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 boulardi* (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-tra*^{F} (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 *tra*^{F} 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 *tra*^{F} maternal provision in an arrhenotokous system could open the possibility for an endosymbiont to overtake its sex determination. If inducing *tra*^{F} 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 *tra*^{F} in their arrhenotokous mode.

Many questions remain about the mechanisms with which *Wolbachia* takes over its host sex determination. Endosymbiont-induced maternal provision of *tra*^{F} is one possibility, but it requires further testing whether this is a widespread mechanism. Interestingly, maternally controlled *tra*^{F} 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 *tra2*, *tra* and its target *dsx* are found in *A. tabida* (Chapter 4), *A. japonica* (Chapter 6) and *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^F) starts female development. Yet, each species exhibits specific deviations from the *N. vitripennis* sex determination mechanism, in particular in their maternal provision of specific *tra* and *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.