Parental and endosymbiont effects on sex determination in haplodiploid wasps
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Chapter 7

General discussion
The conserved outcome of sex determination, i.e. two distinctly differentiated sexes, is in sharp contrast with the underlying wealth of genetic mechanisms and genes involved. It is also remarkable how endosymbionts can seemingly easily take over control of sex determination mechanisms to enhance their own transmission. Two central questions that I have addressed throughout this thesis are (1) to what extent is there conservation in the insect sex determination cascade and (2) how can endosymbionts interfere with the expression of key sex determination genes?

Identification of sex determination genes

The emergence and establishment of Next Generation Sequencing (NGS) techniques facilitates the discovery of sex determination genes. These comprise orthologs of known sex determination genes, transformer (tra), transformer-2 (tra2) and doublesex (dsx), in new study systems as well as new candidate genes in the sex determination cascade. Orthologs of tra and dsx can easily evade detection by PCR with degenerate primers due to their fast sequence divergence. Conserved TRA/TRA2 binding sites have aided detection in Diptera, but knowledge of such conserved motifs is lacking in other insect orders. Identification of primary signals (on top of the cascade) is an even more challenging endeavour because of lack of conservation at this level of the cascade as each species or closely related group of species may have another gene as its primary signal.

A significant number of discoveries have been made since the putative detection of tra and dsx homologs that we reported in Chapter 2, marking a period of less than three years. Tra homologs were detected in various Hymenoptera (Jia et al., 2016) and investigations were started for presence of possible tra homologs outside insects, resulting in the identification of a tra homolog without sex-specific splicing in the hemichordate Saccoglossus kowalevskii (Suzuki et al., 2015). Possible orthologous dsx sequences were identified across a range of hexapod species (Price et al., 2015). A notable lack of sex-specific dsx splicing was found in Sciara flies, which display alternative dsx splice forms that are present in both sexes (Ruiz et al., 2015). Seemingly every newly examined species reveals new features in sex determination regulation, which calls for an extensive screen of the conservation of the sex determination cascade and the functionality of the genes therein.

Our studies in Asobara tabida (Chapter 4), Asobara japonica (Chapter 6) and Leptopilina clavipes (Chapter 5) were facilitated by the assembly of genomic and transcriptomic datasets. The combination of these genomes and transcriptomes with molecular identification of transcripts ensures correct identification of sex determination genes as variable as tra and dsx (see box 7.1). Whereas the availability of genomes and transcriptomes allowed us to identify the sex determination genes tra, tra2 and dsx, it did not enable us to identify primary signals in these species. Upstream additions to the sex determination cascade may only be conserved within closely related species and no homologs of any candidate primary signals are found in these hymenopterans. Still, genome sequence screening yielded a novel addition to the cascade in L.
the duplication transformerB (traB), but this gene requires functional testing before its putative role as primary signal in the sex determination mechanism can be confirmed. Possible approaches are parental RNA interference (pRNAi) to assess its necessity as a maternally provided mRNA, and Yeast-2-Hybrid assays to examine a possible protein-level interaction with tra2. In general, the identification of primary signals requires comparative transcriptomics, and potentially short RNA screens, of early embryos of the study species.

Conservation and evolution of the insect sex determination cascade

The central switch of insect sex determination mechanisms directs development into the female or male mode (Bopp et al., 2014). In insects, three key genes known to be functionally conserved in this process are tra, tra2 and dsx. The presence of this switch is required for the proper development of two sexes in a sexually reproducing species. As asexual reproduction requires at least the possibility of female differentiation, presence of female-determining elements of the sex determination cascade are expected, such as ensuring the maintenance of functional TRA by autoregulation of female specific tra mRNA splicing.

Sex determination studies on haplodiploid species were at the start of this project limited to the Complementary Sex Determination (CSD) mechanism of Apis mellifera and the Maternal Effect Genomic Imprinting Sex Determination (MEGISD) of Nasonia vitripennis. Screens for CSD within the Hymenoptera and the appearance of thelytokous parthenogenesis in many branches of the order suggested a wide variety of sex determination mechanisms, and potentially of sex determination genes. I aimed to identify the sex determination genes in various hymenopteran species and their regulation gain more insight in the evolution of haplodiploid sex determination mechanisms.

The features of dsx, tra2 and tra are described in box 7.1. In this thesis I focus on the tra/tra2 level of the sex determination cascade, as dsx regulation appears conserved in Hymenoptera. We documented a conserved ortholog of dsx in A. tabida (Chapter 4) that features the characteristic sex-specific splicing of this sex determination switch gene (Table 7.1). Dsx does require further attention in these systems as it is the first element in the sex determination cascade that appears shared in functionality between all studied hymenopteran sex determination mechanisms. Its sequence with potential binding sites for the TRA/TRA2 complex and the timing of its appearance in zygotic transcription may provide grips for further elucidation of sex determination mechanism.

The often neglected tra2 has thus far only been studied in Apis mellifera among Hymenoptera (Nissen et al., 2012). This thesis adds conserved orthologs of tra2 in N. vitripennis (Chapter 3), A. tabida (Chapter 4) and A. japonica (Chapter 6). Within Asobara a remarkable pattern of female-specific splicing of tra2 was found in A. japonica, but not in A. tabida. Moreover, tra2 sequences demonstrated limited conservation between these two species, indicating that even this previously considered conserved element of the sex determination cascade can show surprising variability.
Table 7.1. Presence per species of tra(fem), paralogs of tra (csd and traB), tra2 and dsx mRNA in adult females, adult males and as maternal provision in embryos prior to zygotic transcription. mRNA marked in black are abundantly present, mRNA marked in grey are rare. These rare mRNA in tra and dsx may signify splicing towards the default male mode. mRNAs between brackets are predicted and require further testing.

<table>
<thead>
<tr>
<th>Nasonia vitripennis</th>
<th>Apis mellifera</th>
<th>Asobara tabida</th>
<th>Asobara japonica (arrhenotokous)</th>
<th>Leptopilina clavipes (arrhenotokous)</th>
<th>Asobara japonica (thelytokous)</th>
<th>Leptopilina clavipes (thelytokous)</th>
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Nasonia vitripennis: Oliveira et al. 2009, Verhulst et al. 2010a, this thesis (chapter 3)
Asobara tabida: This thesis, chapter 4
Asobara japonica: This thesis, chapter 6
Leptopilina clavipes: This thesis, chapter 5
* A.H. Rensink, unpublished data
† W-J. Ma, thesis box 7.1
‡ F. Chen, unpublished data
§ E. Geeverink, unpublished data
Tra acts at the same level as tra2 in the sex determination cascade and the products of these two genes are suggested to form a protein/protein complex. Whereas tra2 is easily recognized by its high sequence conservation, tra is not when searching in sequence databases of (closely) related species. Only one motif in the TRA protein, the Ceratitis-Apis-Musca (CAM) domain (Hediger et al., 2010), is a discriminating feature. The CAM domain is shared between different insect orders that possess an autoregulatory loop of tra splicing and is notably absent from D. melanogaster tra (Kato et al., 2010), that has no tra autoregulation (Siera & Cline, 2008). Therefore, the CAM domain has been suggested to be a regulating motif for tra autoregulation. Indeed, male specific TRA isoforms containing a CAM-domain have not been documented in males of any insect species. A putative CAM domain is, however, present in non-sex-specific isoforms of TRA in A. tabida (Chapter 4), A. japonica (Chapter 6) and L. clavipes (Chapter 5). In both Asobara species the non-sex-specific tra (traNSS) mRNAs are transcribed from the orthologous tra locus, while L. clavipes transcribes non-sex-specific sequences encoding a putative CAM domain at a paralogous locus. Aside from these non-sex-specific versions of tra, all three species possess female-specific traF and male-specific traM splice variants that match conserved patterns of tra splicing in Hymenoptera and other insects (Table 7.1).

Maternal provision of sex determination genes

Maternal provision of female-specific tra and non-sex-specific tra2 mRNA by females to their oocytes is a conserved feature of insect sex determination. Maternal traF provision has been reported for Ceratitis capitata (Pane et al., 2002), the Anastrepha genus (Ruiz et al., 2007b), the Bactrocera genus (Lagos et al., 2007; Morrow et al., 2014), Lucilia cuprina (Concha & Scott, 2009), Musca domestica (Hediger et al., 2010), and Tribolium castaneum (Shukla & Palli, 2012a), but it is not part of the CSD mechanism of Apis mellifera (Gempe et al., 2009). It is a central feature of the MEGISD mechanism of Nasonia vitripennis (Verhulst et al., 2010a). We found that maternal provisioning of traF mRNA is absent in A. tabida (Chapter 4) and in arrhenotokous individuals of A. japonica (Chapter 6) and L. clavipes (Chapter 5). Therefore, maternal provision of traF splice variants can no longer be considered a conserved feature in insect sex determination. The lack of maternal traF provisioning in Asobara and Leptopilina will however require a different explanation than the absence in A. mellifera. Alternative tra transcripts are maternally provided to arrhenotokous Asobara and Leptopilina oocytes. An intriguing option is that these splice variants would fulfill a role in activating zygotic tra transcription. The alternative tra maternal provision consists of the non-sex-specific tra splice variants in the investigated Asobara species (Chapter 4 and 6) and of traB in L. clavipes (Chapter 5 and discussed below). Table 7.1 provides an overview of different genes and splice forms present in males, females and embryos of various hymenopteran species. Complementary sex determination (CSD) has been excluded for A. tabida and A. japonica (Beukeboom et al., 2000; Ma et al., 2013), suggesting a different role for non-sex-specific CAM domain encoding
TRA isoforms in these systems. Knockdown of these tra variants by pRNAi would illuminate a possible role in the activation of the female sex determination pathway.

In Hymenoptera maternal provision of tra2 occurs in A. mellifera, where it is required in early embryonic stages to regulate fem pre-mRNA splicing into the female mode in diploid eggs and into the male mode in haploid eggs (Nissen et al., 2012). Our pRNAi study in N. vitripennis (Chapter 3) demonstrates its conserved role in sex determination as a maternally provided component. The clear provision of tra2 in both A. tabida and A. japonica suggests that this maternal effect may be shared in all Hymenoptera. Tra2 is spliced into multiple forms in a large number of species, but this pattern is not sex-specific except for the male germline splice form in D. melanogaster (Mattox et al., 1990). A peculiar pattern of female-specific tra2 splicing emerged from our studies in A. japonica (Chapter 6). The longer tra2 splice forms are specifically present in the female A. japonica germline (M. van Leussen, unpublished data). Further studies are needed to assess how interactions between TRA and TRA2 have been modulated and whether these splice variants are interacting with other elements in the sex determination cascade.

Duplications of tra in Hymenoptera

Duplications of tra have thus far only been observed in the order of Hymenoptera (Hasselmann et al., 2008a; Schmieder et al., 2012; Privman et al., 2013; Koch et al., 2014). Recently, the superfamilies Chalcidoidea (Jia et al., 2016) and Cynipoidea (Chapter 5) families were added. It is unknown whether these copies, other than the csd gene of the honeybee, are involved in sex determination. No functional studies have been performed on e.g. ants or bumblebees. The paralog traB copy of L. clavipes, as well as the traB and traC copies in Ceratosolen solmsi (Jia et al., 2016), encode a putative CAM domain. An important distinction between these two species is that the tra paralogs of C. solmsi are female-specifically expressed, whereas the traB paralog of L. clavipes is present in all life stages of both sexes (Table 7.1). Conserved domains encoded on a paralogous locus are also seen in A. mellifera, where the complementary sex determiner (csd) gene transcribes splice variants resembling the female specific fem(tra) forms (Hasselmann et al., 2008a; Gempe et al., 2009). Even though L. clavipes shares with A. mellifera a duplication event of tra, L. clavipes appears to have a sex determination mechanism without complementary loci, based on its gamete duplication type of thelytoky and lack of increased male ratios under inbreeding (Pannebakker et al., 2004b) (K. Kraaijeveld, pers. comm.). Additionally, in A. mellifera csd transcripts are not maternally provided, yet the paralogous traB transcripts in L. clavipes are maternally provided to all eggs (Chapter 5). Taken together, this suggests a different role for the tra duplication in L. clavipes compared to A. mellifera.