variable (weighted by the brood size via the logit function), with a quasi-binomial error structure to correct for over-dispersion (Crawley, 2007). To compare diploid male numbers between antibiotic treatments, a glm was used in which the number of diploid males was the response variable and antibiotic concentration a qualitative explanatory variable with a log link function and a quasi-poisson error structure to correct for over-dispersion (Crawley, 2007). Multiple comparisons of diploid male numbers among different antibiotic treatments were performed with the Tukey test, as implemented in the R package multcomp for general linear hypotheses (Hothorn *et al.*, 2008).

A glm was used to compare the *Wolbachia* titer among females of the various antibiotic treatments, measured as the gene quantity of *gatB* relative to the endogenous controls. As *Wolbachia* titers were normally distributed, this glm used an identity link function and a gaussian error structure. *Wolbachia* titer was used as the response variable and the antibiotic concentration as a quantitative explanatory variable. To test whether *Wolbachia* titer differed between individuals with different ploidy levels, diploids (females and males) were compared with haploid males using a Wilcoxon rank-sum test. The *Wolbachia* titer between diploid females and diploid males was analysed in a similar way.

Glm were also used to compare brood sizes and daughter proportions among introgression generations, with antibiotic concentration as a quantitative explanatory variable. Brood size was modeled as the response variable with a quasi-poisson error structure to correct for over-dispersion, and proportion of female offspring as the response variable with a quasi-binomial error structure to correct for over-dispersion respectively (Crawley, 2007). To check for the possibility of hybrid breakdown causing male mortality during the introgression experiment, male offspring numbers from virgin sexual and virgin introgressed F1 (50% sexual–50% thelytokous) females were compared with a glm model. In this glm, female genotype is the explanatory variable and the male offspring number the response variable with a quasi-poisson error structure to correct for over-dispersion (Crawley, 2007).

5.3 Results

5.3.1 Frequency and ploidy of males

All tested thelytokous *A. japonica* females (n=16, 4 per strain) were infected with *Wolbachia*, as shown by PCR using primers for the *Wolbachia*-specific *wsp* gene. Males were found at frequencies of 0.7%, 1.2% and 1.2% (n=approximately 2,500 offspring per strain) among the progenies of females from the thelytokous strains SPP, HR and TK, respectively. For the KG strain, a total of 220 males (1.2%) was found among 18,000 offspring (Table 5.1). These results corroborate the results of Reumer *et al*. 2012. Flow cytometry revealed that 73% of these males (218 out of 298) were haploid and 27% (80 out of 298) were diploid, with proportions of diploid males ranging from 7-39% among different thelytokous strains (Table 5.1).

Table 5.1 Number and ploidy level of males in progenies of thelytokous females of four different strains of *A. japonica*.

Line	No. haploid males	No. diploid males	No. total males	Prop. diploid males
SPP	11	7	18	39%
KG	153	67	220	30%
HR	26	4	30	13%
TK	28	2	30	7%
Total	218	80	298	27%

5.3.2 Effect of *Wolbachia* titer

To investigate whether male production depended on *Wolbachia* titer of parental females, thelytokous females were offered host larvae that had been fed different antibiotic concentrations. The vast majority of emerged wasps from these antibiotics-treated hosts were females. These females were then offered untreated host larvae for oviposition and again the offspring were counted and sexed. The proportion of male offspring (haploid and diploid combined) gradually increased with higher concentrations of antibiotics administered to the

hosts, ranging from 0% to approximately 60% (glm, $F_{1, 91}$=158.88, P<0.0001, Figure 5.1). *Wolbachia* titer in mothers was verified by qPCR and revealed that higher antibiotic concentrations indeed resulted in lower *Wolbachia* titers (glm, $F_{1, 19}$=63.70, P<0.0001; Figure 5.2). Diploid males occurred at very low frequencies among the progeny of all antibiotic-treated thelytokous females, and there was no significant difference among antibiotic treatments in the number of diploid males (glm, $F_{9, 91}$=0.916, P=0.341; Table 5.2).

Table 5.2 Number of haploid and diploid males among progenies of thelytokous HR strain females treated with different antibiotic concentrations. RIF = rifampicin, see text for concentrations.

Treatment	**RIF0**	**RIF1**	**RIF2**	**RIF3**	**RIF4**	**RIF5**	**RIF6**	**RIF7**	**RIF8**	**RIF9**
Haploid males	0	2	4	0	0	1	6	13	13	21
Diploid males	0	0	0	0	1	0	0	2	1	1
Total tested	0	2	4	0	1	1	6	14	14	22

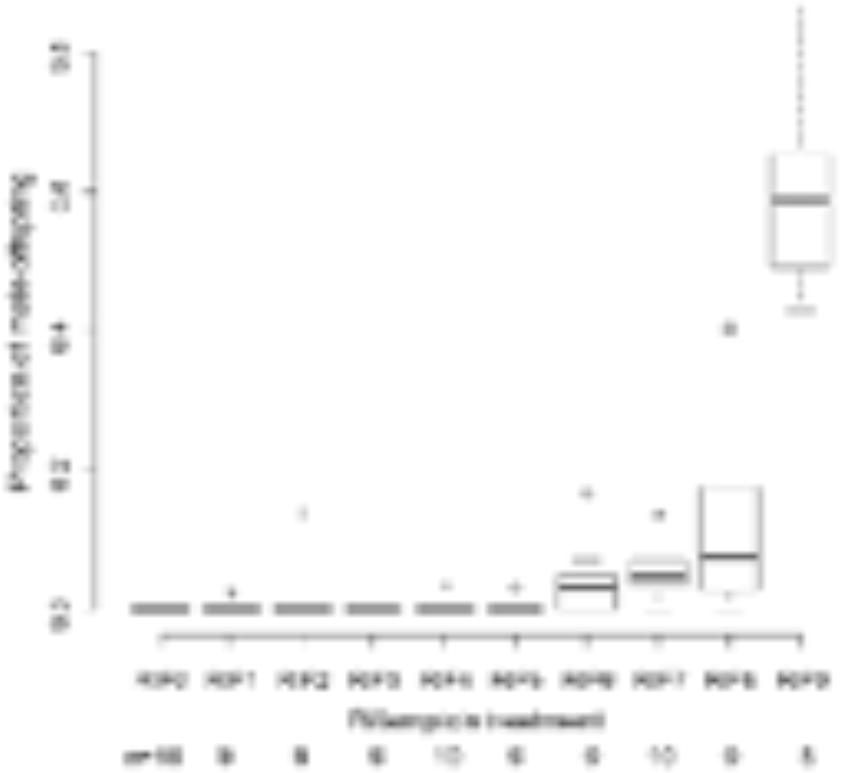

Figure 5.1 Proportion of males among progenies of thelytokous HR strain females treated with different concentrations of rifampicin (RIF). RIF0 is the no antibiotics treatment control, RIF1 to RIF9 depict the series of different concentrations of rifampicin (0.00001, 0.00005, 0.0001, 0.001, 0.005, 0.01, 0.05, 0.1 and 1mg/g respectively). Sample sizes (n) are given for each treatment.

We then assessed the association between *Wolbachia* titer and ploidy, and between *Wolbachia* titer and the gender of thelytokous offspring: haploid males, diploid males and diploid females. All tested diploid females and diploid males were infected by *Wolbachia*. In contrast, only 7 out of 12 of haploid males were infected. The *Wolbachia* titer of diploid individuals (females and males pooled) was significantly higher than that of haploid males (Wilcoxon test, W=5, $P<0.0001$; Figure 5.3), suggesting that a minimum *Wolbachia* titer is required to cause diploidization of unfertilized haploid eggs. Moreover, among the diploid individuals the *Wolbachia* titer of females was significantly higher than that of males (Wilcoxon test, W=59, $P=0.0088$; Figure 5.3), further suggesting an additional role for *Wolbachia* titer in the feminization of diploid embryos.

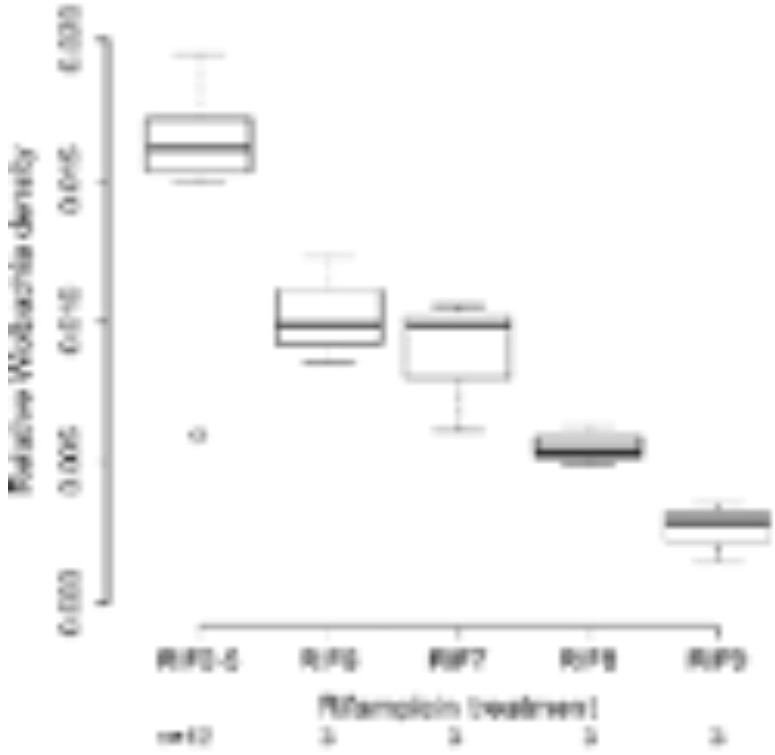

Figure 5.2 *Wolbachia* titer in parental females as a function of rifampicin (RIF) treatment. Treatments RIF0 to RIF5 were pooled for qPCR analysis as they did not differ in the number and proportion of males among progenies. *Wolbachia* titer is determined by qPCR of the *gatB* gene relative to the reference genes *COI* and *ITS2*. Sample sizes (n) are given for each treatment.

5.3.3 *Wolbachia* effect on host sex determination

We hypothesized that if the host's female sex determination pathway was no longer be exposed to selection and therefore has lost functionality, replacing the sexual genome by the thelytokous genome in the absence of *Wolbachia* may cause diploid fertilized eggs to develop into males rather than females. Upon introgression, diploid males are expected to develop from fertilized eggs of females from the first introgression generation onward if the mutations in the sex determination pathway are dominant, if mutations are recessive an increasing proportion of diploid males is expected when more thelytokous alleles are introgressed.

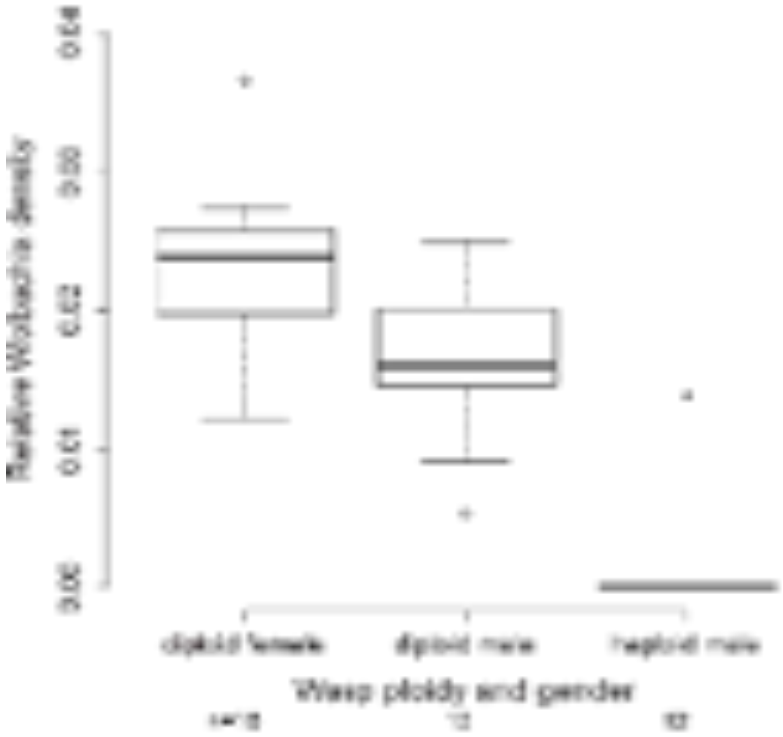

Figure 5.3 *Wolbachia* titer in diploid females, diploid males and haploid males in the untreated thelytokous KG strain. *Wolbachia* titer is determined by qPCR of the *gatB* gene relative to the reference genes *COI* and *ITS2*. Sample sizes (n) are given for each treatment.

An introgression experiment was performed to test for possible defects of the female sex determination pathway in the thelytokous genome as a consequence of *Wolbachia* infection. If this were the case, we expected that the proportion of diploid sons from fertilized eggs wouldincrease with an increasing representation of the thelytokous genome introgressed in the sexual line. The production of diploid sons would then generate a corresponding decrease of the proportion of daughters, since some fraction of fertilized eggs would develop into diploid males instead of females. No diploid individual was found in a random sample of 51 males from the F1 introgression generation. Absence of diploid males is likely not due to mortality, because average F1 brood size in the introgression experiment did not differ from that of the sexual control crosses (80.9±2.4 versus 88.0±3.1, t-test, t=1.8, df=96.0, P=0.076). Introgressed females of generations F1 and G2 produced a higher proportion of female offspring than sexual controls when mated with a male from the thelytokous KG strain (glm, F1 and G2: P<0.009; Figure 5.4),

and a similar pattern was found when these females had mated with sexual AO males (glm, Turkey contrast, all $P<0.001$).

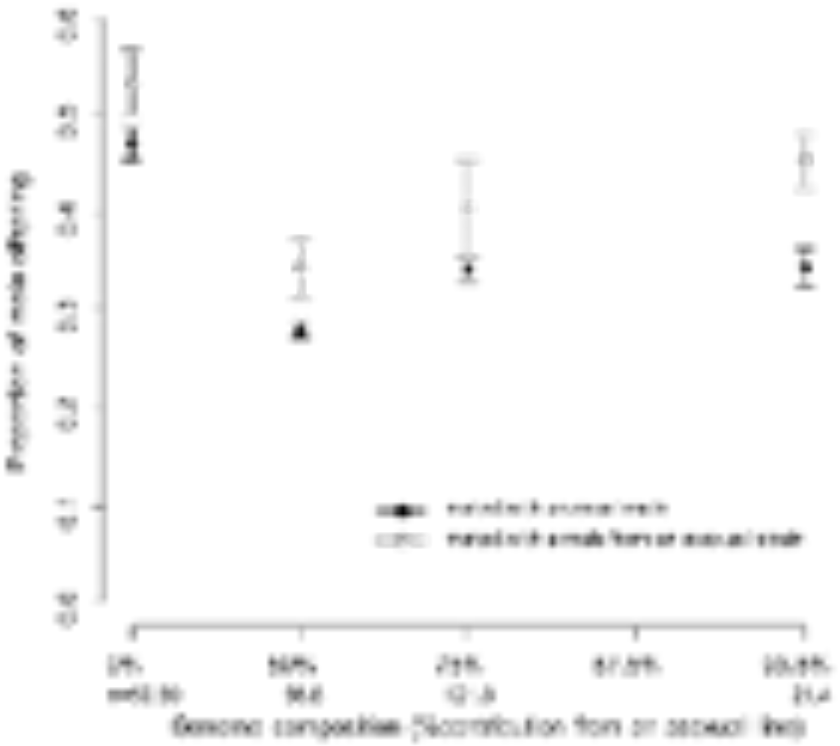

Figure 5.4 The proportion of male offspring in progenies of females with at least one daughter (i.e., females producing only sons are excluded) for different categories of sexual-asexual hybrid females and pure sexual females (100% sexual genome). Females are mated with males from the thelytokous KG (in black) or the sexual AO strain (in grey, as a control). Bars indicate standard errors, *n* indicates the sample size for each generation and F1, G2, and G4 refer to the progressive introgression generations.

The low offspring sex ratios are unlikely to stem from mortality of males as a consequence of hybrid breakdown because all-male broods of virgin sexual females and F1 introgressed females were equal in size (82.3±2.2 versus 82.0±3.0 males; glm, $F_{1,139}=0.006$, $P=0.94$). In addition, females of generation G4 with the highest representation of the thelytokous genome (estimated 93.8%) produced a similar proportion of daughters as the control sexual cross (glm, t=-0.172, $P=0.863$). These combined data provide further evidence that the host sex-determination pathway did not decay under thelytoky as a result of the endosymbiont infection,

and thus not responsible for diploid male occurrence. Rather, this supports the notion that *Wolbachia* is responsible for manipulating host feminization process.

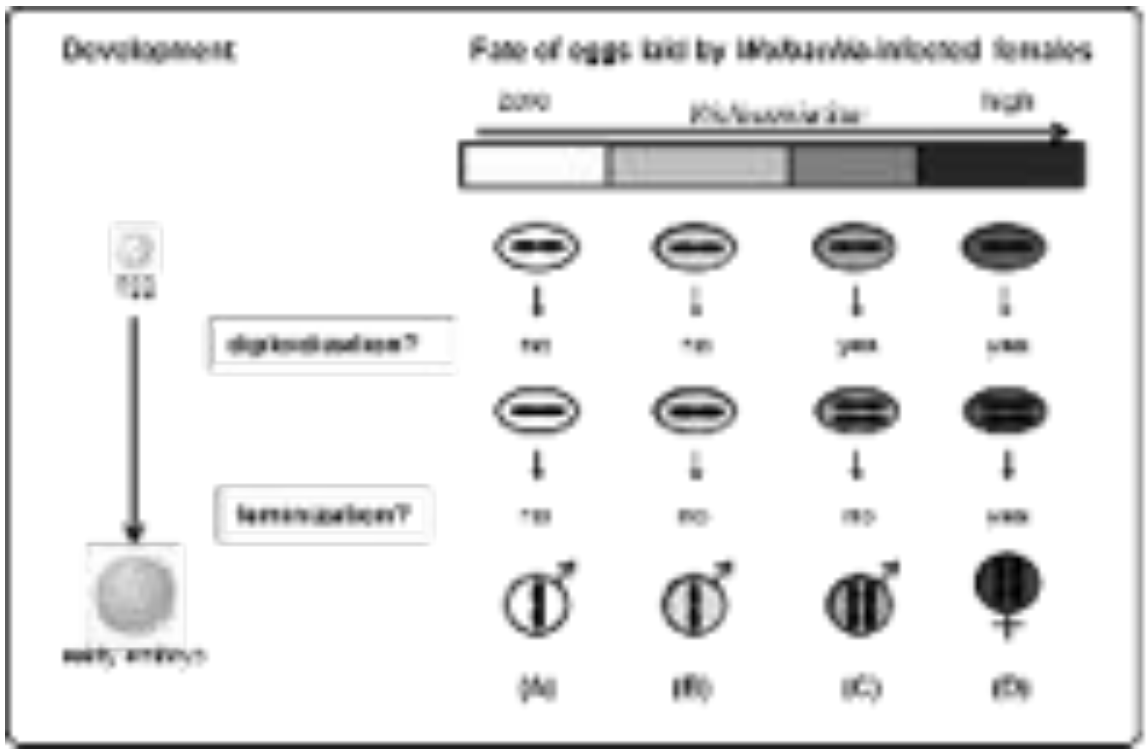

Figure 5.5 A two-step-mechanism model for how endosymbionts induce thelytoky in haplodiploid species. Endosymbionts are considered to induce female development via two distinguished steps: diploidization of the egg followed by feminization of the embryo. Each step relies on a certain threshold of endosymbiont titer during early embryonic development when sex is determined. There are four scenarios: (A) Absence of endosymbionts leads to haploid male development; (B) Low endosymbiont titer fails to initiate both diploidization and feminization, leading to development of haploid males as well; (C) Intermediate number of endosymbionts succeed in diploidization, but are insufficient to induce feminization and result in diploid males; (D) High number of endosymbionts cause both diploidization and feminization, leading to diploid female development. Grey shading indicates endosymbiont titer, ranging from zero (white) to high (black).

5.4 Discussion

In agreement with previous reports (Kraaijeveld *et al*., 2011; Reumer *et al*., 2012), we observed the frequent occurrence of males (0.7-1.2%) in four thelytokous strains of the parasitoid *A. japonica*. Although males are rare or absent in the majority of thelytokous species (Mirab-balou and Chen, 2010; Schwander *et al*., 2013; reviewed in van der Kooi and Schwander, 2014), male frequencies of thelytokous *A. japonica* are high. We found that a significant proportion (7-39%) of these males are diploid rather than haploid. We investigated the possible causes for this high occurrence of males (both haploid and diploid) to develop insights into how *Wolbachia* induces thelytoky in its host.

The most basic explanation of endosymbiont-induced thelytoky is that all-female progenies are the result of diploidization of unfertilized haploid eggs through the action of the endosymbiont (Stouthamer and Kazmer, 1994; Gottlieb *et al*., 2002; Pannebakker *et al*., 2004a). Diploid eggs would then develop into females following the host's haplodiploid sex determination. However, the spontaneous occurrence of diploid males in the progeny of thelytokous females (e.g. Tulgetske, 2010; this study), as well as upon removing of the endosymbiont (Giorgini *et al*., 2009) calls for a reconsideration. Occasional development of haploid males in thelytokous strains has been considered to be the result of (partial) failure of endosymbiont reproductive manipulation, due to maladaptation between two co-evolved parties, or to recent infection or stochastic loss of the bacteria (Heath *et al*., 1999; Reumer *et al*., 2012). However, how diploid males can arise in the broods of thelytokous females has thus far been unexplained.

In this study, we found an effect of *Wolbachia* titer in parental females on male production (haploids and diploids pooled). Similar dosage-dependent effects of *Wolbachia* were found on male production in the thelytokous parasitoid *Muscidifurax uniraptor* (Zchori-Fein *et al*., 2001), on cytoplasmic incompatibility in *Drosophila* (O'Neill and Karr, 1990), and on feminization in the leafhopper *Zyginidia pullula* (Negri *et al*., 2009) and the mosquito *Aedes aegypti* (Lu *et al*., 2012). However, we did not find a similar *Wolbachia* dosage effect in parental females on the production of diploid males, although the probability of detecting such an effect was low given that we found few diploid males overall. Caution should also be taken on the correlation between parental mother's *Wolbachia* titer and diploid males, because of the stochasticity in *Wolbachia* titer in each individual egg and the possibility of delayed antibiotic effects. Future

experiments should quantify *Wolbachia* titer at different stages of embryonic development to reveal the window of *Wolbachia* action leading to diploid males. Haploid males were often not infected at all or, when infected, they carried a much lower *Wolbachia* titer than diploid individuals. This suggests that a minimal *Wolbachia* titer is required for the diploidization of unfertilized haploid eggs. The observation that diploid females carried significantly more *Wolbachia* than diploid males suggests a correlation between the *Wolbachia* density and gender of diploid wasps. To further corroborate this conclusion requires manipulation of *Wolbachia* titer in eggs or early embryos (after sex is determined), which is difficult to perform in practice. Note that we implicitly assume that the measured *Wolbachia* titer in adults is proportional to the titer in their embryonic stage, when the endosymbiont exerted its action. This may be a valid assumption given that Landmann *et al*. (2010) found in nematodes that *Wolbachia* density does not change much during embryonic and early larval developmental stages but proportionally increased with age towards the adult stage. Another possible explanation is that diploid female adults have more bacteria because *Wolbachia* tend to proliferate more in female ovaries than male testes (Landmann *et al*., 2010).

Thus far there is very little knowledge on the mechanisms by which *Wolbachia* induce thelytoky. One possible explanation for the diploid male occurrence is that female sex determination is degenerating under endosymbiont-induced thelytoky. This has been observed in the lepidopterans *Ostrinia scapulalis* (Sugimoto and Ishikawa, 2012) and *Eurema mandarina* (Narita *et al*., 2007). However, our introgression of alleles from a thelytokous into a sexual strain did not result in any diploid males, but led to higher proportions of females among the progenies of multiple generations of introgression. These results indicate that the observed diploid males did not result from disruptions in the female sex determination pathway of thelytokous genomes, and therefore do not support a dependency on *Wolbachia* for proper sex determination in thelytokous *A. japonica*.

Studies of Giorgini *et al*. (2009) and Tulgetske (2010) have implied that diploidization and feminization are separate steps in endosymbiont-induced thelytoky. Our results on thelytokous *A. japonica* corroborate this notion but take it a step further to suggest a causative role of endosymbiont density. We propose a two-step mechanism for the induction of thelytoky by *Wolbachia* in *A. japonica* based on endosymbiont density (Figure 5.5): diploidization of the unfertilized egg is followed by feminization, in which each step critically relies on a different threshold of endosymbiont titer. In this two-step model, the first step involves diploidization of the unfertilized haploid egg. Absence or very low density of endosymbionts in eggs would result

in haploid male development (Figures 5.5A and B). A higher density would result in diploid zygotes that can either develop into males or females (Figures 5.5C and D). This is supported by the observation that haploid males are uninfected or have a low *Wolbachia* titer compared to diploid individuals. The second step involves feminization of diploidized embryos that requires a high *Wolbachia* titer. If this titer is too low, failure of feminization occurs and embryos develops into diploid males (Figure 5.5C). Only a sufficiently high bacterial titer leads to successful diploidization of unfertilized haploid eggs and subsequent feminization of diploid embryos (Figure 5.5D). Note that the window of bacterial titer inducing diploidization but not feminization is assumed to be narrow, given that diploid males are rare overall.

This proposed two-step model is useful as an approach towards understanding the mechanisms of endosymbiont-induced thelytoky, because it generates clear predictions that can be tested in different ways. For example, the study of endosymbiont titers at different embryonic stages can provide more detailed information on the specific timing of reproductive manipulation and its dependence on the bacterial number. Immunohistology and *in situ* hybridization techniques can be used to monitor *Wolbachia* distribution during early egg/embryo development (e.g. Landmann *et al*., 2010; Fischer *et al*., 2011). RNA-seq approaches may be useful to compare gene expression among haploid males, diploid males and diploid females. According to our model, if endosymbiont titers are responsible for diploidization and feminization separately, certain densities might also result in the production of gynandromorphs or intersexes. Gynandromorphs were indeed observed in infected thelytokous *Trichogramma kaykai*, the feminized butterfly *Eurema hecabe* and the moth *Ostrinia scapulalis* (Hiroki *et al*., 2002; Kageyama *et al*., 2003; Narita *et al*., 2007; Pereira *et al*., 2010; Tulgetske and Stouthamer, 2012). Unfortunately, it was not possible to determine the presence of gynandromorphs in our study, as in *A. japonica* both sexes are morphologically similar, except for the presence of an ovipositor in females (C. van Achterberg, personal communication). A remaining question is whether this two-step mechanism of host manipulation applies to more species with endosymbiont-induced thelytoky. It could be much more general than previously thought, given that ploidy levels of rare males have hardly been checked in thelytokous species (Gottlieb, 2009).

Little is known about the underlying molecular basis of parthenogenesis. Cytological studies have revealed that endosymbionts induce diploidization of haploid eggs either via gamete duplication, which generates completely homozygous progeny (Stouthamer and Kazmer, 1994; Gottlieb *et al*., 2002; Pannebakker *et al*., 2004a), or via functional apomixis, which maintains

heterozygosity (Weeks and Breeuwer, 2001; Adachi-Hagimori *et al*., 2008). There are many gene products that endosymbionts could target to change the chromosome constitution of the egg, e.g. histone deposition that affects cell cycle checkpoints in the male pronucleus (Landmann *et al*., 2009), or Argonaute proteins and other genes coding for cell cycle proteins (Schurko *et al*., 2009; Kraaijeveld *et al*., 2012). Similarly, little information is available about the mechanism by which endosymbionts can cause feminization of diploid embryos. They might interfere with the sex-specific splicing of sex determination genes (like *doublesex*) as observed in lepidopterans (Narita *et al*., 2007; Sugimoto and Ishikawa, 2012). It is also possible that endosymbionts act by altering the epigenetic programming of host development, such as changing DNA methylation (Negri *et al*., 2009; Negri and Pellecchia, 2012; Ma *et al*., 2014a). Future experiments need to resolve these mechanistic details.

With this two-step model, there are at least three different ways in which endosymbionts can induce thelytoky in haplodiploids. In the first scenario, the endosymbiont causes diploidization of haploid eggs leading to female development following the host's haplodiploid sex determination. Absence or failure of the endosymbiont leads to haploid male development and diploid males cannot occur. This type has been suggested traditionally, for instance in *Leptopilina clavipes* (Pannebakker *et al*., 2004a) and *Muscidifurax uniraptor* (Gottlieb *et al*., 2002). Under the second scenario, diploidization of eggs is under host control and endosymbionts only feminize diploids. This type appears to apply to *Cardinium*-induced thelytoky in *Encarsia hispida,* in which curing of the bacteria leads to diploid rather than haploid male progenies (Giorgini *et al*., 2009). Our new model suggests a third scenario, which implies that *Wolbachia* cause both diploidization of unfertilized haploid eggs and subsequent feminization of diploid embryos. Curing of thelytoky-inducing endosymbionts will then lead to haploid males or diploid males depending on the degree to which the bacteria are removed. In addition to *A. japonica*, this type may also be present in thelytokous *Trichogramma kaykai* infected with *Wolbachia* (Tulgetske, 2010). Future work needs to clarify how widespread these different forms of thelytoky induction occur and to which extent they are associated with specific endosymbiont species, such as *Wolbachia*, *Cardinium* and *Rickettsia*.

The realization that there exist at least three ways at which endosymbionts can induce thelytoky refines our perspective on the phylogenetic association between infectious thelytoky and host sex determination. It also provides a novel window to study the yet-poorly known haplodiploid sex determination mechanisms. The evolution of thelytoky is tightly intertwined with the mechanism of sex determination in haplodiploids (Heimpel and de Boer, 2008; Asplen

et al., 2009; Ma *et al*., 2014a), and it has been frequently suggested that certain forms of sex determination are incompatible with infectious thelytoky because of the association with homozygosity of the genome. Two empirically confirmed sex-determination mechanisms have been documented in the Hymenoptera, Complementary Sex Determination (CSD), reported from over 60 hymenopteran species across most major superfamilies (Wilgenburg *et al*., 2006; Heimpel and de Boer, 2008; Asplen *et al*., 2009), and maternal effect genomic imprinting sex determination (MEGISD), thus far only described for *Nasonia* (Verhulst *et al*., 2010). The view that CSD is incompatible with endosymbiont-induced thelytoky by gamete duplication, because homozygous diploid eggs would develop into males under CSD rather than females, has to be reconsidered by the newly identified forms of manipulation. Thelytokous reproduction that relies on active feminization of diploid eggs by the endosymbiont regardless of the allelic state at the CSD locus, rather than 'passive' reliance on host sex determination uncouples thelytoky from homozygosity, and implies that CSD species can also become infected. Another sex-determination mechanism described for the parasitoid *Nasonia* is based on maternal effect genomic imprinting sex determination. Under maternal effect genomic imprinting sex determination, female development depends on activation of the *transformer* gene in the zygote by a *trans* factor that is transcribed from one of the parental genomes (Verhulst *et al*., 2010, 2013). It requires specific modifications in order to be compatible with female development after gamete duplication, as femaleness depends on a parentally contributed chromosome complement (for a detailed discussion see Ma *et al*., 2014a; Chapter 2). *A*. *japonica* does not have a CSD mode of sex determination (Ma *et al*., 2013; Chapter 3) but it is currently not known whether it has maternal effect genomic imprinting sex determination. Further studies are needed on the interaction between sex determination and endosymbiont-induced thelytoky in this and many other species. This will help to further clarify the phylogenetic association between sex determination mechanisms and infectious thelytoky in haplodiploids.

Acknowledgements

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Supplementary materials

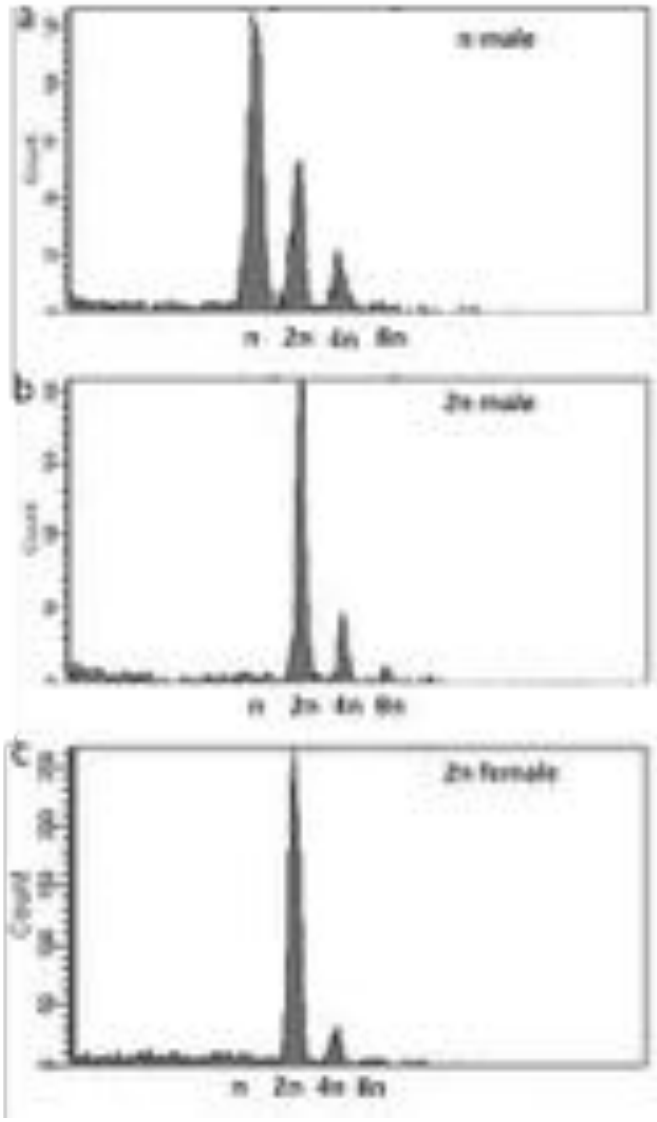

Figure S5.1 Flow cytometric DNA-histograms of a representative (a) haploid male, (b) diploid male and (c) diploid female in the thelytokous KG strain of *A. japonica*. The y-axis depicts the number of nuclei, and the x-axis the fluorescence intensity on a log scale, which converts to ploidy as indicated with the n-value. An excitation wave length of 488 nm and a band pass filter of 585 nm were used to detect propidium iodide fluorescence.

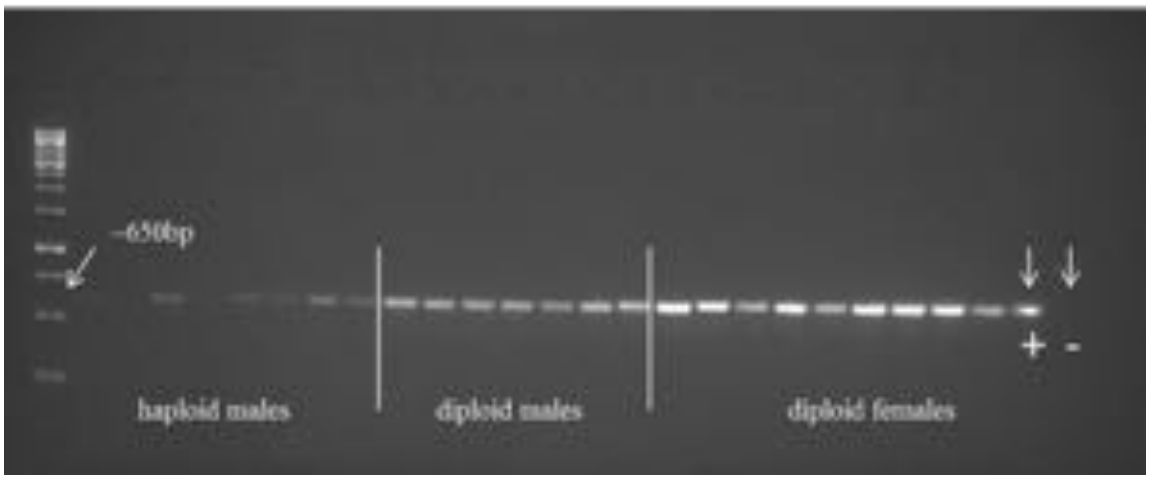

Figure S5.2 PCR assay of *Wolbachia* infection status among haploid males, diploid males and diploid females of the untreated thelytokous KG strain of *Asobara japonica*, using *Wolbachia*-specific *wsp* gene primers.

Box 2

Sexual functionality of diploid males in *Asobara japonica* with *Wolbachia*-induced parthenogenesis

Wen-Juan Ma

1. Background

Males are typically absent or very rare in species with endosymbiont-induced thelytoky (Heath *et al*., 1999; Stouthamer *et al*., 2010; Kraaijeveld *et al*., 2011; Reumer *et al*., 2012). However, we observed a frequent occurrence of males (both haploid and diploid) in hymenopteran parasitoid *Asobara japonica* with *Wolbachia*-induced thelytoky. In Chapter 5, we have investigated the genetic causes of the occurrence of males in thelytokous *A. japonica*. Here we address the question whether these males, and in particular the diploid males, are sexually functional, i.e. can they still mate and transfer sperm to females, and if so, what is their fertility and fecundity? To this end, a sexual control strain (AO) and a thelytokous strain infected with *Wolbachia* (KG) of *Asobara japonica* were studied.

2. Results

Diploid KG males were able to mate with sexual AO females and produce daughters. The proportion of females which mated with diploid KG males and produced daughters (33%, n=34), was not significantly different from matings with haploid KG males (49%, n=46; Fisher's exact test, P=0.403). However, the proportion of females which mated with sexual haploid AO males and produced daughters (90%, n=62) was significantly higher than with diploid KG males (Fisher's exact test, P=0.012; Table 1). Daughters from diploid thelytokous KG males were all triploid (n=50), indicating that diploid males transmitted functional diploid sperm. It is unlikely that triploid daughters are the result of (fertilized) diploid eggs, because sexual females did neither produce any diploid eggs during multiple generations of inbreeding, nor in introgression crosses (see Chapters 3 and 6). The triploid females did not produce any offspring (n=80), probably owing to aneuploidy during oogenesis.

The offspring sex ratios (proportion males) of sexual AO females that had mated with diploid KG males (0.69±0.05) were significantly higher than those that had mated to haploid KG males (0.47±0.03) (glm, $F_{1,48}$=16.64, P=0.0002). Interestingly, offspring sex ratios of females that had mated to haploid AO males (0.47±0.02) were not significantly different from females that had mated to KG haploid males (glm, $F_{1,79}$=0.13, P=0.717). The higher proportion of male offspring produced by diploid KG males cannot be explained by a higher triploid female

mortality, because brood sizes did not differ significantly between the three groups of females (glm, $F_{1,48}$=0.77, P=0.385; Table 1).

3. Discussion

In this study, we found that thelytokous *A. japonica* produced both haploid and diploid males that can mate successfully with sexual females. Both types of males produce viable female offspring, but diploid males from the thelytokous strain produced fewer daughters compared to haploid males from both the thelytokous and the sexual strain. Triploid daughters of diploid KG males are infertile, probably because of the high incidence of aneuploidy given the estimated chromosome number of 12-17 (pers. comm., G. Massimo, Chapter 4; also see de Boer *et al*., 2007b).

Under haplodiploidy, diploid males are rare and often inviable or infertile, hence the production of diploid males generates a costly genetic load in populations (van Wilgenburg *et al*., 2006; Heimpel and de Boer, 2008). In many hymenopteran species, diploid males arise because of homozygosity at the Complementary Sex Determination (CSD) locus (Whiting, 1933; Cook, 1993a). In *Asobara japonica* they occur spontaneously in broods of thelytokous females. We found that such diploid *A. japonica* males can produce functional sperm and successfully inseminate females resulting in the production of daughters. Daughters of diploid males are typically triploid and infertile (Table 2). These results indicate that sexual attractiveness and courtship behaviour has remained (at least partially) functional in thelytokous *A. japonica* males. Rare occurrence of haploid males, that are sexually functional, has been reported from a range of thelytokous species. Under asexual reproduction, decay of male sexual traits are not expected to be rapid and dramatic, because they are likely neutral traits and thus under relaxed selection (van der Kooi and Schwander, 2014).

The reasons for the lower proportion of daughters among the offspring of diploid males compared to that of haploid males remain to be investigated. There are several possible explanations. First, diploid sperm produced by diploid males may be less effective in fertilization, as was observed in a leaf-cutting ant (Armitage *et al*., 2010). In addition, diploid males tend to produce a smaller number of sperm compared to haploid males in the honeybee *Apis mellifera* (Woyke, 1963; Chaud-Netto and Kerr, 1980; Duchateau and Mariën, 1995).

Cryptic female choice is another possibility, if females can actively choose to avoid fertilization of their eggs by diploid sperm (Ward, 2000). As the brood size of parental females is not significantly different when mated to haploid males of either the thelytokous or the sexual strain, we can dismiss the possibility of hybrid incompatibility between the two strains. Interestingly, we also found no difference between the sex ratios of offspring fathered by haploid males of the thelytokous and the sexual strains. Kraaijeveld *et al*. (2011) studied the fertility of males that were derived from cured thelytokous females, and found that when these males mated with sexual females more sons among their offspring were produced, compared to when similar sexual females had mated with sexual males. Similar results were found in a study on *Leptopilina clavipes* by Pannebakker *et al*., (2005). One possible explanation for this discrepancy with our study, is that these other studies may have included diploid parental males, being the source of the deficit of female offspring.

Table 1 Mating success and offspring ploidy level of sexual females when mated with males from the sexual and thelytokous strain.

Traits	**Sons of thelytokous females**		**Sons of sexual females**
	haploid	**diploid**	**haploid**
Total number of females tested	46	34	62
No. females that produced daughters	23 (49%)	11 (33%)	56 (90%)
Ploidy of female offspring	diploid	triploid	diploid
Brood size (mean±s.e.)	85±3.2	80±3.7	88±3.1
Offspring sex ratio (mean±s.e.)	0.47±0.03	0.69±0.05	0.47±0.02

Table 2 Overview of studies on the fertility of diploid males in other haplodiploid species (partially modified from (Elias *et al*., 2009).

Species (family)	Chromosome number	Fertility of diploid males	Ploidy of daughters	Fertility of daughters	Reference
Athalia rosae (Tenthredinidae)	8	fertile	3N	sterile	Naito and Suzuki, 1991
Apis mellifera (Apidae)	16	unknown	unknown	unknown	Woyke, 1963
Asobara japonica (Braconidae)	12-17	fertile	3N	sterile	this study
Bombus terrestris (Apidae)	18	fertile	3N	sterile	Ayabe *et al*., 2004
Cotesia vestalis (Braconidae)	10	fertile	3N	sterile	de Boer *et al*., 2007b
Cotesia glomorata (Braconidae)	10	fertile	2N	fertile	Elias *et al*., 2009
Diadromus pulchellus (Ichneumonidae)	11	fertile	2N	fertile	Agoze *et al*., 1994
Euodynerus foraminatus (Vespidae)	unknown	fertile	2N	fertile	Cowan and Stahlhut, 2004
Habrobracon hebetor (Braconidae)	10	sterile	unknown	unknown	Petters and Mettus, 1980
Lasius sakagamii (Formicidae)	15	unknown	3N	fertile	Yamauchi *et al*., 2001
Nasonia vitripennis (Chalcidoidea)	5	unknown	3N	fertile	Kamping *et al*., 2007
Neodipiron nigroscutum (Diprionidae)	7-8	fertile	3N	sterile	Smith and Wallace, 1971
Polistes dominulus (Vespidae)	23-31	fertile	3N	unknown	Liebert *et al*., 2005
Solenopsis invicta (Formicidae)	16	fertile	3N	sterile	Hung *et al*., 1974
Trichogramma kaykai (Chalcidoidea)	8	fertile	3N	unknown	Tulgetske, 2010

4. Materials and Methods

One sexual (AO, from Amami-oshima island, Japan) and one thelytokous (from Kagoshima, Japan) strain were used to test male fertility. These two strains are closely related (Murata *et al*., 2009; Reumer *et al*., 2012), which minimizes the probability of genetic incompatibility. Virgin sexual AO females, sexual AO males (only haploid) and thelytokous KG males (both haploid and diploid), which are frequently produced by thelytokous females, were collected from mass cultures. The ploidy level of parental males and their daughters was determined by flow cytometry (for details, see Chapters 3 and 5). Males from both strains were individually placed with a sexual AO female for 24 hours, after which the females were allowed to oviposit on approximately 100 *Drosophila* host larvae. A parental male was considered as having successfully mated and sperm transferred if the parental female produced at least one daughter.

The proportions of females that produced at least one daughter after mating to diploid or haploid thelytokous KG males or haploid sexual AO males, were compared using Fisher's exact test. In addition, the offspring sex ratios (the proportion of male offspring) produced by these females were compared using a generalized linear model (glm) with a logit link function and a quasi-binomial error structure to correct for over-dispersion. The proportion of males was the response variable (weighted by brood size via the logit function) and male type (diploid or haploid thelytokous KG males, or haploid sexual AO males) the explanatory variable. Offspring number was compared using a glm model with a log link function and a quasi-poisson error structure to correct for over-dispersion (Crawley, 2007). Offspring number was used as the response variable and male type as the explanatory variable. All statistical analyses were performed with R 2.13.0 (R Development Core Team, 2011).

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Chapter 6

Genetics of decayed sexual traits in a parasitoid wasp with endosymbiont-induced asexuality

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Abstract

Trait decay may occur when selective pressures shift, due to changes in environment or life style, rendering formerly adaptive traits non-functional or even maladaptive. It remains largely unknown if such decay would stem from multiple mutations with small effects or rather involve few loci with major phenotypic effects. Here, we investigate the decay of female sexual traits, and the genetic causes thereof, in a transition from haplodiploid sexual reproduction to endosymbiont-induced asexual reproduction in the parasitoid wasp *Asobara japonica*. We take advantage of the fact that asexual females cured of their endosymbionts produce sons instead of daughters, and that these sons can be crossed with sexual females. By combining behavioural experiments with crosses designed to introgress alleles from the asexual into the sexual genome, we found that sexual attractiveness, mating, egg fertilization and plastic adjustment of offspring sex ratio (in response to variation in local mate competition), are decayed in asexual *A. japonica* females. Furthermore, introgression experiments revealed that the propensity for cured asexual females to produce only sons (because of decayed sexual attractiveness, mating behaviour and/or egg fertilization) is likely caused by recessive genetic effects at a single locus. Recessive effects were also found to cause decay of plastic sex-ratio adjustment under variable levels of local mate competition. Our results suggest that few recessive mutations drive decay of female sexual traits, at least in asexual species deriving from haplodiploid sexual ancestors.

6.1 Introduction

Due to environmental or life style changes and associated shifts in selective pressures, formerly adaptive traits may become non-functional or even maladaptive, and as a consequence they might decay (Fong *et al*., 1995; Wiens, 2001; Ellers *et al*., 2012). Trait decay has been observed for morphological, behavioural and physiological features. Examples include reduced wings in flightless birds (McNab, 1994), loss of eye function, decay of pigmentation in cave-dwelling animals (Jeffery, 2009; Protas *et al*., 2011) and loss of lipid synthesis pathways in parasitoid wasps (Visser *et al*., 2010). Such trait decay, especially when occurring in parallel in independent lineages, highlights the importance of natural selection for the maintenance of adaptations (Fong *et al*., 1995; Wiens, 2001; Lahti *et al*., 2009). Despite the fact that trait decay is a common evolutionary phenomenon, its underlying genetic mechanisms are poorly understood. It is largely unknown if trait decay typically stems from multiple mutations with small effects, or rather has a simple genetic architecture involving few loci with major phenotypic effects (Jeffery, 2009; Lahti *et al*., 2009).

In particular, many traits are expected to decay following a transition from sexual to asexual reproduction, making this transition of special interest for studies of trait decay and the genetic causes thereof (e.g., Carson *et al*., 1982; Pannebakker *et al*., 2004b; Jeong and Stouthamer, 2005; Kraaijeveld *et al*., 2009; Russell and Stouthamer, 2011; Schwander *et al*., 2013). For example, any trait specific to the male sex is useless under asexual reproduction in all-female species. The same holds for sexual traits expressed in females, such as those involved in attracting mates, mating behaviour and egg fertilization.

Here, we investigate the decay of female sexual traits and its genetic basis in the asexual wasp *Asobara japonica*. *A. japonica* is a parasitoid wasp that uses *Drosophila* larvae as host. It consists of both sexual and all-female asexual strains (Murata *et al*., 2009). Genetic analyses can be difficult in asexual organisms due to the inability to perform crosses. An exception to this constraint applies to those species in which asexuality is induced by infection with bacterial endosymbionts, such as *Asobara japonica* (Kremer *et al*., 2009; Reumer *et al*., 2012). Endosymbiont-induced asexuality is mainly found among wasps and among other groups in which sexual species are characterized by haplodiploid sex determination (Werren, 1997; Werren *et al*., 2008; Mateo Leach *et al*., 2009; Giorgini *et al*., 2010; Kageyama *et al*., 2012; Ma *et al*., 2014a), although it has also been suggested to occur in species with other sex determination systems (e.g., Pike and Kingcombe, 2009). Under haplodiploidy, females develop from fertilized, diploid eggs,

while males develop from unfertilized, haploid eggs (Whiting, 1933). However, unfertilized haploid eggs laid by endosymbiont-infected females undergo diploidization in the absence of fertilization with sperm (Suomalainen *et al*., 1987; Stouthamer *et al*., 1990; Werren, 1997; Werren and O'Neill, 1997; Gottlieb and Zchori-Fein 2001; Pannebakker *et al*., 2004b). In species with endosymbiont-induced asexuality, the genetics of traits involved in sexual reproduction can be studied because infected asexual females can often be cured of their endosymbionts via treatment with antibiotics. Such cured asexual females produce males, and these males can be crossed with females from related sexual strains (Pannebakker *et al*., 2005; Jeong and Stouthamer, 2005; Russell and Stouthamer, 2011). In addition, asexual females occasionally produce males under natural conditions and in the absence of antibiotic treatments. This is possibly caused by incomplete endosymbiont transmission, or by the occasional inability of endosymbionts to manipulate host reproduction (Heath *et al*., 1999; Reumer *et al*., 2012). Although these males usually do not have any mating opportunities or success in the asexual populations, they can be mated with females of sexual strains, similar to the males induced by antibiotic treatment.

We investigated four female sexual traits for signs of decay in asexual *Asobara japonica* and studied the genetic architecture of decayed traits via introgression of alleles from the asexual strain into the sexual one. As the expressed level of decay should depend on the degree of introgression of the asexual genome, we can make inferences about the genetic architecture underlying sexual trait decay, using simple genetic models to estimate the number of loci involved. Specifically, we investigated attractiveness to males, mating behaviour and egg fertilization in two different contexts specific to sexual reproduction in haplodiploid parasitoids. We tested which fraction of females with different levels of sexual-asexual admixture would still fertilize eggs, and to what extent these females would adjust the proportion of fertilized eggs (i.e., the proportion of females among their offspring) to different conditions. Females of many parasitoid species adjust the sex ratio of their offspring adaptively when mating occurs among the offspring of a few females in isolated patches ("local mate competition", hereafter LMC; Hamilton, 1967). If only a single female is present in a patch, her sons will compete with each other to mate with their sisters. Hence it is in the female's interest to produce the minimum number of sons required to fertilize all her daughters and allocate more energy into the production of daughters (Hamilton, 1967). When several females are present in a patch, sons from different females will compete, favoring a larger investment in sons. Sexual females are therefore predicted to produce a smaller proportion of sons when ovipositing alone than when other females are present in the same patch (Hamilton, 1967; Werren, 1980), a pattern we also found in a sexual *A. japonica* strain in a pilot experiment. This

plasticity in sex ratio adjustment makes it an interesting sexual trait to consider in the context of decaying sexual functions under asexual reproduction in parasitoid wasps, as there is no selection for the maintenance of plasticity under asexuality. Our results point to a surprisingly simple genetic architecture underlying the decay of female sexual traits, which most likely facilitated the spread of reduced sexual traits in the asexual population.

6.2 Materials and methods

6.2.1 Wasp culturing and collection of individuals

A sexual and a *Wolbachia*-induced, asexual strain of *Asobara japonica* were used in this study. Both strains were collected from Japan and have been cultured in the laboratory since 2009; the sexual strain originated from the island of Amami-oshima and the asexual strain from Kagoshima on the mainland of Japan. These two strains are closely related (Murata *et al*., 2009; Reumer *et al*., 2012), which minimizes the probability of genetic incompatibility in crosses. *Asobara japonica* was cultured on second-instar *Drosophila melanogaster* larvae as hosts at 25°C, with a 16L: 8D light-dark cycle and 60% relative humidity (for details see Ma *et al*., 2013).

We used five different classes of virgin individuals: sexual females, sexual males, asexual females cured of their *Wolbachia* infection via antibiotic treatment, untreated asexual females and males produced by asexual females. The males produced by asexual females were either induced via antibiotic treatment or directly collected from our mass culture with the highest incidence of accidental males (we collected 40-70 males among approximately 6000 individuals). After using these males in our experiments, we verified, via flow cytometry, that they were haploid (as expected under normal haplodiploid sex determination in Hymenoptera; for details see Ma *et al*., 2013). Virgin sexual males and females were also collected directly from standard laboratory cultures. Sexual males were collected first because they emerge one or two days earlier than females. After the emergence of males, sexual females were collected by individually isolating wasp pupae in plastic vials (diameter 2.4 cm, height 7.5 cm) containing a layer of agar to control humidity (Ma *et al*., 2013).

Wolbachia-infected asexual females were cured of their bacteria with antibiotics applied to the *Drosophila* host larvae. 10mg of rifampicin was added to 1g yeast powder, which was mixed with water to feed the second-instar *Drosophila* larvae. Rifampicin treatment has been shown to have

little impact on the development of *Asobara* wasps (Dedeine *et al*., 2001), and no effects on life-history traits, such as brood size and pupal mortality, were found in our experiments (data not shown). Female wasps that emerged from rifampicin-fed *Drosophila* hosts were individually collected in plastic vials containing a layer of agar. To confirm complete removal of *Wolbachia* by antibiotic treatment, 23 emerged females were allowed to lay eggs in antibiotic-free host larvae, and the production of only male offspring was verified.

6.2.2 Observation of courtship behaviour and spermatheca dissections

To investigate sexual attractiveness and mating behaviour of females, we set up 109 no-choice mating trials. For each trial, asexual females (n=20 cured, 40 untreated) or sexual females (n=49) were individually paired with a sexual male for 20 minutes. We evaluated female attractiveness to sexual males by scoring if the male attempted to court the females (i.e., displayed wing vibration and actively approached the female). For female mating behaviour, we scored if females responded to the copulation attempts of sexual males with escape behaviour or acceptance. A successful copulation was scored when it lasted at least seven seconds. As these experiments revealed that cured asexual females were relatively unattractive to sexual males (see results), we evaluated if decreased attractiveness could stem from lineage divergence that is unrelated to the asexual mode of reproduction (i.e., as could be observed between two sexual species). To this end, we repeated similar tests with males produced by asexual females, since there is no lineage divergence between these males and asexual females.

To investigate if cured asexual females fertilize their eggs and produce daughters when mated with sexual males, two experiments were performed. First, we paired each of 20 cured asexual females with a sexual male for 24 hrs. Second, to increase the chance of obtaining any mated asexual females, we paired each of 13 additional cured asexual females with a group of at least 50 sexual males in one mating vial for 24 hrs. Females were then offered 50-100 second-instar host larvae for egg laying for approximately 24 hrs (both experiments), to test if they produce any daughters, indicative of successful egg fertilization. Next, the spermathecae of all asexual females were dissected to check for the presence of sperm, as an indication of successful mating and sperm transfer.

For spermatheca dissections, an individual female wasp was placed in a drop of *Drosophila* Ringer's solution (Rajaram *et al*., 2005) on a microscope slide. The wasp abdomen was first separated from the rest of the body using a very fine needle. The spermatheca was then carefully separated from the rest of the abdomen. The spermatheca was isolated under a Zeiss Stemi SV 6

stereo microscope (with 25× 2.4 magnification, Carl Zeiss AG, Oberkochen, Germany), and a cover slide was gently put on top of it. Motile sperm were scored as present or absent under a Zeiss Axio Lab.A1 binocular microscope (with 10 × 40 magnification, Carl Zeiss GmbH, Göttingen, Germany).

6.2.3 Introgression experiment

To determine the genetic architecture of decayed female sexual traits, we introgressed alleles from the asexual into the sexual strain. Upon emergence of virgin sexual females, they were individually paired with a male from the asexual strain for 24 hours. Each female was then offered approximately 100 second-instar *D. melanogaster* larvae for oviposition during 36 hours. After 12-14 days, wasp pupae were isolated from parasitized hosts to prevent matings between individuals upon emergence. Females emerging from these pupae (the F1 hybrid generation) were collected and individually paired with a male from the asexual strain to produce the offspring for the next generation (see Ma *et al*., 2013 for further details; Chapter 3). This experimental procedure was continued for successive generations of introgressions. Because almost no daughters were produced after the 4th generation ("G4") of introgression (because females with high proportions of their genome stemming from the asexual strain do not produce daughters, see results), the introgression experiment was stopped after the 4th generation of introgression. We repeated the same introgression experiment three times independently over the course of three months, because only a few males that were produced by asexual females were available on specific days for crosses. Because there was no difference for brood size or offspring sex ratio or any other parameter between different experiments (analyses not shown) all data were pooled for further analysis. Given our crossing design, the proportion of asexual alleles in each generation increased from 50% in F1 hybrid females, to 75% in females of G2, 87.5% in G3 and a final 93.8% in females of G4 (Figure 6.1). For each generation of introgression, the emerging wasps were anaesthetized with CO_2, counted and sexed (Ma *et al*., 2013).

The combination of decayed sexual attractiveness, mating behaviour and egg fertilization results in cured asexual females producing only sons. Therefore, we monitored the proportion of admixed females producing only sons across successive generations of introgression. For each generation of introgression, a subset of hybrid females (those not used to continue the introgression crosses) were paired with a sexual male for 24 hrs followed by oviposition for 36 hrs. The sex ratio of offspring produced during these 36 hrs was then determined for each female

to infer the proportion of females producing only sons. Using the same experimental conditions, we also determined the sex ratio produced by sexual females and cured asexual females.

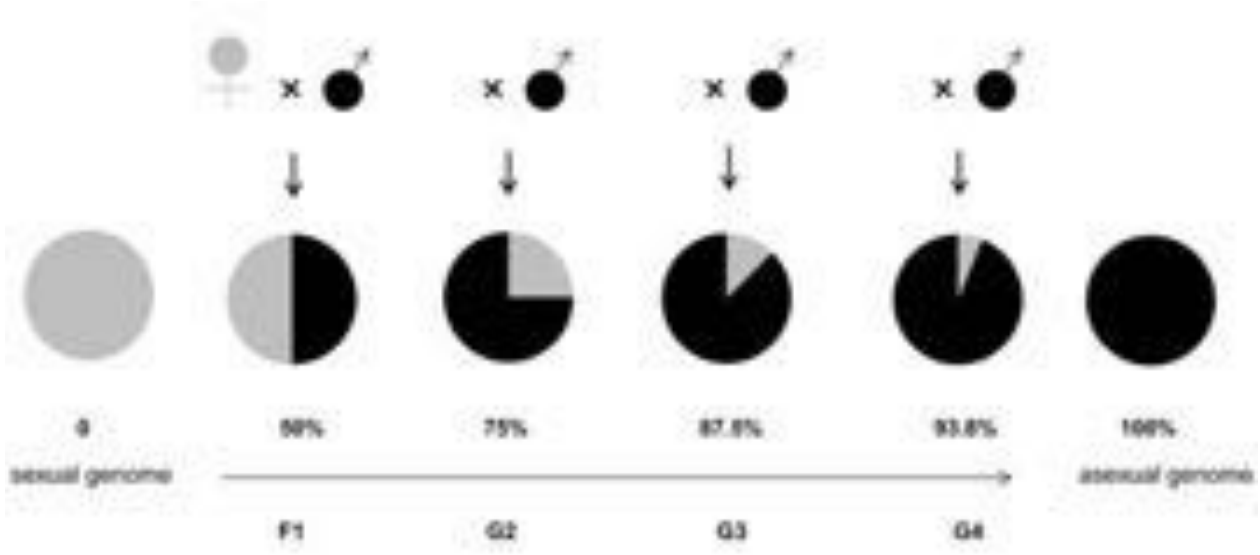

Figure 6.1 Introgression scheme. Grey and black areas in the pie charts depict sexual and asexual genome proportions respectively, F1 and G2-G4 are successive generations of introgression. The number of percentage represents the relative proportion of asexual genome in females.

6.2.4 Local Mate Competition experiment

To investigate if *A. japonica* females produce different offspring sex ratios (proportion of male offspring) in response to different situations of LMC, we investigated offspring sex ratios produced by females that oviposited alone or in groups of three females per patch. LMC theory predicts that the proportion of sons increases with increasing numbers of females in a patch. A pilot experiment (data not shown) showed that sexual females do indeed change the sex ratio among their offspring depending on the number of females (one or three) per patch. Thus we tested if sexual females, cured asexual females and females from different introgression generations would produce different offspring sex ratios when alone in a vial relative to when in a group of three. To avoid any interactions between females in different vials (Shuker *et al*., 2007), the vials were spaced 5cm apart. As not all hybrid females mate, which would constrain their sex allocation, females were observed while paired with a sexual male and only those females that copulated were used to test for sex ratio adjustments. Prior to this experiment, each female was

offered approximately 50 host larvae to get oviposition experience in order to minimize super-parasitism (oviposition in already-parasitized hosts; van Alphen and Nell, 1982; Ma *et al*., 2013). To control for effects of host larva density on wasp offspring sex ratio, we adjusted the number of the host larvae to the number of females, i.e. 50 larvae were offered to a single and 150 to a group of three females for approximately 15 hours. Fifty larvae per female are an excess as a single female can handle a maximum of 30-40 larvae in 15 hours (W.-J. Ma, personal observation). Offspring were counted and sexed upon their emergence from the host pupae.

6.2.5 Statistical analyses

Fisher's exact tests were used to compare the proportion of courting attempts by males, rejection rates of males and the frequency of successful copulations of sexual and cured asexual females. Offspring sex ratios of females from different generations of introgression were compared with logistic regressions specified in generalized linear models (glm). The proportions of females producing only sons between different introgression generations were tested using glm models with a logit link function, and a quasi-binomial error structure to correct for over-dispersion (Crawley, 2007). The proportion of asexual genome in hybrid females was used as a quantitative explanatory variable and the progeny type (either only sons or at least one daughter) as the response. To pinpoint significant differences between introgression generations, a sequential multiple comparison was used. To conduct the multiple comparison, generation had to be used as a categorical variable. For comparison of offspring sex ratios of females producing at least one daughter among different generations of introgression, a glm model was used with a logit link function and a quasi-binomial error structure to correct for over-dispersion (Crawley, 2007). In this model the proportion of males was used as the response variable (weighted by brood size via the logit function) and generation as the explanatory variable. A similar glm model was used for comparing the proportion of male offspring in the LMC experiments, i.e. between single and triple ovipositing females. All statistical analyses were performed with R 2.13.0 (R Development Core Team, 2011), multiple comparisons of traits among generations were done using the Tukey test as implemented in the R package multcomp for general linear hypotheses (Hothorn *et al*., 2008).

6.3 Results

6.3.1 Sexual traits in asexual females

First, we evaluated whether sexual traits were functional in *Wolbachia*-cured asexual *A. japonica* females. Specifically, we tested whether cured asexual females were able to attract sexual males, mate and store sperm in their spermathecae, and, if so, whether they fertilize eggs and display plastic sex-allocation behaviour under different levels of LMC.

No-choice mating trials (20 min) revealed that cured asexual females were less attractive to sexual males than sexual females were. Sexual males courted a significantly smaller percentage of asexual (55%; 11 out of 20) than sexual females (88%; 43 out of 49; Fisher's exact test, P=0.008, Table 6.1). In addition, among the females that were courted by sexual males, cured asexual females rejected all mounting and copulation attempts of males (100%, n=11), whereas rejections were significantly less frequent for sexual females (23%; 10 out of 43, Fisher's exact test, P<0.0001, Table 6.1). As a consequence, not a single one out of 20 cured asexual females successfully copulated with a sexual male during the 20 min observation period, as compared to over 67% (33 out of 49) sexual females (Fisher's exact test, P=0.0003, Table 6.1). The reduced attractiveness and copulation propensity of asexual as compared to sexual females are unlikely to be caused by the treatment with antibiotics, as untreated asexual females were also unattractive to sexual males; only three out of 40 untreated asexual females (8%) were courted, compared to 43 out of 49 sexual females (88%; Fisher's exact test, P=0.0001, Table 6.1). The comparison of cured and untreated asexual females further revealed that sexual males are more inclined to court the former (Fisher's exact test, P=0.0001). Reduced attractiveness of asexual females to sexual males is unlikely to stem from divergence of functional sexual signals between sexual and asexual strains (as could be expected for comparisons between two diverged sexual strains). This is revealed by the fact that males produced by asexual females also courted sexual females more often (79%; 50 out of 63) than cured asexual females (52%; 11 out of 21; Fisher's exact test, P=0.024).

To investigate if cured asexual females were able to fertilize their eggs and produce daughters, we conducted two experiments to increase the chances of finding mated asexual females, given the above-described 20min no-choice trials did not result in any copulations. In the first experiment, 20 cured asexual females were each paired with a sexual male for 24hrs, but all 11 females that produced offspring had only sons (40-60 offspring per female, over 400 offspring in total). The remaining nine females produced no offspring at all (for unknown reasons, subsequent dissections revealed a normal egg load and apparently developed ovaries in all females). In the second

experiment, we paired each of 13 cured asexual females with groups of at least 50 sexual males for 24hrs. Again, nine females produced only sons (40-60 offspring per female, over 300 offspring in total), even though dissecting them after the experiment revealed that three of these contained sperm in their spermathecae. The remaining four females, of which one contained sperm in her spermatheca, did not produce any offspring (despite having active ovaries). Thus, even in the rare cases where asexual females do mate and store sperm, sperm is apparently not used to fertilize eggs.

Table 6.1. Summary of sample sizes and results for the different sexual traits evaluated in asexual *A. japonica* females. Court: proportion of females courted by sexual males, Accept: proportion of females accepting to copulate, Mate: proportion successfully mated females, Repl.: number of replicates.

Sexual trait evaluated	**Female sexual attractiveness**		**Female copulation behaviour**		**Female mating success**		**Spermatheca status (with or without sperm) and proportion of females producing only male offspring**		
	Repl.	**Court (mean ± s.e.)**	**Repl.**	**Accept (mean ± s.e.)**	**Repl.**	**Mate (mean ± s.e.)**	**Repl.**	**Nr. females with sperm**	**Prop. male offspring**
Untreated asexual female	40	0.08 ± 0.04	3	0	40	0	/	/	/
Cured asexual female	20	0.55 ± 0.11	11	0	20	0	20*	0	1
							13**	3	1
Sexual female	49	0.88 ± 0.13	43	0.77 ± 0.07	49	0.67 ± 0.07	/	/	/

* 20 cured females were individually paired with a single sexual male for 24 hours.
**13 cured females were individually paired with a group of more than 50 sexual males for 24 hours.

6.3.2 Patterns of sexual trait change across different levels of introgression

The combination of low attractiveness to males, low copulation propensity and the absence of egg fertilization, leads to cured asexual females producing only sons under conditions where the majority of sexual females (89.3%; Figure 6.2) produce daughters in addition to sons. Hence, we monitored the proportion of females producing only sons, as a measure of sexual trait decay, across increasing levels of introgression of the asexual genome into the sexual one. As expected given the phenotype of the sexual and asexual strains for this trait, the proportion of females producing only sons increased with increasing levels of introgression (Figure 6.2, glm, $F_{1,405}$=72.3, P<0.0001). Importantly, the first significant increase was in the second generation (glm, t=-4.5, P<0.0001); no significant increase was observed in the first generation (glm, t=1.0, P=0.340; Figure 6.2). This indicates that recessive genetic effects cause females to produce only sons.

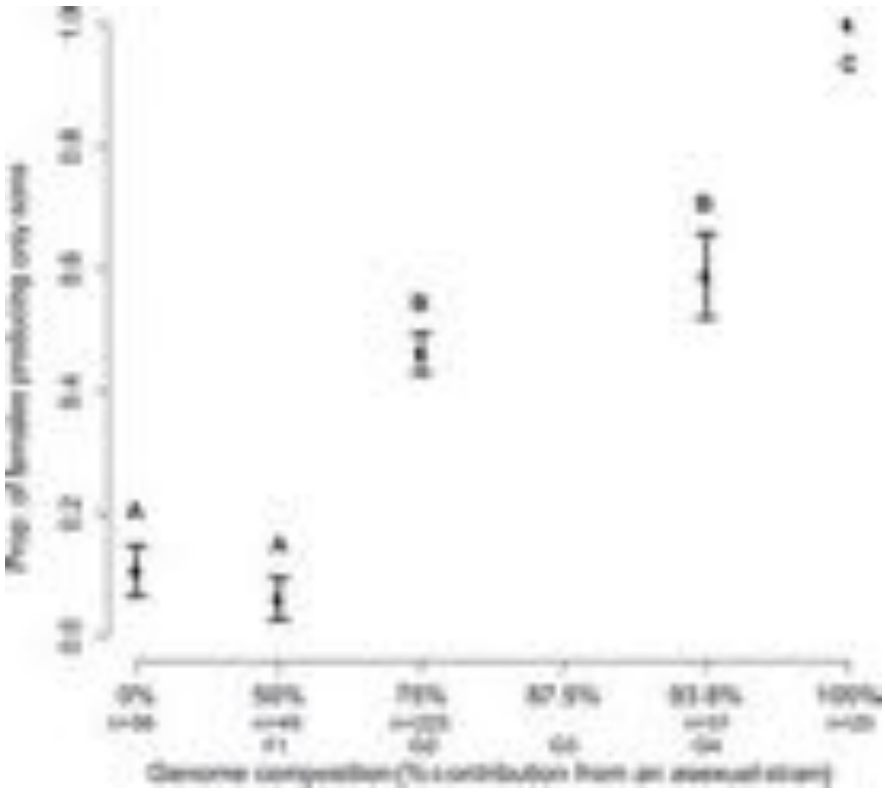

Figure 6.2 Proportion of females producing only sons when provided with a sexual male mating partner, for different categories of sexual-asexual hybrid females and *Wolbachia*-cured asexual females (100% asexual genome). Bars indicate standard errors. The x-axis represents the proportion of alleles stemming from the asexual genome for each consecutive generation. Different capital letters indicate statistical difference (P<0.01), n indicates the sample size for each generation and G1-4 refers to the different introgression generations. For logistic reasons, phenotyping of the G3 generation was not possible and therefore no data are provided.

Table 6.2 Inferring the number of loci involved in the decay of a female sexual trait in asexuals. Predicted proportion of sexual-asexual hybrid females (75% of the genome from the asexual strain) producing only sons for one to three unlinked loci, and the binomial test values for the comparison of each model with the observed data (0.46; 104 out of 225 females producing only sons, data from Figure 6.2).

Number of loci **Parental genotypes** **(F1 hybrid female x asexual male)**	**1 locus** **Aa x a**	**2 loci** **AaBb x ab**	**3 loci** **AaBbCc x abc**
Expected genotypes of offspring females (f= expected frequency); in bold: the genotype producing only sons	Aa; **aa** (f= 0.50 each)	AaBb; Aabb; aaBb; **aabb** (f= 0.25 each)	AaBbCc; AaBbcc; AabbCc; Aabbcc; aaBbCc; aaBbcc; aabbCc; **aabbcc** (f= 0.125 each)
Binomial test	P=0.286	P<0.00001	P<0.00001

Note: The predictions are based on two alleles at each locus: A and a, B and b, C and c respectively. Capital letters denote dominant alleles, lower-case letters recessive alleles. Fixed homozygosity at each locus is assumed for the parental populations (for the dominant alleles in the sexual, the recessive alleles in the asexual population) and there is no epistasis among different loci. The predictions of recessive allele frequency from two and three-loci genetic model are significantly lower than the observed data, and higher number of loci would predict an even lower frequency.

More precisely, the quantitative increase of the proportion of females producing only sons in the second generation is consistent with a simple genetic architecture of this trait (Table 6.2). Indeed, for hybrid females with 75% of their genome stemming from asexual strains, 46% (104 out of 225) produced only sons, which is in agreement with the expected 50% under a single-locus model, but differs significantly from the expected frequencies under models with two or more loci (Table 6.2).

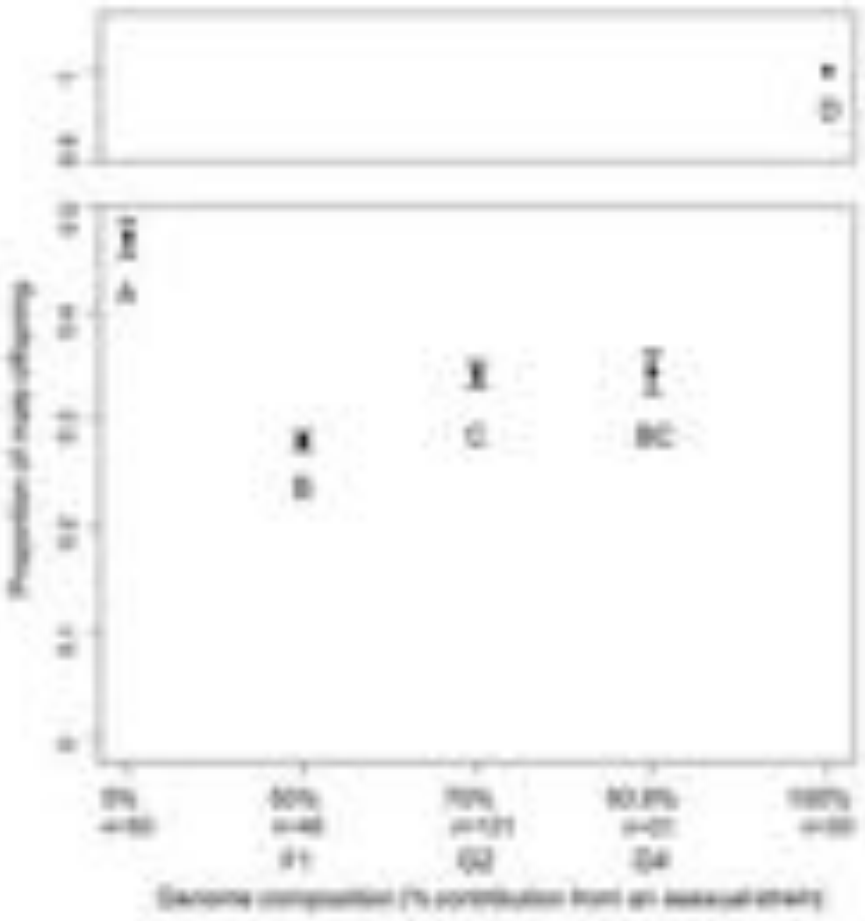

Figure 6.3 Offspring sex ratios produced by females with at least one daughter (i.e., females producing only sons are excluded) for different categories of sexual-asexual hybrid females and *Wolbachia*-cured asexual females (100% asexual genome), when mated with sexual males. Bars indicate standard errors. Different capital letters indicate statistical difference (P<0.01), n indicates the sample size for each generation and F1, G2, G4 refers to the different introgression generations.

Given that in addition to the females producing only sons, there was an (decreasing) fraction of females producing daughters in addition to sons, we also monitored how the

offspring sex ratios of these females changed across increasing levels of introgression. However, in contrast to the proportion of females producing only sons, we had no specific prediction for how this trait would change with an increased representation of the asexual genome, given that this trait would derive from the sexual rather than the asexual genome. Indeed, as revealed by the experiments described above, cured asexual females do not fertilize any of their eggs, even if they have copulated. In our introgression experiment, we needed hybrid females for initiating each successive generation, hence we indirectly selected for females that copulated and fertilized at least some of their eggs. Following this indirect selection, we found that the sex ratio among offspring of females with at least some daughters fluctuated across increasing levels of introgression (Figure 6.3). While females from all different introgression levels produced significantly more female-biased sex ratios than 'pure' sexual females, the most female-biased offspring sex ratios were found for the F1 hybrid females (Figure 6.3, glm, Tukey contrasts, all $P<0.001$). Even though these females also produced significantly fewer offspring (76.6±2.6) than 'pure' sexual females (88±3.1; Welch t-test, $t_{91.6}=2.9$, $P=0.004$), the low sex ratios are unlikely to stem from high mortality of males as a consequence of hybrid breakdown. Hybrid breakdown should cause increased mortality of sons produced by F1 hybrid females independently of rearing conditions, yet we found no indication of hybrid breakdown among the females producing only sons (described above), and there was also no evidence for it in the LMC experiment (see the next paragraph). Hence the reason for the initial drop in sex ratio and offspring number of F1 hybrid females remains unknown.

As asexual females produce only daughters, there is no selection for plastic sex allocation in response to different levels of LMC in asexual strains. We first verified that sexual *A. japonica* females indeed displayed plastic sex allocation. This was the case, as revealed by sexual females producing a significantly larger proportion of sons when in groups of three than when alone (Figure 6.4, glm, $F_{1,57}=13.9$, $P<0.001$). A similar response was also displayed by sexual-asexual F1 hybrid females (Figure 6.4, glm, $F_{1,22}=5.5$, $P=0.029$). However, in the second generation (females with 75% of their genome stemming from asexual strains), females did not adjust their offspring sex ratio to different levels of LMC (all of these females produced at least one daughter; Figure 6.4, glm, $F_{1,10}=0.2$, $P=0.66$). We did not perform the LMC experiment for G3 and G4 introgression generations because plastic sex allocation was already completely lost in generation G2. In combination, these patterns suggest that there is decay of sex allocation plasticity in asexual females, and that this decay is

due to recessive genetic effects, similar to the decay of attractiveness and copulation propensity. However, an estimation of the number of loci affecting this trait cannot be provided, given that the presence vs. absence of sex allocation plasticity is evaluated qualitatively at the group level.

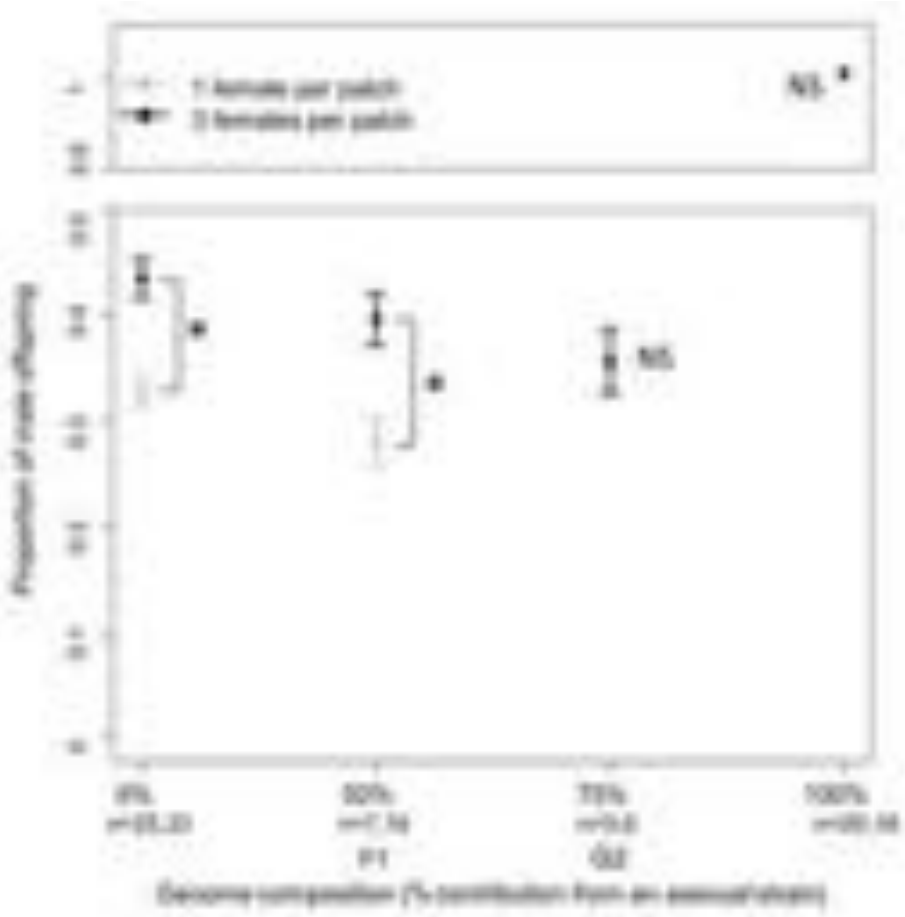

Figure 6.4 Offspring sex ratios produced by different sexual-asexual hybrid females and *Wolbachia*-cured asexual females (100% asexual genome), when alone (in grey) or in groups of three (in dark). Bars indicate standard errors. Proportions labeled with stars differ significantly (P<0.01), NS indicates non-significant differences, n with two numbers indicates the sample sizes for the single females and the groups of three females per patch respectively, and F1, G2 refers to the different introgression generations.

6.4 Discussion

In this study, we used the parasitoid wasp *A. japonica* with *Wolbachia*-induced asexuality to investigate if female sexual traits decay under asexuality, and if so, whether trait decay is caused by many loci with small effects or by only a few major-effect loci. A combination of behavioral experiments with crosses designed to introgress alleles from the asexual into sexual genome revealed decay of all investigated sexual traits and suggested a surprisingly simple genetic architecture underlying trait decay.

We found evidence for moderate decay of sexual attractiveness and extensive decay of mating and egg-fertilization behaviour in asexual *A. japonica*. Asexual females were always less likely to be courted by males than sexual females, but the percentage of asexual females that were courted depended on whether these females were infected with *Wolbachia* (8%) or cured of their infection by antibiotic treatment (55%). This suggests that *Wolbachia* negatively affect the sexual attractiveness of infected females via an unknown mechanism. *Wolbachia* may, for example, modify sexual signals expressed in asexual females. Bacteria are known to alter pheromone production in some insects, such as commensal bacteria in *Drosophila melanogaster* (Sharon *et al*., 2010), and gut bacteria in the desert locust *Schistocerca geraria* (Dillon and Charnley, 2002). In *D. melanogaster*, curing individuals from endosymbiont infection decreases levels of mate discrimination between populations by about 50%, an effect likely mediated by the effect of endosymbionts on female pheromone production (Koukou *et al*., 2006; see also Sharon *et al*., 2010). *Wolbachia* could also affect female attractiveness by altering cuticular hydrocarbon profiles, which function as mating cues in many insect species (e.g. Ivy *et al*., 2005; Yew *et al*., 2009).

Mate attraction is often associated with significant costs in sexual species (Daly, 1978), because of resource investment in the production of mate attraction signals and/or because it can increase the risk of predation (e.g. Zuk *et al*., 2006). As a consequence, mate attraction is expected to be under strong negative selection in asexual species where it is superfluous, a prediction largely supported by empirical data (e.g. Lehmann *et al*., 2011; reviewed by van der Kooi and Schwander, 2014). However, once mate attraction is low, for example as a consequence of endosymbiont infection as we found for asexual *A. japonica*, the strength of selection for decreased expression of signals involved in mating interactions might be reduced. This may explain why asexual *A. japonica* females cured of their endosymbionts were still somewhat attractive to sexual males, although less than sexual females.

In contrast to the moderate decay of sexual attractiveness, mating behaviour is largely disrupted in asexual females. This disruption is not caused by *Wolbachia* infection. Both infected and cured asexual females rejected all mating attempts by males under conditions where the majority of sexual females (88%) would accept mates and copulate. The mechanisms underlying disrupted mating interactions in asexual females remain to be investigated.

We also aimed at investigating if asexual females fertilize their eggs upon mating with a sexual male. In order to obtain any mated asexual females, we had to pair cured asexual females individually with groups of over 50 sexual males. The four asexual females that contained sperm in their spermathecae either produced no offspring at all (one female) or did not fertilize their eggs and produced only sons (three females). Although the sample is small, this pattern suggests that the ability to produce fertilized embryos decayed in asexual *A. japonica* females. Decay of egg fertilization can have different causes, for example egg modifications such as impermeability to sperm, and/or a lack of structures for sperm maintenance in the spermatheca. In the asexual parasitoid wasp *Muscidifurax uniraptor*, an essential spermatheca-associated muscle is completely absent (Gottlieb and Zchori-Fein, 2001). Similarly, non-reproductive workers of many ant species have degenerated spermathecae with a flattened reservoir epithelium with few organelles (Gobin *et al*., 2008).

Different mechanisms have been suggested to drive the decay of female sexual traits. In *A. japonica* and other asexual species deriving from haplodiploid sexual ancestors, low copulation propensity and the absence of egg fertilization lead to cured asexual females producing only sons even when presented with mating opportunities, a pattern referred to as "functional virginity" (Jeong and Stouthamer, 2005; Stouthamer *et al*., 2010; Russell and Stouthamer, 2011). It has been suggested that such "functional virginity" would be favored selectively in sexual populations where only a fraction of females are asexual as a consequence of endosymbiont infection (Stouthamer *et al*., 2010). Because "functional virginity" would enhance male production in populations with female-biased sex ratios, uninfected females might benefit from such a trait, assuming their sons could reproduce sexually with the majority of females in that population. "Functional virginity" may therefore become fixed in the population before the fixation of endosymbiont-induced asexuality. This could explain why female mating and egg-fertilization behaviours decay rapidly in species with endosymbiont-induced asexuality (Stouthamer *et al*., 2010), a pattern reported for many

haplodiploid species (Pijls *et al*., 1996; Arakaki *et al*., 2000; Pannebakker *et al*., 2004b, 2005; Jeong and Stouthamer, 2005; Russell and Stouthamer, 2011; this study).

The spread of "functional virginity" mutations along with endosymbiont-induced asexuality is, however, not the only possible cause leading to decay of mating behaviour and egg-fertilization in asexual females. A more general driving force underlying trait decay under asexuality might be strong negative selection on sexual traits that are expressed in females (Pijls *et al*., 1996; van der Kooi and Schwander, 2014). Indeed, reduced female sexual attractiveness, lower copulation propensity and the absence of egg fertilization are “the norm” in asexual lineages (van der Kooi and Schwander, 2014). The majority of known asexual lineages derive from sexual ancestors with genetic sex determination systems other than haplodiploidy. In these cases virgin sexual females produce no offspring at all (rather than producing sons as under haplodiploidy), such that the decay of their mating behaviour and lack of egg fertilization cannot be explained by the spread of "functional virginity" mutations.

To gain insights into the genetics of cured asexual females producing only sons, we monitored the proportion of such females across increasing levels of introgression of alleles from the asexual into the sexual strain. The proportion of females producing only sons increased gradually with an increasing representation of the asexual genome, indicating that alleles underlying asexuality-specific traits can indeed be introgressed into a sexual genome. The first significant increase was observed in the second generation, indicating that recessive genetic effects underlie an increased propensity of females to produce only sons (Figure 6.2). In addition, the proportion of second-generation (G2) hybrid females producing only sons matched the predicted proportion for a single-locus model (50%, Table 6.2). Cured asexual females produce only sons because of the combination of decreased sexual attractiveness, low copulation propensity and the absence of egg fertilization. Because we did not monitor each of these traits separately across the introgression generations, we cannot infer whether the single locus effect that we found stems from changes at only one or some combination of these traits. Independently of whether the locus acts on a single or multiple traits, our study points to a rather simple genetic architecture underlying trait decay, possibly a single locus. A simple genetic architecture for other decayed sexual traits was also uncovered in three previous studies of independently derived wasp lineages with endosymbiont-induced asexuality. Pannebakker *et al*. (2004b) found a single major locus underlying reduced male fertility using quantitative trait locus mapping. Two other studies investigated the decay of egg fertilization and found evidence for a single mutation with recessive effects in one case

and dominant effects in the other (Jeong and Stouthamer, 2005; Russell and Stouthamer, 2011).

Two of the three studies investigating the genetic basis of female sexual trait decay in endosymbiont-induced asexuals found trait decay to be caused by recessive alleles (Jeong and Stouthamer, 2005; this study). This raises the question of how recessive alleles could spread and fix within a short evolutionary time; asexuality in *A. japonica* evolved very recently, as indicated by little or no molecular genetic differentiation between sexual and asexual populations for mitochondrial DNA (Murata *et al*., 2009; Reumer *et al*., 2012). One possible explanation may lie in the cytological mechanism of *Wolbachia*-induced parthenogenesis. Diploidy of the embryo is frequently restored through gamete duplication, which results in complete homozygosity (e.g. Stouthamer and Kazmert, 1994; Gottlieb *et al*., 2002; Pannebakker *et al*., 2004a). Such homozygosity would facilitate spread of adaptive recessive mutations, similar to dominance favoring the spread of adaptive mutations in diploid species (Otto and Goldstein, 1992; Orr and Otto, 1994). It remains to be investigated whether recessive mutations may also frequently cause trait decay in species with forms of asexuality that maintain heterozygosity or whether trait decay in these cases is mostly caused by dominant mutations.

In addition to the females producing only sons, there was an (decreasing) fraction of females producing daughters in addition to sons across successive introgression generations. Copulation and egg-fertilization behaviours required for production of daughters most likely stem from the sexual rather than the asexual genome, given that cured asexual females almost never copulate, and do not fertilize their eggs in the rare event of copulation. Since we needed daughters for initiating each successive introgression generation, we may have indirectly selected for a portion of the sexual genome favoring copulation and egg fertilization. Importantly, however, such putative indirect selection does not affect our conclusions for simple genetic effects underlying the increased tendency of females to produce only sons across successive introgression generations. Indeed, given the recessive effects underlying this tendency are only expressed in the second introgression generation, indirect selection would not have occurred prior to this generation, and we only used the first two generations to make inferences on genetic architecture. Following indirect selection for copulation and fertilization, we found that sex ratios among offspring of females with at least one daughter fluctuated across increasing levels of introgression, but were always more female-biased than sex ratios produced by ‘pure’ sexual females (Figure 6.3).

We found that sexual *A. japonica* females change the sex ratio of their offspring according to the level of local mate competition (LMC) their sons are exposed to (Figure 6.4), and we therefore monitored sex allocation in single vs multiple female set-ups across successive introgression generations. Plastic sex allocation was displayed by first generation sexual-asexual F1 hybrid females, but not by females with a higher level of introgression of the asexual genome. This indicates that plastic sex allocation behaviour has decayed in asexual females. Similar to the traits discussed above, the decay of plasticity is most likely also caused by recessive genetic effects, given that plasticity was still expressed in the first, but not in the second or any of the later introgression generations. The mechanisms underlying this decay remain a matter of speculation, but might be due to a loss of control over egg fertilization or to a loss of perception of levels of competition.

The decay of plastic sex allocation response under asexuality has not been investigated previously, hence it is impossible to evaluate if it represents a general trend in asexuals derived from haplodiploid ancestors or if such decay is specific to asexual *A. japonica*. Nevertheless, selection experiments in the (sexual) spider mite *Tetranychus urticae* have revealed that plastic sex-allocation behaviour can decay rapidly (within 54 generations) under situations of relaxed selection (Macke *et al*., 2011). Such rapid decay, whatever the causes underlying it, would likely result in decay of plastic sex allocation in most if not all asexual species.

In conclusion, we have investigated the potential decay of four female sexual traits in asexual *A. japonica*: sexual attractiveness, mating behaviour, egg fertilization and plastic sex allocation under different levels of LMC, and we have found evidence for decay of all four traits. We have shown that the propensity for females to produce only sons is likely caused by a recessive allele at a single locus. Recessive genetic effects also caused the decay of sex allocation plasticity in asexuals. Whether trait decay frequently stems from recessive genetic effects, or whether recessive effects may be specific to decay of sexual traits in asexuals characterized by gamete duplication remains to be investigated. Genetic mapping studies in progress, facilitated by next-generation sequencing techniques, will provide insights into this question as well as into the molecular causes of sexual trait decay.

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Chapter 7

Synthesis and afterthoughts

Wen-Juan Ma

7.1 Thelytokous parthenogenesis

Parthenogenesis, or unisexual reproduction, is a form of asexual reproduction where unfertilized eggs can develop into mature individuals. Parthenogenesis can be obligate when species or populations reproduce exclusively in a parthenogenetic way (e.g., many insects and rotifers, reviews in Bell, 1982; van der Kooi and Schwander, 2014), or it can be cyclical when individuals can switch during their life between sexual and parthenogenetic reproduction (e.g., aphids, water fleas; Moran, 1992). Males can be produced by parthenogenesis, for example in many insects with haplodiploid sex determination, where unfertilized eggs produced by virgin females develop into haploid males (Whiting, 1933). Female-producing parthenogenesis, or thelytokous parthenogenesis ("TP" from here on), refers to a form of asexual reproduction where daughters develop from unfertilized eggs. TP has been observed in a large spectrum of organisms, including insects, crustaceans, rotifers, flatworms, snails and reptiles (Bell, 1982; Normark, 2003). Approximately two percent of the documented arthropod species reproduce parthenogenetically (Bell, 1982). Parthenogenesis can either be under the regulatory control of the hosts' genome (e.g., Beukeboom and Pijnacker, 2000; Lattorff *et al*, 2005; Schwander *et al*., 2010; Sandrock and Vorburger, 2011), or induced by intracellular endosymbionts such as *Wolbachia*, *Cardinium* and *Rickettsia* (reviewed in Werren, 1997; Werren *et al.*, 2008; Kageyama *et al.*, 2012; Ma *et al.*, 2014a). Endosymbiont-induced thelytokous parthenogenesis occurs in arthropod lineages with haplodiploid sex determination, such as Hymenoptera, Thysanoptera, some Hemiptera (e.g., scale insects) and some spider mites. These endosymbionts are typically vertically transmitted via the egg cytoplasm, and manipulate host reproduction to increase female production to enhance their own transmission (Werren, 1997; Werren *et al.*, 2008). Several cytological mechanisms of endosymbiont-induced parthenogenesis are known, including apomixis and different forms of automixis, but the genetic regulatory mechanisms remain poorly understood.

In this thesis, I have focused on the genetics, as well as on the evolutionary consequences for the host, of *Wolbachia*-induced TP in the parasitoid wasp *Asobara japonica*. In particular, I have attempted to answer the following questions: What is currently known about the molecular mechanisms of the manipulation of host reproduction by endosymbionts (Chapter 2)? What is the sex determination mechanism of *Asobara*, and how does it interact with endosymbiont-induced TP (Chapters 3 and 5)? Why do males (haploid and diploid) regularly occur in the progenies of some infected species and what does this tell us about the

mechanism of endosymbiont-induced TP (Chapter 5)? After the transition from sexual to TP reproduction following endosymbiont infection, do sexual traits of parthenogenetic hosts decay under either relaxed or negative selection, and if so, what is the genetic basis of the decay (Chapter 6)?

7.2 Convergent evolution of endosymbiont manipulation of host reproduction

Four main types of reproductive manipulation induced by endosymbionts have been documented so far, cytoplasmic incompatibility (CI), male killing (MK), feminization (FM) and thelytokous parthenogenesis (TP). The host species that are infected by these manipulation types have a broad and partially overlapping phylogenetic distribution. For example, CI occurs in nine and MK in six arthropod orders, and among these are five orders for which both CI and MK have been reported (see Chapter 2, Table 2.1). In addition, a large spectrum of endosymbionts induce these four reproductive manipulation types, including *Wolbachia*, *Cardinium*, *Rickettsia*, *Spiroplasma* and *Arsenophonus* bacteria, microsporidia and viruses (review in Kageyama *et al.*, 2012). An interesting question is whether the different manipulation types and the diversity of endosymbionts involved, reflect convergence or horizontal gene transfer between endosymbionts. Answering this question can help a deeper understanding of bacterial-host coevolution both at the mechanistic level and in a historical context. The recent sequencing of a CI-inducing *Cardinium* genome suggests that CI has an evolutionary independent origin in *Wolbachia* and *Cardinium* bacteria, since no recent horizontal gene transfer between these two endosymbionts could be detected (Penz *et al.*, 2012). Until evidence to the contrary will be obtained, the evolution of similar manipulation types in distantly related endosymbiont taxa and a large spectrum of host species infected with similar reproductive manipulation types can be considered as convergent evolution that has occurred repeatedly (Sasaki *et al.*, 2002, 2005; Jaenike, 2007; Kraaijeveld *et al.*, 2011).

Convergence in not only evident at the phenotypic level, but also at the mechanistic level. Based on a number of studies, frequent transitions between different reproductive manipulation types appear to have occurred, such as between CI and MK, and from MK and TP to FM (also see Chapter 2, Figure 2.2; Sasaki *et al.*, 2002, 2005; Jaenike, 2007;

Kraaijeveld *et al*., 2011). This suggests that the genetic regulatory mechanisms of different manipulation types are at least partially shared, although details of the genetic regulation of host reproductive manipulation by endosymbionts are still poorly understood. More sequencing and annotation of endosymbiont genomes is required to identify the bacterial genes that may interfere with host developmental processes. For example, ankyrin repeat domain-encoding genes in *Wolbachia* strains are known to be involved in protein-protein interactions, suggesting that they play a role in the molecular regulation of host-bacterial symbiotic interaction (Siozios *et al.*, 2013). These proteins are involved in cell-cycle regulation, transcriptional regulation, host development and sexual differentiation (Al-Khodor *et al.*, 2010). A comparative phylogenetic study of four complete genome sequences of *Wolbachia* revealed changes in the size of the ankyrin repeat domain-encoding genes, due to expansions and contractions controlled by short repeated sequences (Siozios *et al.*, 2013). Similar genomic studies and extensions to other endosymbionts are needed for a better understanding of convergent evolution at the mechanistic level.

The genetic basis of reproductive manipulation is expected to be intertwined with the host sex determination pathway, since altering the sexual fate of a developing embryo will somehow require the involvement of sex determination genes. Most arthropods share a conserved sex determination pathway consisting of primary signals (e.g. chromosomal constitution) that converge downstream to regulate a key sex-determination gene *transformer* (*tra*), which in turn controls sex-specific splicing of the master switch gene *doublesex* (*dsx*) (Bull, 1985; Wilkins, 1995; Sánchez, 2008; Gempe and Beye, 2011). Endosymbionts may target the host's sex determination cascade at different levels (Beukeboom, 2012). They may alter the primary sex determination signal by changing the chromosomal constitution of their hosts, but there is also some evidence that they can directly interfere with sex-specific splicing of *dsx* (Chapter 2, Table 2.1; Sugimoto and Ishikawa, 2012).

Much more research is needed to understand how endosymbionts may impinge on host reproduction. Such knowledge will help to answer the question of convergence of endosymbiont manipulation at the mechanistic level. Comparative genomics of different endosymbionts, such as *Wolbachia*, *Cardinium*, and *Rickettsia* with the same manipulation phenotype in closely-related host species may be the way forward. This approach could focus on sequence similarities among endosymbionts to reveal whether horizontal gene transfer occurred in the history, or whether certain shared protein-coding genes require special attention in further investigations (Koonin *et al.*, 2000). An alternative approach would be to

compare genomes of strains from the same bacteria that induce different manipulation phenotypes, or genomes of endosymbionts of which some strains do induce transitions between two different phenotypes and other strains do not.

7.3 Genetic basis of endosymbiont-induced TP

7.3.1 Host sex determination

As alluded to above, the genetic basis of TP is expected to be tightly associated with the host's sex determination pathway, and studying the mechanisms of these two processes can be informative for each other (Vorburger, 2014). I started with investigating the sex determination mechanism of four *Asobara* species (Chapter 3). In Hymenoptera, complementary sex determination (CSD) is the best-studied sex determination mechanism (van Wilgenburg et al 2006; Heimpel and de Boer, 2008; Asplen *et al.*, 2009). CSD has been shown to control sex determination in various hymenopteran lineages (e.g. Apoidea, Braconidae), but there are also lineages for which it has been ruled out (e.g. Chalcicoidea). Under CSD, the allelic status of one (single locus) or more (multiple loci) genes determines the sex. I showed for all four *Asobara* species that CSD is not the underlying sex determination mechanism (Chapter 3).

One of the *Asobara* species in which CSD was also ruled out is *A. japonica*, in which both sexual and thelytokous populations coexist, the latter being infected with *Wolbachia*. Absence of CSD in *A. japonica* is consistent with the most commonly assumed mechanism of *Wolbachia*-induced parthenogenesis, gamete duplication, which typically results in homozygous females. If CSD were the sex determining mechanism, homozygosity would instead lead to diploid males. A SNP marker analysis of parental females and offspring indeed indicated that thelytokous *A. japonica* females are completely homozygous (see details in Box 1), suggesting that gamete duplication is also the underlying cytological mechanism for diploidization in this species.

Absence of CSD rejects Asplen *et al.*'s (2009) hypothesis of multiple-locus CSD in *Asobara*, and calls for an adjusted phylogenetic reconstruction of CSD in the Hymenoptera. For this, it would be prudent to incorporate some relevant life-history traits, such as mating system and reproductive mode (sexual reproduction or parthenogenesis), because CSD is

shown to be negatively correlated with inbreeding and therefore homozygosity of diploids (e.g. resulting from gamete duplication). Nevertheless, the mutual constraint between CSD and homozygosity-based thelytoky could be evaded if a new mechanism of *Wolbachia*-induced thelytoky evolves (Vorburger, 2014). Detection of a new mechanism, either diploidization that does not lead to homozygosity, or uncoupling of diploidization and feminization (or female development), would break the phylogenetic association between presence of CSD and absence of TP.

The molecular basis of the sex determination of *A. japonica* needs to be further investigated to fully understand the mechanism of *Wolbachia*-induced parthenogenesis in this species (Chapters 3 and 5). The only other thus far known sex determination mechanism in Hymenoptera is maternal effect genomic imprinting sex determination (MEGISD), which has been documented for the chalcidoid *Nasonia vitripennis*. Under MEGISD, female development requires the maternal provision of *transformer* (*tra*) mRNA and the activation of *tra* expression in the zygote (Verhulst *et al.*, 2010). A trans-acting factor (*womanizer* in *Nasonia vitripennis*) likely regulates zygotic *tra* expression and is maternally silenced. Therefore, zygotic *tra* expression is only activated in fertilized eggs by the paternal copy of the trans-acting factor (Verhulst *et al.*, 2013). At this point, MEGISD sex determination remains a potential sex determination mechanism for the *Asobara* genus. The maternal provision of *tra* and the dynamics of *tra* regulation in *Asobara* shows some similarity to *Nasonia* but needs to be further investigated (E. Geuverink, unpublished data).

A functional analysis of the candidate sex determination genes in *A. japonica* is needed to reveal if, under sexual reproduction, *tra* mRNA is maternally provided to the egg and if *tra* expression is activated in fertilized eggs only to maintain an autoregulatory loop. As a first step, the genes *tra* and *dsx* and their sex-specific splicing variants need to be identified and characterized, as these two genes are essential components of the conserved sex determination cascade in insects (Chapters 1 and 3, Figure 1.2; Wilkins, 1995; Schütt and Nöthiger, 2000; Verhulst *et al.*, 2010; Gempe and Beye, 2011). The expression of *tra* and *dsx* can be further quantified at different time points during early development for embryos collecting from both virgin and mated females (Verhulst *et al.*, 2010). However, *A. japonica* being a second-instar-larval endoparasitoid of *Drosophila*, collection and microinjection of embryos or pupae is challenging in practice. An alternative approach to do a functional gene study may be to feed the double-stranded RNA to the *Drosophila* host larvae, assuming that the wasps will ingest it

while feeding of the host's hemolymph, which would initialize the RNA-interference response (e.g. Turner *et al.*, 2006; Chen *et al.*, 2010).

In the study of the molecular basis of sex determination in *Asobara*, and perhaps many other insect species, a crucial bottleneck has been the lack of genomic information and an annotated genome. Lack of a reference genome requires *de novo* assembling of genomic DNA and this is often limited by available bioinformatic expertise. The SNP marker-based genetic linkage map constructed in Chapter 4 can aid in the assembly of the *A. japonica* genome. The SNP-containing contigs can be further ordered and formed into larger scaffolds for each linkage group. The ongoing *Asobara* genome project (Droparcon, http://www.droparcon.org/wiki/DroparconStart) will provide information that can help to elucidate the genetic basis of different traits, including the mechanism of *Wolbachia*-induced parthenogenesis (also see Chapter 5), sex determination and decayed sexual traits (Seyahooei *et al.*, 2009; Mabiala-Moundoungou *et al.*, 2010; de Jong *et al.*, 2011; Chapters 3 and 6).

7.3.2 *Wolbachia* density dynamics during early embryonic development

The phylogenetic distribution of TP in arthropods is restricted to haplodiploids, including hymenopterans, thrips and mites. One possible explanation for this limited phylogenetic distribution is that TP evolves more easily in haplodiploids. Under haplodiploidy, males develop from unfertilized eggs and the pre-existing developmental machinery required for spontaneous egg development (without sperm involvement) is already present which could then be easily altered for the production of females from unfertilized gametes (Normark, 2003). Thus, the only additional requirement for female development in TP is the production of diploid instead of haploid gametes (Schwander and Keller, 2012; Neiman *et al.*, 2014). For haploidiploid arthropods, several different endosymbionts have evolved the ability to alter the ploidy level of the host eggs, converting haploid eggs that would develop into males, into diploid eggs that develop into parthenogenetic females.

Males are absent or very rare in the majority of thelytokous species. However, a high frequency of males (both haploid and diploid) was observed in several thelytokous populations of *A. japonica* (Reumer *et al.*, 2012; Chapters 5 and 6). To elucidate the mechanism of *Wolbachia*-induced parthenogenesis in *A. japonica*, I investigated the causes of male occurrence in parthenogenetic populations (Chapter 5). I found a distinct *Wolbachia* dosage effect on both the ploidy level and the gender of wasps. Diploid individuals, either male or female, have a significantly higher *Wolbachia* titer than haploids (only males), and

among diploids, females harbour more bacteria than males. A two-step model for the induction of parthenogenesis by *Wolbachia* was proposed based on the results of *A. japonica*: diploidization of the unfertilized egg is followed by feminization, and each step depends on a threshold titer of endosymbionts during a specific embryonic developmental stage. Below (section 7.3.5) I discuss how this two-step mechanism of parthenogenesis can be reconciled with the mechanism of host sex determination in *A. japonica.* Diploidization is established during the first mitotic division of the egg (e.g., Stouthamer and Kazmer, 1994; Gottlieb *et al.*, 2002; Pannebakker *et al.*, 2004a), and feminization occurs at a later stage of development. Therefore, these two critical steps likely reflect different stages in *Wolbachia* density dynamics during early embryonic development (Hiroki *et al.*, 2002; Hornett *et al.*, 2009; Sugimoto and Ishikawa, 2012).

One open question is how the bacteria are distributed in the early embryo and how their numbers change during embryonic development. Study of endosymbiont titers at different embryonic stages will provide more detailed information on the specific timing of reproductive manipulation and its dependence on the bacterial number. Immunohistology and *in situ* hybridization techniques can be used to monitor *Wolbachia* distribution during early egg/embryo development (e.g. Landmann *et al.*, 2010; Fischer *et al.*, 2011). Furthermore, RNA-seq techniques can be used to compare total gene expression between embryonic developmental stages, between haploids and diploids, as well as between sexual and thelytokous individuals. This may yield information on genes that are affected by *Wolbachia* to alter ploidy and gender of wasps, and then a candidate gene approach can be used to follow up and narrow down the list of genes that are potentially associated with *Wolbachia*-host interaction. Furthermore, the technique chromatin immunoprecipitation sequencing (ChIP-seq) can be suitable to study the genome-wide distribution and associations of DNA-binding proteins, such as transcription factors and histone-modification proteins involved in epigenetic mechanisms, which *Wolbachia* might employ (Park, 2009). ChIP-seq then can be followed by functional characterization of these gene products by the RNAi technique.

7.3.3 Possible target genes of *Wolbachia*-induced two-step TP

As I demonstrated, the first step in *Wolbachia*-induced TP of *A. japonica* involves diploidization of the unfertilized haploid egg. Gamete duplication is the so far documented cytological mechanism of chromosome duplication induced by *Wolbachia* in thelytokous Hymenotpera (i.e. Stouthamer and Kazmer, 1994; Gottlieb *et al.*, 2002; Pannebakker *et al.*,

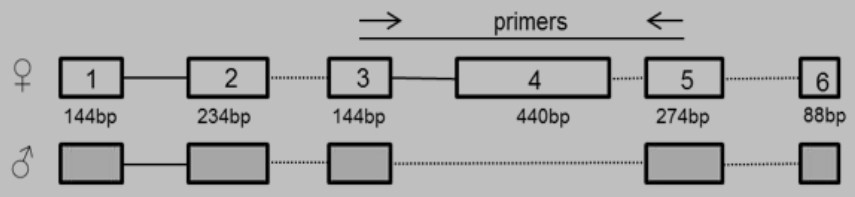
2004a; Box 1). However, little is known about the underlying molecular regulatory mechanism of this meiotic alteration. Candidate genes are those that can impair proper digestion of cohesions controlling chromosome separation, and those that can inhibit the M checkpoint in the cell cycle (Schurko *et al.*, 2009; Kraaijeveld and Bast, 2012).

Similarly, little information is available about the molecular basis of how endosymbionts feminize host embryos. In the woodlouse *Armadillidium vulgare* (Isopoda) (Bouchon *et al.*, 2008) endosymbionts prevent development of the androgenic gland, which produces a hormone that is essential for male development, in genotypically male embryos. However, in insects, sex determination is generally considered a cellular genetic process and hormonal signaling is less important (Steinmann-Zwicky *et al.*, 1989; Schütt and Nöthiger, 2000; Negri and Pellecchia, 2012). There is some evidence that endosymbionts can direct the sex-specific splicing of host sex determination genes (e.g. *dsx*) to the female-specific form, as observed in lepidopterans (Narita *et al.*, 2007; Sugimoto and Ishikawa, 2012). In addition, *Wolbachia* might interfere with splicing of *tra* in thelytokous *Leptopilina clavipes*, where *Wolbachia* promotes the production of the female splice variant and suppress the male splice variant in thelytokous females. In sexual females the male splice variant is also expressed at low levels next to the female splice variant (pers. comm. E. Geuverink). I also found preliminary evidence that *Wolbachia* interferes with splicing of *dsx* in thelytokous *A. japonica* (see for more details Box 7.1). A similar pattern might also apply to the regulation of *tra* in *A. japonica* (pers. comm. E. Geuverink).

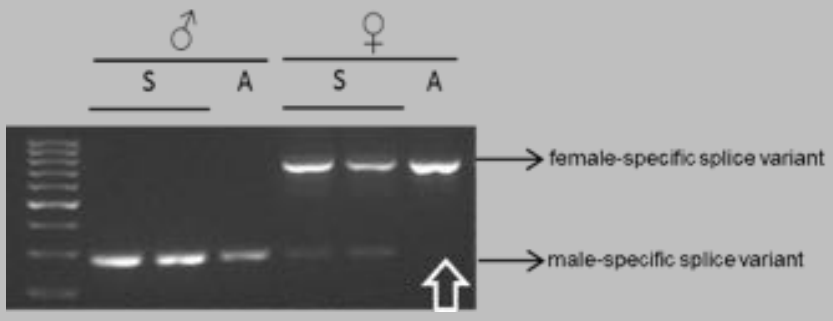
In my introgression experiment, where I replaced 94% of the sexual genome by the asexual genome (see details in Chapter 5 and 6), the female sex determination pathway could still be activated without *Wolbachia* presence, suggesting that this trait remains functional in the asexual genome despite *Wolbachia* infection. If *Wolbachia* indeed interfere with the splicing of *tra* or *dsx* in thelytokous *A japonica*, it has not led to a functional loss of the sex determination pathway, which could be due to the relatively young *Wolbachia* infection (Murata *et al.*, 2009; Reumer *et al.*, 2012). Another possible reason for no sign of degeneration of the female sex determination pathway is that the genes involved have pleiotropic functions; they may also be involved in other important developmental pathways. For example, *dsx* also regulates the complex wing patterns, colours and structures required for mimicry in the butterfly *Papilio polytes* (Kunte *et al.*, 2014).

Box 7.1 *Wolbachia* interfere with the splicing of *dsx* in *A. japonica*

I used the homolog of the *A. tabida dsx* gene for the genomic BLAST (version ncbi-blast-2.2.26+) search against *A. japonica* assembled contigs from the ongoing *Asobara* genome project (Droparcon, http://www.droparcon.org/wiki/DroparconStart). Primers were designed for the validation of the gene predictions in the relevant contigs including all putative *dsx* gene of *A. japonica* (using PerlPrimer 1.1.21). Using 5' and 3'RACE and RT-PCR, one female-specific and one male-specific splice variant of *A. japonica dsx* were identified. A schematic representation of part of the *dsx* gene is shown in Figure 7.1a. Primers were designed to identify sex-specific splice variants, spanning exon 3 to exon 5. Since exon 4 is absent in the male-specific splice variant, the female-specific amplicon size is 721bp and the male specific amplicon size is 280bp; Figure 7.1a). RT-PCR showed that in thelytokous females, only the female-specific splice variant was present in contrast to sexual females that showed both splice variants with the female-specific splice variant being predominant (Figure 7.1b). More samples are needed to further verify this conclusion. Both sexual males and males from a thelytokous strain show only the male-specific splice variant (Figure 7.1b). Leakage of the male-specific splice variant in females is commonly observed (pers. comm. E. Verhulst). These results show that *Wolbachia* presence leads to constitutive female-specific splicing of *dsx*, suggesting an active role of *Wolbachia* in *dsx* splicing process with an unknown mechanism.



(a)



(b)

Continued.

Figure 7.1 RT-PCR of the sex-specific splice form of the sex determination gene *doublesex* in *A. japonica* female and male adults. (a) The putative gene structure of *dsx* of *A. japonica*, based on Sanger-sequencing results. The arrows show the locations of the primers. Primer F: 5'-GGGTACTGCTGGATTACAGTG-3', Primer R: 5'-CTGCATTGACAGCGGTTGTG-3'. (b) Agarose gel electrophoresis of the female-specific and male-specific splice variant from females and males from a sexual and a thelytokous strain. The white arrow indicates the complete absence of the male-specific splice variant in asexual females compared to a low level in sexual females. "A" and "S" indicate the TP and the sexual population, respectively.

7.3.4 Does manipulation of host reproduction by *Wolbachia* involve epigenetic modification?

Recently, evidence has grown for epigenetic control of developmental processes in insects, including the process of sex determination (Verhulst *et al.*, 2010; Lyko and Maleszka, 2011; Gómez-Díaz *et al.*, 2012; Zwier *et al.*, 2012). There are many different epigenetic mechanisms known from insects. Chromatin remodeling can result in the alteration of chromosomal behaviour and in variation in gene expression or splicing processes. Manipulation phenotypes that include alteration of chromosomal constitution, such as CI and TP, may therefore involve some form of genomic imprinting (Werren and Beukeboom, 1998; Negri and Pellecchia, 2012; Rabeling and Kronauer, 2013). Another type of epigenetic mechanism is methylation, which has been documented to be involved in FM, as *Wolbachia* change the host genome methylation from male to female patterns in feminized genetic males of the leafhopper *Zyginidia pullula* (Negri *et al.*, 2009). Although a role of epigenetics in host manipulation by endosymbionts is plausible, we are only at the beginning of elucidating the possible molecular and biochemical pathways involved.

Interestingly, in the offspring of thelytokous *A. japonica*, diploid females and diploid males have identical, completely homozygous, genotypes (Box 1). However, diploid males harbour a lower *Wolbachia* titer (Chapter 5). Hence, epigenetic regulation of host development that depends on *Wolbachia* titer may (partly) explain male versus female development for these identical genotypes. As a first step to look into a possible role of epigenetics, I performed a pilot experiment for methylation involvement in *Wolbachia*-induced TP. Methylation could be performed by *Wolbachia* but also by *Asobara*, as preliminary data show that the *A. japonica* genome contains the DNA methylthransferase genes *Dnmt2* and *Dnmt3*, although it lacks *Dnmt1* (pers. comm., E. Geuverink). To disrupt

DNA methylation, I fed the methylation inhibitor 5-Aza-2'-deoxycytidine (5-aza-dC) to freshly emerged thelytokous and sexual females (see details in Box 7.2). The preliminary results showed no significant effect on either brood size or male production of thelytokous females compared to the sexual control. So, we cannot yet assess a role of methylation in *Wolbachia* manipulation of *A. japonica* reproduction.

Box 7.2 Epigenetic regulation of TP by *Wolbachia*?

As a first step to test whether *Wolbachia* induce TP in *A. japonica* via an epigenetic mechanism, the methylation inhibitor 5-Aza-2'-deoxycytidine (5-aza-dC) was fed to freshly-emerged adult thelytokous females (KG, HR strains) and sexual females (AO strain) as control for reproductive mode difference. 5-aza-dC functions as a hypomethylation agent, which hypomethylates DNA by inhibiting DNA methyltransferase and as a consequence the associated methylation genes become activated (Christman, 2002). One study shows that *Wolbachia* is associated with the host genome methylation pattern shift from the male to the female form in feminized genetic males of the leafhopper *Zyginidia pullula* (Negri *et al.*, 2009). This leads us to hypothesize that *Wolbachia* affect host sex determination in thelytokous *A. japonica* by altering methylation patterns, and diploid male occurrence is due to the failure of manipulation of the host methylation pattern. Thus, it is predicted that thelytokous KG females produce sons, because the 5-aza-dC treatment disrupts all methylation gene expression, but there will be no such effect in the sexual AO strain. To monitor if the adults fed successfully on the medium containing 5-aza-dC, red food colorant was added to the mix with the chemical agent 5-aza-dC; red food colorant was indeed found in the treated females' abdomen. Brood size (glm_KG: $F_{1,21}$=0.986, *P*=0.333; ANOVA_HR: F=0.970, *P*=0.337; ANOVA_Am, F=0.011, *P*=0.918) and offspring sex ratio did not differ between the treated and control replicates (glm, $F_{1,14}$=0.081, *P*=0.781). The treatment also did not affect the proportion of females that produced sons (Fisher's exact test, KG, *P*=1.000; HR, *P*=1,000). Thus, no support was found for a role of methylation in *Wolbachia*-induced thelytoky in *A. japonica*. In another parasitoid wasp *Apanteles galleriae*, a 100-fold higher concentration of 5-aza-dC than used in this study led to significantly decreased longevity and adult size, as well as an increased proportion of male offspring due to pre-adult female mortality. This suggests that 5-aza-dC is affecting life-history traits of this parasitoid, including the processes of fertilization, but the presence of diploid males was not investigated (Uçkan *et al.*, 2007). However, there may also be toxic effects of 5-aza-dC at high concentrations. More experiments with different concentrations of this methylation inhibitor are needed in *A. japonica*, as well as quantifying the effects on methylation inhibitor levels and gene expression changes at the genetic level, to establish whether *Wolbachia* affect host genome methylation patterns.

7.3.5 TP-inducing endosymbionts and host sex determination: an extended two-step model

In Hymenoptera, sex is determined by a variety of primary signals, including CSD, MEGISD and likely additional yet-unknown mechanisms (see details in Chapters 2 and 3). These primary signals direct the sex-specific splicing of *tra,* which in turn regulates sex-specific splicing of *dsx* that controls sexual differentiation; female development needs tra^F (the female splice form) to be transcribed and spliced, while the male splice form tra^M leads to male development (Wilkins, 1995; Schütt and Nöthiger, 2000; Beye *et al.*, 2003; Verhulst *et al.*, 2010; Bopp *et al*., 2014). Based on studies by both Giorgini *et al.* (2009) and Tulgetske (2010), it has been suggested that diploidization and feminization are separate processes in TP induction. My results on thelytokous *A. japonica* confirm this notion, and take it further to point out that the two separate steps require specific *Wolbachia* titer thresholds. In this section I combine the current knowledge on mechanisms of diploidization and sex determination to propose a model endosymbiont induction of TP in Hymenoptera (Figure 7.2).

My results (Chapter 5) indicate that diploidization only occurs above a certain endosymbiont titer, as haploid males are uninfected or have a low endosymbiont titer compared to diploid individuals. Thus, if endosymbionts are absent or present at very low density in unfertilized eggs, no diploidization occurs. In terms of sex determination, the female-specific splice variant of *tra* (tra^F) is not expressed in the unfertilized eggs and the male-specific *dsx* splice variant (dsx^M) is produced, resulting in haploid embryos that develop as males (Figure 7.2 A and B). Diploidization of haploid embryos occurs at higher endosymbiont titers (Figure 7.2 C and D). There are several possible outcomes of diploidization. A low titer of *Wolbachia* could potentially result in diploid males while the second step of feminization is not initiated, because of insufficient endosymbiont threshold (Figure 7.2 C). In some species, diploidized eggs may autonomously develop into females following the haplodiploid sex determination, without further interference of the endosymbiont (Figure 7.2 D1). In other species an additional step of feminization induced by the endosymbiont may be required for female development. My study results indicate that both the diploidization and the feminization steps are required in *A. japonica* and that both steps rely on a sufficiently high level of bacteria, while intermediate levels result in diploidization alone. In terms of sex determination, in such diploid embryos there is successful initiation of zygotic *tra* expression and the downstream female splicing of dsx^F (Figure 7.2 D). Depending on the specific mechanism of host sex determination (CSD,

MEGISD or a yet-unknown mechanism) feminization by endosymbionts can occur in different ways. For hosts with a MEGISD mechanism, it is speculated that the endosymbiont may either manipulate the maternal imprinting of an yet unknown trans-acting factor of tra^{Mat}, in order to initiate tra^{F} for female development (Figure 7.2 D2). For host species with a CSD mechanism, the endosymbiont might overcome the constraint of homozygosity by manipulating the regulation of tra^{F} to cause female development (Figure 7.2 D3). In some host species, endosymbionts only feminize the diploid (male) embryos after the host genome has diploidized the haploid eggs by some unknown mechanism, e.g. *Cardinium* induced thelytokous *Encarsia hispida* (Giorgini *et al.*, 2010; Figure 7.2 D4).

Some scenarios of endosymbiont induced parthenogenesis in the extended two-step model are supported by experimental evidence, whereas others are more speculative. There is empirical evidence for a role of *Wolbachia* titer on the diploidization (A, B, C; Zchori-Fein *et al.*, 2000; Kraaijeveld *et al.*, 2011; Chapter 5) and feminization process (Chapter 5). Evidence for female development following the diploidization of unfertilized eggs (D1) is found in e.g. *Muscidifurax uniraptor* and *Leptopilina clavipes* (Gottlieb *et al.*, 2002; Pannebakker *et al.*, 2004a). Thelytokous female development under scenarios D2 and D3 remain a matter of speculation in terms of sex determination. In *A. japonica*, for which a CSD mechanism was ruled out (Ma *et al.*, 2013; Chapter 3), feminization might occur through alteration of MEGISD and therefore scenarios D2 and D3 are worth further investigating. Evidence for scenario of D4 has been reported from parthenogenetic *Encarsia hispida* infected with *Cardinium* (Giorgini *et al.*, 2009).

It should be noted that the traditional scenario (D1) is based on cytological data only. Occurrence of diploid males and effects of endosymbiont density on ploidy level and gender have typically not been investigated (Gottlieb, 2009). Hence, more thelytokous species may exist in which parthenogenesis is induced via a two-step mechanism. Thus, more screens for diploid male occurrence in endosymbiont-induced thelytokous species should be performed to judge the relative frequencies of the different scenarios of the model. The evolution of endosymbiont-induced thelytoky is often regarded as being constrained by the sex determination mechanism (Vorburger, 2014). For instance, it has long been assumed that gamete duplication is not compatible with CSD, because homozygosity leads to diploid male development under CSD rather than females. The two-step mechanism of parthenogenesis partly alleviates the phylogenetic constraints of CSD and infectious thelytoky. Moreover, it opens a new window to study sex determination mechanisms within the Hymenoptera. For

instance, one could consider introducing a homozygosity-inducing endosymbiont by microinjection into a sexual species, and check their whether ploidy and gender of individuals are consistent with CSD (Vorburger, 2014). Challenges that need to be overcome for this approach are to extract *Wolbachia* from live thelytokous individuals, inject them into sexual females, and ensure effectiveness of *Wolbachia* manipulation in the new host.

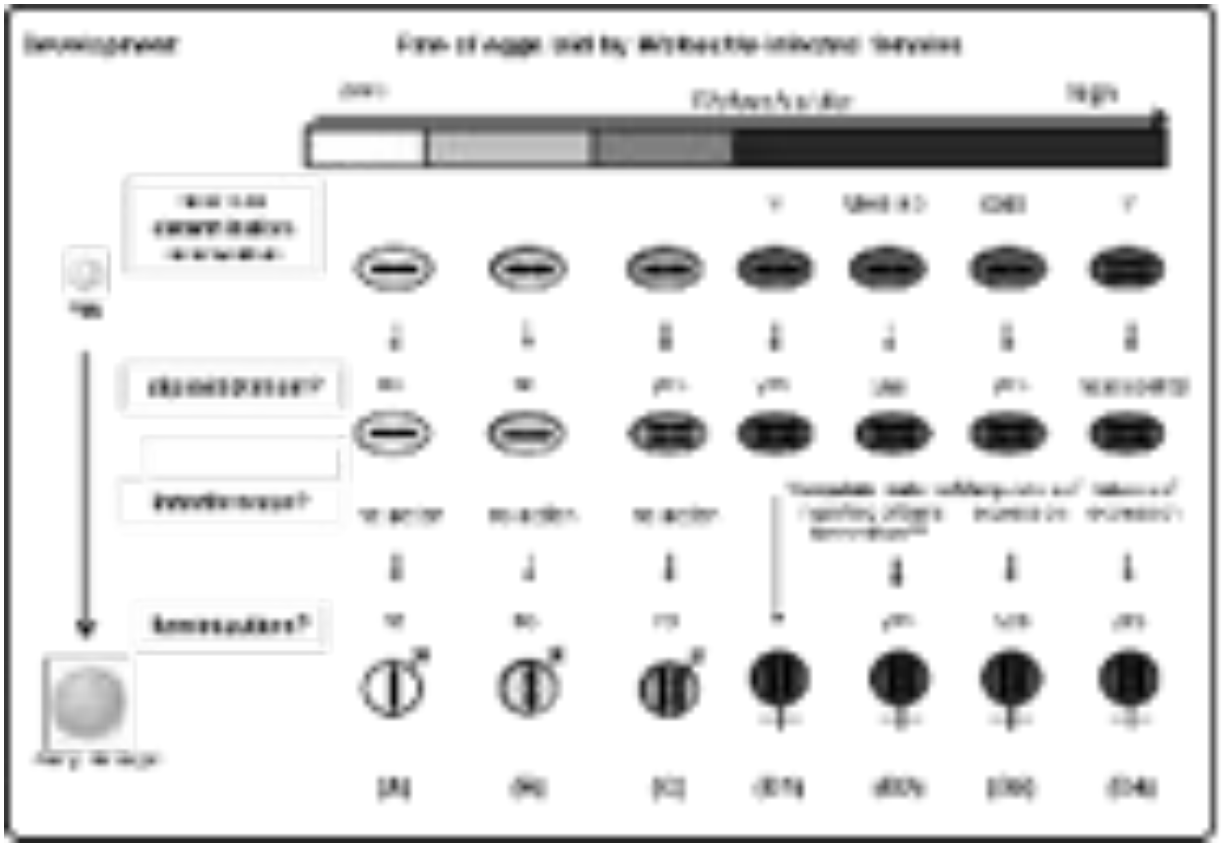


Figure 7.2 An extended two-step model for how endosymbionts can induce thelytoky in Hymenoptera. Endosymbionts can either diploidize the unfertilized eggs and female development follows because of haplodiploid sex determination, or they can have a dual effect: diploidization followed by feminization of the embryo. In the latter case, both steps rely on different endosymbiont titer thresholds during early embryonic development. The host sex determination mechanism affects the fate of diploidized eggs under TP induction. There are several scenarios: (A) Absence of endosymbionts leads to standard haploid male development; (B) Low endosymbiont titer fails to initiate diploidization and feminization, leading to haploid males; (C) Intermediate numbers of endosymbionts succeed in diploidization, but are insufficient to induce feminization and result in diploid males. (D1) for some host species female development follows directly from diploidization of

unfertilized haploid eggs; Endosymbionts might feminize diploids by interfering with the female sex determination pathway in different ways: (D2) for hosts with a MEGISD mechanism, high endosymbiont titers successfully induce feminization by either manipulating the maternal imprint of an unknown *trans* factor of tra^{Mat} on the duplicated chromosome set following diploidization to activate the zygotic expression of *tra*, resulting in the development of diploid females; (D3) for hosts with gamete duplication and a CSD mechanism, endosymbionts might alter the splicing of *tra* towards females (tra^{F}) leading to female development; (D4) for hosts in which diploidization of unfertilized eggs is under host (nuclear genome) control, the endosymbiont is only responsible for feminizing of diploid embryos by causing tra^{F} splicing for female development.

7.4 The evolutionary consequences of *Wolbachia*-induced TP

7.4.1 Genetic architecture of decayed sexual traits

Former adaptive traits might become maladaptive under relaxed selection, or decay under negative selection, due to the shifts in selection pressure following a change in environmental or lifestyle (Fong *et al.*, 1995; Wiens, 2001). Another aim of my study was to investigate the fate of sexual traits in a TP species after it abandons its sexual lifestyle. The genetic changes associated with decay of sexual traits are largely unknown, whether it for example involves few loci with major effects or many loci each with minor effects. I performed an introgression experiment between a sexual and a TP strain and monitored the fate of several female sexual traits, such as sexual attractiveness, courtship behaviour and fertilization ability. The results of this introgression experiment revealed a simple genetic architecture, i.e. a single recessive locus with major effect for the trait of females producing only sons (because of decayed sexual attractiveness, mating behaviour, egg fertilization or any combination of the three traits; Chapter 6). As *Wolbachia* is a relatively young infection in *A. japonica* (Murata *et al.*, 2009; Reumer *et al.*, 2012), a remaining question is how this recessive allele can have spread in thelytokous populations within a short evolutionary time. One possible explanation is related to the mechanism of diploidization under *Wolbachia*-induced parthenogenesis. Diploidization of the embryo is likely restored through gamete duplication and therefore results in complete homozygosity. Such homozygosity would facilitate spread of these recessive alleles in TP popuations, similar to dominance favoring the spread of adaptive mutations in diploid species (Otto and Goldstein, 1992; Orr and Otto, 1994). Studies on

decayed sexual traits in more species are needed to draw general conclusions on genetic architecture of the decayed traits. In addition, it remains to be investigated whether recessive alleles may also frequently cause trait decay in species with forms of asexuality that maintain heterozygosity, or whether trait decay in these cases is mostly caused by dominant mutations.

In the introgression experiment, I also found an interesting result that *Wolbachia* can negatively affect the females' sexual attractiveness towards sexual males (Chapter 6). This suggests that *Wolbachia* might also be able to promote pre-zygotic isolation by preventing gene flow between populations, next to the often studied *Wolbachia*-induced post-zygotic isolation under cytoplasmic incompatibly (i.e. Breeuwer and Werren, 1993; Vavre *et al.*, 2000; Hunter *et al.*, 2003; Brucker and Bordenstein, 2013). Bacteria are known to alter pheromone production in insects (i.e. Dillon and Charnley, 2002; Koukou *et al.*, 2006; Sharon *et al.*, 2010), or affect female attractiveness by altering cuticular hydrocarbon profiles, which function as mating cues in many insect species (i.e., Ivy *et al.*, 2005; Yew *et al.*, 2009). It will be an interesting follow up to investigate the molecular genetic basis of how *Wolbachia* affects female sexual attractiveness.

In addition, I found that asexual *A. japonica* lost their plastic sex allocation ability in response to variation in local mate competition. Sex allocation persisted in females of the F1 hybrid generation, but it was lost in the second hybrid generation, which is consistent with recessive genetic effects (Chapter 6). However, an estimation of the number of loci affecting this trait could not be obtained, given that the presence or absence of sex allocation plasticity is evaluated qualitatively at the group level. The mechanisms underlying the decay of this trait remain a matter of speculation, but might include a loss of control over egg fertilization or the perception of levels of competition among founding females after switching to thelytokous reproduction. Because males have no functional role in asexual species, plastic sex allocation may not be maintained by selection. The decay of plastic sex allocation response under asexuality has not been investigated previously, hence it is at this moment impossible to evaluate if it represents a general trend in asexuals derived from haplodiploid sexual ancestors, or if such decay is specific to asexual *A. japonica*.

In addition to decay of sex allocation plasticity, I also showed in chapter 6 that recessive effects at a single locus likely cause the propensity of females to produce only sons, following introgression of alleles from the asexual to the sexual genome. The logical next step is to elucidate the underlying molecular basis of these decayed sexual traits. This could be done by quantitative trait loci (QTL) mapping, or by direct genomic comparison. The genetic linkage

map of SNP markers constructed in Chapter 4 can facilitate QTL analyses in this species. After the genomic regions for the decayed sexual traits have been determined, candidate genes may be identified and functionally characterized, for example by RNAi (i.e. Verhulst *et al.*, 2010) or clustered regularly interspaced short palindromic repeats (CRISPR; Wiedenheft *et al.*, 2012).

7.4.2 Genomic signature of *A. japonica* with *Wolbachia*-induced TP

In the previous section, I discussed some possible approaches to uncover the genes that code for the decayed female sexual traits of thelytokous *A. japonica* (Chapter 6). Another evolutionary question related to thelytokous reproduction is to what extent lack of recombination and selection on sexual traits leaves signatures in the genome after a species abandons its sexual mode of reproduction. Muller's Ratchet theory predicts that asexual species would eventually be driven to extinction due to the accumulation of deleterious mutations following lack of recombination (Muller, 1932, 1964). In thelytokous *A. japonica*, deleterious mutations are expected to have been accumulating in the genome since the sexual and thelytokous strains separated approximately 0.2-0.8 million generations ago (Reumer *et al.*, 2012). Thelytokous lineages are predicted to have an elevated mutation rate in genes coding for traits that are no longer under selection, such as female sexual attractiveness, courtship behaviour and sex allocation. However, sexual recombination in the sexual strain might result in relatively mutation-free genotypes over generations. The variance of the genetic load between the two strains with different reproductive mode provides materials for selection to act on. Thus, comparative genomic analysis between sexual and thelytokous genomes could be used to assess the ratio of non-synonymous versus synonymous mutations (dN/dS ratio), sequence variation in duplicated genes and proportions of pseudogenes, etc. This would provide an interesting insight into genomic signature on the thelytokous reproduction due to *Wolbachia* infection, and might be a valuable alternative approach towards revealing the genomic and genetic basis of decayed sexual traits. Such genome-wide tests of Muller's Ratchet have not yet been performed to my knowledge.

7.5 Final remarks

I am fascinated by the fundamental evolutionary questions of coevolution between endosymbionts and their hosts. In this thesis I have focussed on the topic of *Wolbachia*-induced thelytokous parthenogenesis (TP), and tried to understand the genetic basis and evolutionary consequences of TP. Approximately 1 out of 1000 living organisms develops from an unfertilized egg through thelytokous parthenogenesis, which makes it an important alternative mode of reproduction compared to sexual reproduction.

By reviewing literature of the various types of host reproduction manipulation by endosymbionts, I found that convergent evolution likely occurs at both the phenotypical and mechanistic level. Additionally, I argued that the host sex determination mechanism is the first to be investigated in order to understand the evolution of infectious parthenogenesis. By revealing important details of the sex determination mechanism of *Asobara* wasps, I could rule out CSD in four species with diverse life-history traits, and call for an adjusted perspective on the phylogenetic distribution CSD within the Hymenoptera. The MEGISD sex determination mechanism is a suitable candidate to explain the how sex is determined in *A. japonica*.

Investigation of the genetic causes of regular male occurrence in thelytokous *A. japonica*, led me to propose a two-step model that captures the different forms of *Wolbachia*-induced TP. It led to the insight that the traditionally considered phylogenetic constraints between host sex determination and infectious parthenogenesis within Hymenoptera need not be that strict. My study calls for more comparative data on the association between sex determination and reproductive mode and opens up new opportunities to investigate host sex determination. To further understand the genetic basis of TP, a combination of comparative genomics of these endosymbionts and ChIP-seq technique may generate a list of candidate genes that endosymbionts interfere with to manipulate host reproduction.

By introgression of alleles from a thelytokous to a sexual strain for multiple generations, I found that female sexual traits decayed under neutral or negative selection. I found evidence for a major-effect recessive locus for some investigated traits, as well as recessive genetic effects for the loss of plastic sex allocation under thelytoky. To uncover the specific genes encoding these decayed sexual traits, QTL mapping and genomic analyses should be further performed. Furthermore, I confirmed that gamete duplication is the likely mechanism to

induce TP in *A. japonica*. This might have facilitated the rapid spread of recessive alleles that underly the trait decayns.

Finally, I hope that my study will contribute to a further understanding of the mechanisms of endosymbiont-induced parthenogenesis and the evolution of asexuality in general.

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Chapter 8

Epilogue

Wen-Juan Ma

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