provision of traF mRNA is induced by Wolbachia.

In arrhenotokous A. japonica the female-specific splice form of Aj-tra starts to appear in diploid embryos of 12-14 h after parasitization, indicating that zygotic transcription of Aj-tra starts around this time (Figure 6.3). It is not known how the female-specific splicing of the Aj-tra transcript is effectuated in the absence of maternally provided Aj-traF. The haploid arrhenotokous embryos do not express Aj-traF mRNA at any point in their development. This pattern is also seen in the embryos from cured thelytokous wasps that develop into males. Such embryos match the arrhenotokous male embryos in all stages, again indicating the involvement of Wolbachia in maternal provisioning of Aj-tra.

Identification and splicing variation of tra2 in A. japonica

A homolog of tra2 (Aj-tra2) was detected in the A. japonica genomic assemblies. It consists of eight exons, but there are three alternative splice forms (Figure 6.5), that differ by the inclusion or exclusion of exons 3 and 4. These exons contain duplicated regions, which also are present at the start of exon 5. This region is 72 base pairs long (Figure 6.6) and, hence, its inclusion causes no frameshift but results in an arginine/serine-rich region of varying length, depending on the number of included exons. A homolog of this region in A. tabida tra2, present in the middle of its exon 2, is aligned against the Aj-tra2 duplicated regions (Figure 6.6). Tra2 is a highly conserved gene which barely shows amino acid modifications within genera, but a surprising level of divergence is present in the Asobara genus (Table 6.1).
Identification, splicing and maternal provision of \( \text{tra} \) and \( \text{tra2} \) in arrhenotokous and thelytokous \emph{A. japonica}

---

**Figure 6.5.** Exon-intron structure of the splice variants of \( \text{tra2} \) in \emph{A. japonica}. White boxes represent the 5' and 3'UTR's, black boxes contain the coding sequence. The RBD is plotted in grey on the exons. Incomplete intron lengths are marked by breaks in the line.

**Figure 6.6.** Alignment of repeated regions of \( \text{Aj-tra2} \) against each other and against the homologous sequence of \( \text{At-tra2} \).

### Presence of \( \text{Aj-tra2} \) splice variants during adult stages

All splice variants (\( \text{Aj-tra2}^A \), \( \text{Aj-tra2}^B \), \( \text{Aj-tra2}^C \) and \( \text{Aj-tra2}^D \)) are found in adult females, regardless of their arrhenotokous or thelytokous mode of reproduction (Figure 6.7, Table 6.2). \( \text{Aj-tra2}^C \) and \( \text{Aj-tra2}^D \) are found in low abundance. Males of either reproductive type transcribe the \( \text{Aj-tra2}^A \) and \( \text{Aj-tra2}^C \) splice variants abundantly, but \( \text{Aj-tra2}^B \) only sporadically (26 independent bacteria colonies containing either an \( \text{Aj-tra2}^B \) or an \( \text{Aj-tra2}^C \) fragment were sequenced and only 2 transcripts of \( \text{Aj-tra2}^B \) were detected). No transcripts of \( \text{Aj-tra2}^D \) were detected in males (Figure 6.7, Table 6.2). Therefore, \( \text{Aj-tra2}^D \) can be regarded as female specific. Removal of \emph{Wolbachia} does not change the splicing pattern of \( \text{Aj-tra2} \) in adult thelytokous females, unlike \( \text{Aj-tra} \) which is changed to more male-biased splicing.

### Presence of \( \text{Aj-tra2} \) splice variants during embryonic development

Maternal provision of \( \text{Aj-tra2} \) reveals the same pattern in developmental stages of both reproductive modes (Figure 6.7, Table 6.2). Both thelytokous and arrhenotokous females supply
the female-specific \( Aj-tra^D \) and the non-specific \( Aj-tra^B \) to their eggs. A lower amount of \( Aj-tra^A \) and \( Aj-tra^C \) transcripts is present in these early embryos, which contrasts to its abundant presence in adults, particularly in males. The pattern of splicing is similar in all developmental stages and changes rapidly from the maternally provided \( Aj-tra^D \) and \( Aj-tra^B \) transcripts in early embryos to a higher level of \( Aj-tra^A \) at later stages. This universal maternal provision is in contrast with that of \( Aj-tra \) mRNA, which is only maternally provided in thelytokous females in the presence of \textit{Wolbachia}.

Figure 6.7. Splice variants of \( Aj-tra2 \) during embryo development and in adults of thelytokous and arrhenotokous \textit{A. japonica}. \textit{D. melanogaster} larvae and no template reactions are included as negative controls.

Table 6.2. Presence of \( Aj-tra2 \) splice variants in embryos and adults. Presence of \( Aj-tra^B \) and \( Aj-tra^C \) mRNA cannot be scored on gel due to equal length of PCR products. These bands were cloned and sequenced to score the abundance of each variant. Numbers of each clone (number/total) are denoted between brackets.

<table>
<thead>
<tr>
<th>Category</th>
<th>Wolbachia</th>
<th>( Aj-tra^A )</th>
<th>( Aj-tra^B )</th>
<th>( Aj-tra^C )</th>
<th>( Aj-tra^D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrhenotokous females</td>
<td>No</td>
<td>Yes</td>
<td>Yes (15/16)</td>
<td>Yes (1/16)</td>
<td>Yes</td>
</tr>
<tr>
<td>as embryos</td>
<td></td>
<td>Yes</td>
<td>Yes (19/20)</td>
<td>Yes (1/20)</td>
<td>Yes</td>
</tr>
<tr>
<td>Arrhenotokous males</td>
<td>No</td>
<td>Yes (2/3)</td>
<td>Yes (11/13)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>as embryos</td>
<td></td>
<td>Yes (19/20)</td>
<td>Yes (1/20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thelytokous females</td>
<td>Infected</td>
<td>Yes (13/16)</td>
<td>Yes (3/16)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>as embryos</td>
<td>Yes</td>
<td>Yes (11/12)</td>
<td>Yes (1/12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thelytokous males</td>
<td>No</td>
<td>Yes (0/13)</td>
<td>Yes (13/13)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>as embryos</td>
<td>Yes</td>
<td>Yes (5/7)</td>
<td>Yes (2/7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thelytokous females*</td>
<td>No</td>
<td>Yes (13/15)</td>
<td>Yes (2/15)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*This category is fed antibiotics during their adult life. This removes the \textit{Wolbachia} infection and changes their splicing pattern of \( Aj-tra \). No embryos of equivalent category can be produced, as these females produce male eggs without \textit{Wolbachia}.
DISCUSSION

Structural conservation of tra and tra2

We identified orthologs of the central sex determination genes tra and tra2 in the genomes of arrhenotokous and thelytokous A. japonica. Aj-tra has sex-specific splice variants consistent with other insects (Boggs et al., 1987; Pane et al., 2002; Ruiz et al., 2007b; Lagos et al., 2007; Hasselmann et al., 2008a; Concha & Scott, 2009; Verhulst et al., 2010a; Hediger et al., 2010; Schmieder et al., 2012; Shukla & Palli, 2012a; Morrow et al., 2014; Peng et al., 2015; Jia et al., 2016; Laohakieat et al., 2016), but whereas tra2 normally does not exhibit sex-specific splicing, we found Aj-tra2 to be sex-specifically spliced. In addition, Aj-tra exhibits additional non-sex-specific isoforms that contain a duplication of sequences coding for the CAM domain, which has a putative role in the splicing autoregulation of tra. A similar feature was found in the transformer splice forms of A. tabida (Chapter 4), in which TRA variants also possess a putative copy of the CAM domain adjacent to a short arginine-serine region. In A. tabida these additional splice variants had a much shorter transcript, with an alternate poly-A tail compared to the sex-specific splice variants (Chapter 4). In A. japonica these transcripts appear to share the same C-terminal exons as Aj-tra5 and Aj-traM. These alternative splice variants, conserved between the two Asobara species, may be involved in tra regulation as well. The transcripts of these extra forms are present in all assessed developmental stages of both females and males. Moreover, the presence of transcripts containing a CAM-like domain in males has not yet been documented for any other insect and hints at additional complexity of the sex determination pathway.

The structure of TRA2 is conserved in A. japonica compared to other insects, with an RBD domain flanked by two arginine/serine regions on either side. An unexpected feature was found in the N-terminal arginine/serine region. This region consists of different lengths of an SR-rich region, as a result of differential splicing. The shortest SR-rich region is primarily found in males and the longest only in females. The N-terminal arginine/serine region in TRA2 is a more potent splicing activator (Sciabica & Hertel, 2006). A splice variant unique to females is a novelty for the presumed highly conserved tra2. It is maternally provided to the embryos, but appears absent during early larval development of females (24-26h and 48-52h). This could suggest a function in oogenesis and early embryogenesis, in which case the signal from the adult female is potentially germline-restricted.

Though specific domains and regions within the tra and tra2 genes are conserved between the two Asobara species, the overall sequences are strongly diverged (Table 6.1). This sequence divergence is reflected in changes in splicing. The short non-sex-specific transcripts of A. tabida tra (At-traNLS) with an alternative polyA-tail are suggested to be a derived feature, as their parallel transcripts in A. japonica still demonstrate an extended 3’UTR. The sex-specific splicing of Aj-tra2 is not seen in A. tabida, where in fact no alternative splicing of tra2 is present. Additional data from closely related species would be required to distinguish whether this is a loss in A. tabida or a gain in A. japonica.
**Alternative maternal provision**

The *N. vitripennis* MEGISD model for sex determination consists of a maternally silenced *womanizer* factor (Verhulst et al., 2013) combined with the maternal provision of female-specific *tra* mRNA (Verhulst et al., 2010a) and non-sex-specific *tra2* mRNA (Chapter 3). *Womanizer* is imprinted on the maternal genome and ensures male development in haploid unfertilized eggs. Its paternal equivalent is not silenced and activates female-specific *tra* splicing in diploid fertilized eggs. In arrhenotokous *A. japonica* an epigenetic distinction between the maternal and paternal chromosome would be required to start autoregulation of the female developmental pathway in diploid fertilized eggs (Figure 6.8). This potential paternally active factor initiating the female sex determination cascade cannot be identified in the current study. The other requirement, a combination of *tra* and *tra2* mRNA provided by the mother to her eggs, displays unique patterns in the *A. japonica* system.

A key feature of the *Asobara* system is the absence of maternally provided female-specific *tra* mRNA in eggs, which in all studied insects (with the exception of *A. mellifera* and *D. melanogaster*) serves as the maternal effect factor in sex determination. The lack of maternal *Aj-tra*\(^F\) provision in arrhenotokous wasps suggests an alternative activator of the *tra* autoregulatory loop to direct female development in *A. japonica* (Figure 6.8). This is consistent with the pattern in arrhenotokous *A. tabida* (Chapter 4), which do not provide At-*tra*\(^F\) to their eggs. As proposed for *A. tabida* (Chapter 4), the maternally provided non-sex-specific splice forms of *tra*, that contain an alternative CAM domain, may serve as the start of the autoregulatory loop.

*Tra2* is more conserved than *tra* in its shared role as maternally provided activator of the female developmental pathway. *A. japonica* provides all splice variants of *tra2* to its eggs, but particularly *Aj-tra2*\(^B\) and *Aj-tra2*\(^D\), the latter of which is not found back in males after the earliest developmental stages. This maternal input suggests a switch in role from *tra* to *tra2* as a female-specific cofactor activating the sex determination cascade. The female-specific *Aj-tra2* mRNAs however do not appear to be necessary in maintaining the female-specific autoregulation of *tra*, as they disappear after 12-14 hours of development. The difference between the *TRA2* isoforms is not as distinct as the difference between *TRA* male and female isoforms. Where male *tra* mRNA codes for a premature stop-codon before the CAM domain, caused by a frameshift due to the inclusion of male-specific exons, all *tra2* versions contain a complete ORF. One interpretation of these results is that each isoform has kept some functionality, but their interaction with TRA or other targets may differ.

**Wolbachia effect on *tra* and *tra2* regulation**

Splicing patterns of *Aj-tra2* do not differ between thelytokous and arrhenotokous wasps, neither during embryonic development nor adulthood. Furthermore, maternal provision of *Aj-tra2* transcripts does not change upon removal of *Wolbachia* infection (Figure 6.8). It suggests that *Wolbachia* does not interfere with *Aj-tra2* in the sex determination cascade to feminize the
Identification, splicing and maternal provision of \( tra \) and \( tra2 \) in arrhenotokous and thelytokous \( A. japonica \)

The thelytokous offspring. This is consistent with the proposed other role of \( tra2 \) in early developmental processes. In other Hymenopterans inhibition of \( tra2 \) maternal input or early zygotic transcription leads to high lethality (Nissen et al., 2012) (Chapter 3). If \( Wolbachia \) would start manipulating maternal provision of \( tra2 \) it could interfere with its other functions and lead to increased mortality of infected individuals in early development. Instead \( Wolbachia \) appears to target maternal \( Aj-tra \), which in thelytokous \( Wolbachia \) infected individuals is maternally provided as female-specific transcripts. If \( Wolbachia \) is removed, there is no maternal input of \( Aj-tra^F \) and only non-sex-specific transcripts of \( Aj-tra \) are provided. Splicing patterns of \( Aj-tra \) in adults suggest an overexpression of \( Aj-tra \) in infected thelytokous females, specifically of \( Aj-tra^F \). This is evident from a completely biased amplification of \( Aj-tra^F \) in these individuals. However, if only the male-specific splice variants are amplified, some product is still obtained, suggesting that splicing cannot be completely altered by \( Wolbachia \). This pattern matches the \( traA \) splicing of the \( L. clavipes \) system (Chapter 5). It is at this moment not yet clear whether \( Wolbachia \) targets the splicing mechanism of \( Aj-tra \) or enforces an elevated expression of \( Aj-tra \).

**Mechanism of Wolbachia feminization**

\( Wolbachia \) is operating in \( A. japonica \) according to a two-step model (Ma et al., 2015). The first step is diploidization of the haploid gamete, which results in the development of diploid males. An elevated \( Wolbachia \) titer can ensure additional feminization, resulting in diploid female development. This suggests that the threshold of \( Aj-tra^F \) is dependent on \( Wolbachia \) titer to exert its feminizing effect. A lowered titer of \( Wolbachia \) in the female germline would then result in an absence or reduction of \( Aj-tra^F \) provision, leading to diploid male or gynandromorph offspring. Notably, it is not possible to detect gynandromorphism in \( A. japonica \), as the presence of an ovipositor is the only external morphological distinction between females and males (Ma, unpublished data).

The mechanism with which \( Wolbachia \) induces thelytoky in \( A. japonica \) could consist of one or multiple elements. In its simplest form, the maternal input of \( tra^F \) alone would be sufficient to start and maintain female development. If an autoregulatory mechanism on \( tra \) exist in \( A. japonica \) to enforce zygotically transcribed \( tra^F \), an introduction of \( tra \) mRNAs and/or TRA protein in the pre-zygotic transcription stage could be sufficient to start this loop. However, as another potentially paternally activated factor would be necessary to start \( tra^F \) in arrhenotokous female development, a more complicated system could be present in the thelytokous mode as well. If the same activator of the arrhenotokous female mode is needed in the thelytokous system, \( Wolbachia \) should remove the imprinted signal of the maternal genome, as thelytokous reproduction occurs without a paternal genome.
Chapter 6

Figure 6.8. Model of the sex determination cascade in arrhenotokous and thelytokous *A. japonica* compared to *N. vitripennis* and *A. mellifera*. Diploid female development is depicted starting from an heterozygotic genetic primary signal in *A. mellifera* and a proposed imprinting difference between the parental chromosome sets in *N. vitripennis* and *A. japonica*. The heterozygosity between the paternal and maternal chromosome of *A. mellifera* is illustrated by the different colours and the epigenetic differentiation between the paternal and maternal chromosome of *N. vitripennis* is illustrated by the dotted and solid lines around the chromosome sets. Arrhenotokous and thelytokous chromosomes of *A. japonica* are depicted in different colours and the proposed epigenetic difference between paternal and maternal chromosome set is depicted alike the *N. vitripennis* illustration. Maternal mRNA transcripts provided to the embryo are displayed for *tra* (*fem* is not maternally provided) and *tra2*. A proposed cascade of female sex determination displays the different isoforms activating *tra* transcription, followed by the complex of TRA\(^{F}\)/TRA2 regulating the splicing of *dsx* in the female mode.

The way in which *Wolbachia* manipulates the sex determination cascade is not known. Recent studies have revealed that *Wolbachia* can use host microRNAs (miRNAs) to manipulate their host. Such miRNAs can affect cellular localization of an Argonaut protein (Hussain et al., 2013; Zhang et al., 2013). Argonaut proteins are involved in a wide array of cellular processes, which include cell division and transposon control (Carmell et al., 2002; Thomson & Lin, 2009). Epigenetic regulation of host processes by methylation may be another possibility. Negri et al. (2009) and Ye et al. (2013) reported that *Wolbachia* changed the methylation state of the genomes of *Wolbachia*-infected hosts indicating a reprogramming of imprinting. It is however unknown to what extent *Wolbachia* manipulate host reproduction by altering gene expression or splicing through miRNAs and DNA methylation. As epigenetic effects are known to play a role...
in hymenopteran sex determination (Verhulst et al., 2010a), manipulating methylation patterns may be a way for *Wolbachia* to control the sex determination pathway of its host. Methylome sequencing of ovipositing females and transcriptomic comparisons of early embryos are required to identify candidate genes and pathways for *Wolbachia* to act upon.

**Acknowledgements**

We thank Maria Chaplinska for collecting the developmental embryo series and sequencing the 5’RACE *Aj-tra* fragment, Rogier Houwerzijl and Peter Hes for assistance with wasp and fly culturing.