Parental and endosymbiont effects on sex determination in haplodiploid wasps
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Box 7.2 DNA methylation as a possible epigenetic control of sex determination

Imprinting mechanisms in sex determination

Sex determination in haplodiploid species can be governed by either the allelic state of one or multiple, so called complementary sex determination (CSD) loci, or alternative signals. One alternative model of haplodiploid sex determination is genomic imprinting sex determination (GISD) (Poirie et al., 1992; Beukeboom, 1995), it is based on epigenetic differences between the maternal and paternal chromosome set. To determine whether DNA methylation as epigenetic control of sex determination is possible in *Asobara* and *Leptopilina*, their genomes were searched for the presence of potential DNA methyltransferase genes.

DNA methylation in insects

DNA methylation is one of the most widespread forms of epigenetic modification (Suzuki & Bird, 2008; Jones, 2012) and has been documented for many insect genomes (Glastad et al., 2011, 2014). DNA methyltransferases (DNMTs) are considered to be key factors responsible for DNA methylation (Goll & Bestor, 2005). *Dnmt1* is implicated in DNA methylation maintenance during cell division cycles. Conversely *Dnmt3* is suggested to be a *de novo* methyltransferase indicating that it creates new methylation patterns (Goll & Bestor, 2005). Both *Dnmt1* and *Dnmt3* vary in their copy number, even within insects (Glastad et al., 2011). *Dnmt3* consists of 2 copies in the hemipteran *Acyrthosiphon pisum* (The International Aphid Genomics Consortium, 2010; Walsh et al., 2010), but is otherwise detected as a single homolog. More variation is present in the copy number of *Dnmt1*, which switches even in Hymenoptera from a single copy in various ant species (Bonasio et al., 2010; Smith et al., 2011a, 2011b; Suen et al., 2011; Wurm et al., 2011) to two copies in the honeybee *A. mellifera* (Wang et al., 2006) to three copies in the wasp *N. vitripennis* (Werren et al., 2010).

In insects, absence of *Dnmt1* orthologs has thus far only been recorded for a number of dipteran species (Hung et al., 1999; Tweedie et al., 1999; Holt et al., 2002; Nene et al., 2007), in which case they also all lack *Dnmt3*. In both early embryos and the adult stage of *Drosophila melanogaster*, DNA methylation appears absent, just as in adults of *T. castaneum* (Zemach et al., 2010; Raddatz et al., 2013). The latter does possess a *Dnmt1* ortholog, but, again, misses a *Dnmt3* copy (Richards et al., 2008; Zemach et al., 2010). *Dnmt3* losses are widespread in holometabolous insects, including the branch containing Coleoptera, Diptera and Lepidoptera (Glastad et al., 2011). Furthermore, losses of *Dnmt3* have been recorded for the order Phthiraptera in the paraneopteran insects (Kirkness et al., 2010; Werren et al., 2010). Absences of hymenopteran *Dnmt3* genes were documented in the paper wasps *Polistes canadensis* and *Polistes dominula* (Patalano et al., 2015; Standage et al., 2016). The syntenic region of the latter
species revealed conservation with the *A. mellifera* Dnmt3 region, but only the Dnmt3 gene itself was missing (Standage *et al*., 2016). Dnmt2, also known as tRNA aspartic acid methyltransferase 1 (Trdmt1), uses the conserved DNA methyltransferase mechanism to methylate tRNA instead (Goll *et al*., 2006; Jurkowski *et al*., 2008). Species which only possess Dnmt2 appear to lack detectable methylation patterns (Raddatz *et al*., 2013). Dnmt2 is not considered a candidate gene for epigenetic control of sex determination based on these features. Furthermore, its presence appears to be conserved in all Hymenoptera, which indicates a different function (unpublished data). This leaves Dnmt1 and Dnmt3 as possible candidates for epigenetic regulation in sex determination and the presence of these genes was investigated for the hymenopteran species described in this thesis.

**The role of DNA methylation in development of Hymenoptera**

Caste-specific patterns of methylation were observed in various social Hymenoptera (Lyko *et al*., 2010; Bonasio *et al*., 2012). Studies in these systems link DNA methylation patterns to alternative splicing (Bonasio *et al*., 2012; Foret *et al*., 2012). Knockdown of Dnmt3 expression in *A. mellifera* larvae that would otherwise develop into worker bees resulted in queen-like development (Kucharski *et al*., 2008). Furthermore, RNA interference of Dnmt3 in adult honeybees changed splicing patterns, particularly linked to exon skipping and intron retention (Li-Byarlay *et al*., 2013).

Though different methylation patterns were associated to caste determination, no link has yet been shown to sex determination. Interesting candidates are the multiple Dnmt genes of *N. vitripennis* (Werren *et al*., 2010). In *N. vitripennis*, Dnmt1a,c and Dnmt3 mRNA are provided maternally to the embryo and Dnmt1a is essential for early development (Zwier *et al*., 2012). Knockdown of maternal mRNA provision of Dnmt1a, however, did not change sex-specific splicing of the key sex determination genes *tra* and *dsx*. This does not exclude a possible role of Dnmt genes in sex determination in *Nasonia* or other systems, as the relative importance of different Dnmt genes is unknown and their presence, copy number and functionality display high variability.

**Presence and absence of Dnmt genes in Braconidae**

*Asobara tabida* and *Asobara japonica* do not possess the complete toolkit for DNA methylation. Tblastn searches with hymenopteran Dnmts only identified Dnmt3 homologs; one copy in each species. Despite the lack of Dnmt1, which is the DNA methyltransferase that is presumed to be responsible for methylation maintenance, the occurrence of global DNA methylation in adult *A. tabida* was confirmed by measuring genome-wide levels of 5-methylcytosine (A. de Haan, unpublished data).

To assess whether a possible absence of Dnmt1 is restricted to the *Asobara* genus or a common feature of the braconid family we searched the published transcriptome of *Cotesia vestalis* (Misof *et al*., 2014) using tblastn for Dnmt homologs. A homolog of Dnmt1 (GAUP02012000 and
GAUP02010942) was detected, but no homolog of Dnmt3, suggesting multiple losses of Dnmts in the family of Braconidae.

**Presence and absence of Dnmt genes in the Leptopilina genus and beyond**

We searched the genome of *Leptopilina clavipes* (Kraaijeveld et al., 2016) for Dnmt homologs. No sequence similarity was found for Dnmt3, however, *L. clavipes* does possess a single Dnmt1 homolog. This single Dnmt1 copy is in stark contrast to the three Dnmt1 genes in its closest relative *N. vitripennis* (Werren et al., 2010), again indicating the variability in Dnmt genes presence and number. The transcriptomes of *L. clavipes* (Misof et al., 2014), *Leptopilina heterotoma* and *Leptopilina bouardi* (Goecks et al., 2013) were screened for Dnmt3 homologs. None were detected, suggesting an absence of Dnmt3 in the entire *Leptopilina* genus. Other species of Hymenoptera, e.g. *Orussus abietinus* and *Tenthredo koehleri* (Misof et al., 2014), possess homologs of Dnmt1 and Dnmt3. However, *Xyela alpigena* (Peters et al., 2014) in the most basal family (Xyelidae) (Peters et al., 2011), appears to lack both Dnmt1 and Dnmt3. As *Leptopilina*, *Cotesia* and *Xyela* are independent branches of Hymenoptera (see Figure 1 in Chapter 1), this would indicate that Dnmt3 has been lost multiple times in the order of Hymenoptera. Moreover, the apparent absence of Dnmt1 in *Xyela* and *Asobara* suggests multiple secondary losses of this DNA methyltransferase gene as well.

**DNA methylation toolkits and functionality of methylation**

Absence of DNA methylation genes does not necessarily imply an absence of DNA methylation (Glastad et al., 2014). DNA methylation has been reported for a range of insects, but when comparing species, different genes and different regions of genes are methylated. Data for insect genomes are currently too fragmented to link an absence of methylation genes to specific epigenetic processes. Especially Dnmt3 is exemplary in its widespread absence from insects. This suggests that Dnmt3 has either a non-essential role in insect genome methylation, or can be replaced by other genes in different insect groups. The absence of Dnmt1 in *Asobara* wasps seems to be an exceptional case of a recent gene loss, as amongst Hymenoptera, only *X. alpigena* appears to also lack Dnmt1. Whether *Asobara*, that lacks Dnmt1, or *Leptopilina*, that lacks Dnmt3, have lost the ability to pass on epigenetic signals to their offspring, and whether this impacts the sex determination mechanism would be an interesting objective for further studies.

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Modes of sex determination and endosymbiont-induced thelytoky

Our results described in chapter 5 and 6 are the first to document possible interference of Wolbachia at the tra level of the sex determination cascade. The unique opportunity to compare arrhenotokous and thelytokous systems allows for study of how endosymbionts can manipulate sex determination regulation. Any difference between the regulation of sex determination genes in the two systems may point at a manipulation by the endosymbiont. Moreover, sex determination regulation can be studied when the endosymbionts are removed from the thelytokous lineage (Chapter 6), and one can observe whether this regulation then reverts to a pattern matching the arrhenotokous system.

Previous studies on Wolbachia interference with the sex determination cascade concerned systems without a conserved copy of tra. The lepidopterans Ostrinia scapulalis and Ostrinia furnacalis are infected with male-killing Wolbachia that act by lethal feminization of genetic males (Kageyama & Traut, 2004; Sugimoto et al., 2010; Fukui et al., 2015). Sex determination regulation is, however, largely unknown in Lepidoptera which makes it difficult to elucidate the Wolbachia action. Dsx is conserved at the bottom of the cascade with sex-specific splice variants (Wang et al., 2014). Tra2 does not regulate dsx splicing (Suzuki et al., 2012) and no homolog of tra has been detected (see also box 7.1 and Chapter 2). Dsx splicing is apparently altered by Wolbachia infection (Sugimoto & Ishikawa, 2012), but further details of sex determination regulation are unknown. Recently, upstream elements of the B. mori sex determination pathway have been identified, consisting of a feminizing piRNA fem which targets the Masc gene that controls both masculinization and dosage compensation (Kiuchi et al., 2014). This Masc gene is also found in O. furnacalis, where it is repressed in the presence of Wolbachia (Fukui et al., 2015). The repression of Masc results in a lack of dosage compensation in Wolbachia infected embryos (Sugimoto et al., 2015). It is not yet identified how Wolbachia interacts with Masc or an upstream factor, or how Masc is connected to dsx.

My study not only illustrates the importance of maternal effects in haplodiploid sex determination but also suggests a link between maternal provisioning and the evolution of thelytoky. Wolbachia interference with sex determination in A. japonica and L. clavipes reveals at least one parallel phenomenon: the shift to maternal provision of female-specific tra mRNA. Maternal provision is a signature of N. vitripennis sex determination (Verhulst et al., 2010a) (Chapter 3) and this feature may be widespread in non-CSD systems. Manipulation of the maternal provision may be the single route by which Wolbachia interacts with the sex determination mechanisms of L. clavipes and A. japonica to induce parthenogenesis. However, the conservation of tra in all but one lineage of Hymenoptera (Box 7.1) is not enough to explain the occurrence of thelytoky within the Hymenoptera. More thelytokous systems need to be studied for their sex determination regulation to understand why some groups show frequent thelytoky whereas others do not.
Compatibility of sex determination mechanisms and endosymbiont-induced female development

Reproductive mode and sex determination are mutually dependent, i.e. some forms of reproduction are restricted by the mechanism of sex determination and vice versa. One question is whether the MEGISD model can be compatible with thelytoky. Under MEGISD female development depends on a paternally provided genome. A maternal silencing mechanism is in place to ensure that only fertilized eggs receive an active paternal allele to start the female developmental pathway. Wolbachia could remove the maternal imprinting or mimic the paternal imprint, as endosymbionts have been documented to change their host’s imprinting pattern (Negri et al., 2009). More mechanistic details are needed about Wolbachia action before a role in imprinting can be substantiated. Another possibility is that the endosymbiont makes the host’s imprinting mechanism obsolete. The Wolbachia-induced maternal provision of Aj-traF (Chapter 6) and Lc-tra (Chapter 5) would be sufficient to start zygotic tra transcription without a paternal genome.

Another open question about thelytokous systems is whether diploidization by Wolbachia directly results in female development as a consequence of host sex determination, or whether Wolbachia needs to secondarily induce feminization. The switch to traF provisioning in the presence of Wolbachia would suggest the latter. In A. japonica both steps (diploidization and feminization) are separately induced by Wolbachia (Ma et al., 2015), but in Encarsia hispida Cardinium bacteria only causes feminization of already diploidized eggs (Giorgini et al., 2009). Such separation of diploidization and feminization needs to be tested in more endosymbiont-induced thelytokous systems.

Wolbachia versus maternal control of sex determination

To what extent is infectious parthenogenesis depended on the sex determination mechanism? My results have shown that an absence of traF maternal provision in an arrhenotokous system could open the possibility for an endosymbiont to overtake its sex determination. If inducing traF mRNA provisioning by the endosymbiont is sufficient to start female development, a range of sex determination mechanisms may be conducive to infectious thelytoky. I predict that those sex determination systems share a lack of maternal provision of traF in their arrhenotokous mode.

Many questions remain about the mechanisms with which Wolbachia takes over its host sex determination. Endosymbiont-induced maternal provision of traF is one possibility, but it requires further testing whether this is a widespread mechanism. Interestingly, maternally controlled traF provision could also be seen as a counter-measure of the host to prevent endosymbiont infection. It would be interesting to survey different branches of Hymenoptera, particularly non-CSD associated lineages, for maternal provision of different tra transcripts. The increasing availability of genomic and transcriptomic data enables such a pursuit. The mechanistic interactions between endosymbiont manipulation of host reproduction and host sex determination promises to be an intriguing field of future research. This should provide
more insight into the directions that the evolutionary arms race between host and endosymbionts can take and make clear who is in control.

CONCLUDING REMARKS

The central switch of sex determination is conserved in haplodiploid systems. Its key actors can however be hard to recognize. Orthologs of the sex determination genes \textit{tra2}, \textit{tra} and its target \textit{dsx} are found in \textit{A. tabida} (Chapter 4), \textit{A. japonica} (Chapter 6) and \textit{L. clavipes} (Chapter 5). Each species displays distinct and characteristic female and male splice forms of these genes and production of female-specific TRA (TRA\textsuperscript{F}) starts female development. Yet, each species exhibits specific deviations from the \textit{N. vitripennis} sex determination mechanism, in particular in their maternal provision of specific \textit{tra} and \textit{tra2} mRNAs. It remains an open question which forces drive this variation in sex determination mechanisms. A tempting possibility is that endosymbionts form such an evolutionary pressure. Solving this question requires further mechanistic research into sex determination and endosymbiont action.