Chapter 7

Does a parthenogenesis-inducing *Wolbachia* induce vestigial cytoplasmic incompatibility?

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Published in Naturwissenschaften (2011) 98: 175-180

Wolbachia is a maternally inherited bacterium that manipulates the reproduction of its host. Recent studies have shown that male-killing strains can induce cytoplasmic incompatibility (CI) when introgressed into a resistant host. Phylogenetic studies suggest that transitions between CI and other *Wolbachia* phenotypes have also occurred frequently, raising the possibility that latent CI may be widespread among *Wolbachia*. Here, we investigate whether a parthenogenesis-inducing *Wolbachia* strain can also induce CI. Parthenogenetic females of the parasitoid wasp *Asobara japonica* regularly produce a small number of males that may be either infected or not. Uninfected males were further obtained through removal of the *Wolbachia* using antibiotics and from a naturally uninfected strain. Uninfected females that had mated with infected males produced a slightly, but significantly more male-biased sex ratio than uninfected females mated with uninfected males. This effect was strongest in females mated with males that had a relatively high *Wolbachia* titer. Quantitative PCR indicated that infected males did not show higher ratios of nuclear versus mitochondrial DNA content. *Wolbachia* therefore does not cause diploidization of cells in infected males. While these results are consistent with CI, other alternatives such as production of abnormal sperm by infected males cannot be completely ruled out. Overall, the effect was very small (9%), suggesting that if CI is involved it may have degenerated through the accumulation of mutations.

Introduction

Wolbachia is a cytoplasmically inherited bacterium, known for its ability to manipulate reproduction in its arthropod hosts (Stouthamer *et al.* 1999). These manipulations include the induction of parthenogenesis (PI), male-killing, feminization and cytoplasmic incompatibility (CI). The first three result in highly female-biased sex ratios, while CI decreases the offspring production of uninfected females by inducing sterility in crosses between infected males and uninfected females. In haplodiploids, only fertilized eggs, which normally develop into diploid females, suffer CI, while unfertilized eggs, which develop into haploid males, do not. Incompatible eggs either die or are converted into males. In both cases, CI results in a more male-biased sex ratio.

Phylogenetically, *Wolbachia* strains inducing different phenotypes do not form monophyletic clusters (Baldo *et al.* 2006), suggesting that switches between phenotypes have occurred repeatedly. Recurrent acquisition and loss of genes involved in reproductive manipulations in *Wolbachia* genomes may lead to rapid changes in the phenotype. Alternatively, all manipulations may be induced by the same *Wolbachia* strain, but their expression may depend on the host genetic background. In support of the latter, several recent studies have shown that some *Wolbachia* can switch rapidly between male-killing and CI (e.g. Jaenike 2007, Hornett *et al.* 2008). For example, Hornett *et al.* (2008) showed that a male-killing *Wolbachia* infecting the butterfly *Hypolimnas bolina* induces CI when introgressed into host strains that are resistant to male-killing, showing it has a latent ability to induce CI. Whether *Wolbachia* that normally induce parthenogenesis or feminization also have the ability to induce CI is unknown.

Hurst *et al.* (2002) showed theoretically that CI-only strains are highly susceptible to invasion by mutants that can manipulate host sex ratios while retaining their CI ability. When sex ratio distortion is complete (i.e. infected females produce no males), as is common for parthenogenesis-inducing strains, CI is not expressed anymore and the ability to cause CI is expected to degrade by selection and/or mutation, resulting in a strain that only distorts the sex ratio (Hurst *et al.* 2002).

In this study, we investigate whether the *Wolbachia* strain *wAjap* that normally induces parthenogenesis in its *Drosophila* parasitoid host *Asobara japonica* (Hymenoptera: Braconidae) can also induce CI. Phylogenetic analysis showed *wAjap* to be closely related to several strains that induce CI (Kremer *et al.* 2009), suggesting that transitions between CI and other phenotypes have occurred in this group. Populations of *A. japonica* on the main islands of Japan reproduce through *Wolbachia*-induced thelytokous parthenogenesis, in which females are produced from unfertilized eggs. Populations from the southern subtropical islands are uninfected and reproduce through arrhenotoky, in which females develop from fertilized, diploid eggs and males from unfertilized, haploid eggs (Mitsui *et al.* 2007, Kremer *et al.* 2009). During routine culturing, infected *A. japonica* females regularly produce

small numbers of males (rarely many), some of which are infected with *Wolbachia*, a situation rarely observed in species infected with PI-*Wolbachia*. This allowed us to investigate whether *wAjap* is able to induce CI.

Materials and Methods

Asobara japonica strains and antibiotic treatment

A. japonica strains were kindly provided by M.T. Kimura from cultures derived from the field samples described in Mitsui *et al.* (2007) and Murata *et al.* (2009). An infected, thelytokous population from Kagoshima and an uninfected, arrhenotokous population from Amami-Oshima were used in all experiments. Culturing and removal of *Wolbachia* were described in Kremer *et al.* (2009). Briefly, parasitoid eggs and larvae were exposed to antibiotics through the host's haemolymph. Most of the antibiotic-treated larvae developed as aposymbiotic females. These females produced only males when allowed to oviposit, indicating they were cured of their *Wolbachia* infection. Few antibiotic-treated larvae developed directly as aposymbiotic males. Hence, we used both males from the first generation, which had been exposed to the antibiotic, and from the second generation, of which only their mother had been exposed.

Mating experiments

To obtain virgin females from the arrhenotokous strain, pupae were transferred individually to PCR tubes just prior to emergence. These tubes were checked twice daily for emerged females, which were then transferred to a small glass tube (2.5 x 8.0 cm) containing a layer of agar. Each female was provided with a single male of one of five types (arrhenotokous and thelytokous refer to the line of origin): uninfected arrhenotokous, uninfected thelytokous antibiotics-exposed, uninfected thelytokous from antibiotics-exposed mother, naturally uninfected thelytokous, infected thelytokous. Males develop faster than females. To ensure that the males were virgin, the culture jars were checked every three hours around the time of emergence and virgin males were transferred to fresh jars and kept in single-sex groups. No males were collected from jars in which females had started emerging. The mating pair was kept together in the glass tube for two days, after which the female was allowed to parasitize about 200 *D. melanogaster* larvae. All males were checked for *Wolbachia* infection through PCR assay (see below). The numbers of male and female offspring emerging from these crosses were scored for the next four weeks.

DNA extraction
DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, using mini spin columns. Before starting the DNA extraction, each wasp was transferred to a new 1.5 ml Eppendorf tube. After evaporation of remaining ethanol, tissue lysis buffer (ATL) was added to the tube and the wasp was crushed using a plastic pestle. The tissue was incubated overnight in proteinase K at 56°C. The DNA was dissolved in 100 µl elution buffer (AE).

Wolbachia detection
Wasps were tested for *Wolbachia* infection by amplifying the *Wolbachia*-specific *wsp* gene, with primers *wsp*-81F and *wsp*-691R (Braig *et al.* 1998, Zhou *et al.* 1998) and the *Wolbachia*-specific *ftsZ* gene (Holden *et al.* 1993, Sinkins *et al.* 1995). Polymerase Chain Reactions (PCR) for both genes were performed in a total volume of 20.0 µl, containing 1x PCR-buffer (Qiagen), 62.5 µM dNTPs, 1 unit Taq polymerase, 250 nM forward primer, 250 nM reverse primer and 1.0 µl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research, Waltham, MA, USA) was used for all PCRs. PCR conditions for the *wsp* gene were as follows: 3 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and finally 5 min at 72°C. PCR conditions for the *ftsZ* gene were as follows: 3 min at 94°C, then 35 cycles of 45 sec at 94°C, 1 min at 55°C and 1 min at 72°C and finally 5 min at 72°C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Real-time quantitative PCR
To test whether the sex ratios induced by infected males were affected by *Wolbachia* density, we conducted real-time quantitative PCR (qPCR) on the DNA samples from the mating experiment. We also compared *Wolbachia* density between infected males and infected females. *Wolbachia* density was assessed by quantifying the copy number of the *Wolbachia*-specific *wsp* gene, using the nuclear *18S* gene to control for DNA concentration. The number of *Wolbachia* cells was determined by using the generalist primers *wsp*-81F/*wsp*-691R which amplified the single-copy *wsp* gene as described in Mouton *et al.* (2004). The multicopy nuclear gene *18S*rRNA was amplified using the primers *18S*.lo1/NS58+2, as described in Mouton *et al.* (2009).

Furthermore, we tested whether *Wolbachia* infection resulted in diploidization of cells in males. We again quantified the nuclear *18S* gene and compared this to the quantity of the mitochondrial *CO1* (*Cytochrome Oxidase I subunit*) gene, which should not be affected by diploidization. In this test, we compared infected with uninfected males. We designed new primers from the alignment of the *CO1* sequences of 16 *A. japonica* strains (Murata *et al.* 2009) especially to optimize qPCR reactions: *Ajap-CO1*-159F (5'- ACC TGT AAT ATT AGG TGG ATT TGG

-3') and *Ajap-CO1*-289R (5'- CCA ACA CCT ACA TTT AAT ATT CCT CT -3'; amplified product 139bp). The PCR conditions consisted of 10 min at 95°C followed by 35 cycles, each consisting of denaturing for 10 sec at 95°C, annealing for 10 sec at 54°C and elongation for 10 sec at 72°C. qPCR reactions were performed on the LightCycler 480 Real-Time PCR System (Roche, Penzberg, Germany). The 10 µL reaction mix contained 200 nM of each primer, 5 µL of LightCycler 480 SYBR Green I Master (Roche), and 1 µL of template DNA.

MLST sequencing

In order to assess whether the *Wolbachia* infecting the male *A. japonica* was indeed *wAjap* (and not an additional strain that had escaped detection in infected females), we sequenced a set of five MLST genes (Multi Locus Sequence Typing) and the *wsp* gene for one infected male and one infected female for each of five thelytokous *A. japonica* strains (Kagoshima, Sapporo, Hirosaki, Sendai and Tokyo; Mitsui *et al.* 2007). The protocols for PCR and sequencing were described in Baldo *et al.* (2006).

Statistical analysis

The sex ratios of the offspring produced by females mated to different types of males were compared using generalized linear models (glm) with a binomial error distribution and an empirically estimated scale parameter. The number of males was the response variable and the total number of offspring the binomial denominator. Significance was assessed by removing explanatory variables from the model and comparing the change in deviance using an F-test. Clutch size was compared using analysis of variance (anova).

Since the data sets for the qPCR experiments did not follow normal distributions (Shapiro test), we used non-parametric Wilcoxon rank sum tests, with $\alpha = 0.05$.

All analyses were performed in R software (version 2.9.2; R Developmental Core Team 2006), except the power analysis, for which we used the sample size calculator available at: http://www.stat.ubc.ca/∼rollin/stats/ssize/n2.html.

Results

Mating experiments

A total of 34 males were produced by infected mothers, of which 18 (53%) tested positive for *Wolbachia* in the PCR assay. The sex ratios produced by arrhenotokous females differed significantly, depending on the type of male they had mated with (Fig. 7.1; $F_{4,64} = 2.81$, $p = 0.03$). Lumping the uninfected male groups did not result in a significant change in deviance of the model (comparison of the model with five male types ($df = 4$) to a model with only infection status ($df = 1$): $F_{3,64} = 0.31$, $p = 0.82$). However, the infection status of the male had a significant effect on the sex ratio of the offspring ($F_{1,67} = 10.61$, $p = 0.002$). The difference

in sex ratio between infected and uninfected groups was 9% (infected: 37% male offspring, uninfected: 28% male offspring). Clutch size was highly variable, but did not differ between females mated to infected or uninfected males (infected males: mean = 114.39 ± 24.91 SD, uninfected males: mean = 104.96 ± 33.91 SD, $F_{1,67} = 1.16$, $p = 0.28$). However, the variability meant that we would have needed to sample about 100 females in each group to detect a 9% difference in clutch size (1-sided test, $\alpha = 0.05$, power = 0.8).

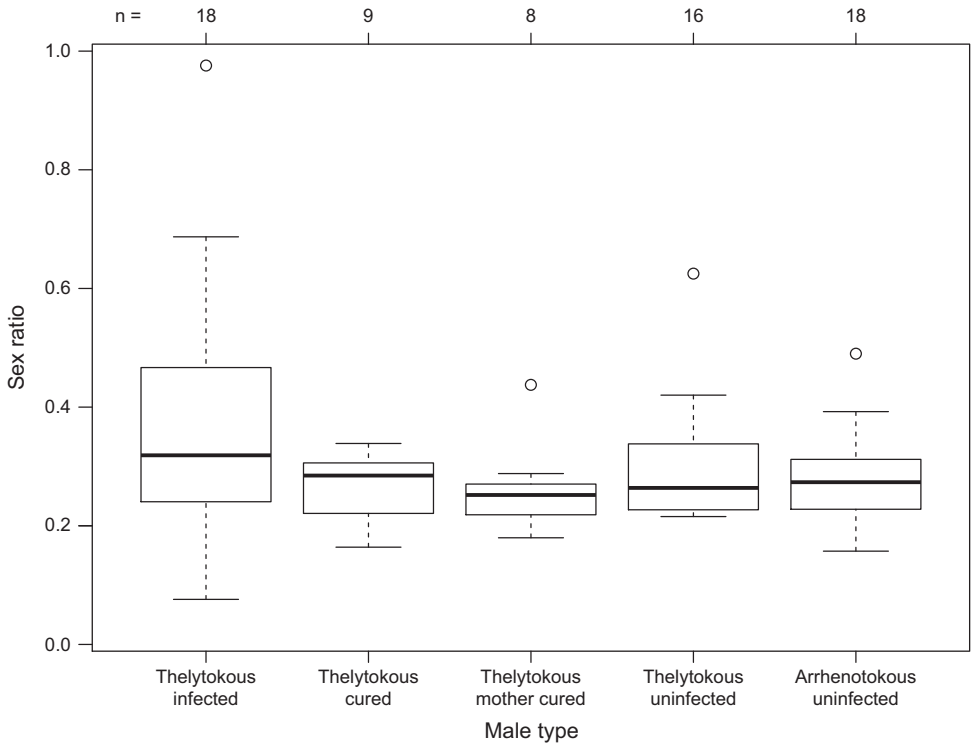

Figure 7.1: Sex ratio (proportion males) of offspring produced by uninfected, arrhenotokous *Asobara japonica* females that mated with various types of males. Sample sizes (n) are indicated above the graph. The horizontal dark lines represent the median sex ratios, the bottom and top of the boxes indicate the 25^{th} and 75^{th} percentiles, the whiskers show up to 1.5 times the interquartile range and the dots represent outliers.

Wolbachia density

Wolbachia density was significantly lower in infected males than in infected females (ratio *wsp/18S*: $n_{males} = 18$, $n_{females} = 20$, Wilcoxon $W = 360$, $p < 0.0001$). Among infected males, *Wolbachia* density correlated with the sex ratio produced by the females they mated with (Fig. 7.2; ratio *wsp/18S*: $F_{1,16} = 10.82$, $p = 0.005$). This correlation was also significant when *CO1* was used instead of *18S* to control for DNA content (ratio *wsp/CO1*: $F_{1,15} = 8.53$, $p = 0.01$). Males with higher *Wolbachia* densities induced more male-biased clutches than males with lower *Wolbachia* titers. There was no difference in the *18S/CO1* ratio between infected and uninfected males from the thelytokous strain, indicating that *Wolbachia* does not cause diploidization of cells in infected males ($n_{infected} = 17$, $n_{unifected} = 13$, Wilcoxon $W = 111$, $p = 0.99$).

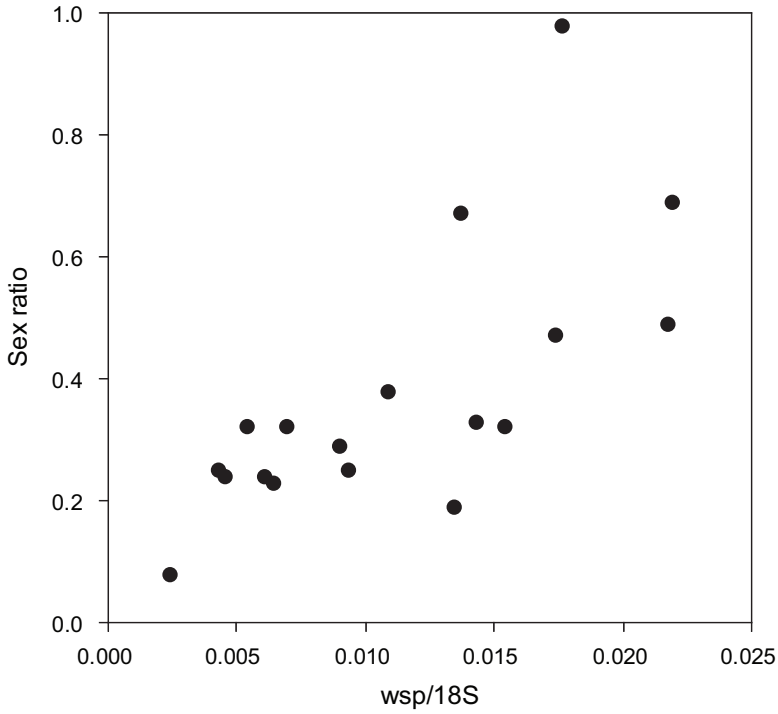

Figure 7.2: Sex ratio (proportion males) of offspring produced by uninfected, arrhenotokous *Asobara japonica* females that mated with males from a thelytokous strain in relation to the *Wolbachia* density in these males. *Wolbachia* density was measured as the ratio between the copy numbers of the *Wolbachia*-specific *wsp* gene and the nuclear *18S* gene.

MLST sequencing
The sequences of *Wolbachia* genes obtained from all males and all females of all strains were identical (GenBank accession numbers: HM241181-HM241186). There was thus no indication that strains other than *wAjap* are infecting *A. japonica*, or that infected thelytokous males harbour a different *Wolbachia* strain than the one that is normally inducing parthenogenesis in this species.

Discussion

Our results show a significant difference between the sex ratios produced by females that were mated to *Wolbachia*-infected males and those of females that were mated to uninfected males. This effect was strongest when the *Wolbachia* density in the infected male was relatively high. Uninfected thelytokous males did not show a reduction in fertilization capacity compared to arrhenotokous males, indicating normal spermatogenesis and absence of nuclear incompatibilities between individuals originating from thelytokous and arrhenotokous populations. Furthermore, *Wolbachia* did not cause (partial) diploidization of infected males. No other symbiont has been detected in *A. japonica* (Kremer *et al.* 2009) and MLST indicated that the *Wolbachia* strain that caused this effect was the same as the strain that induces parthenogenesis in the females. *Wolbachia* infection in males thus causes male-biased sex ratios when crossed with uninfected females.

Different hypotheses can be proposed for explaining this pattern. First, infected males could be mosaics of haploid and diploid cells as happens in *Nasonia* (Kamping *et al.* 2007), leading to production of some diploid sperm. However, infected males did not show an elevated ratio of nuclear versus mitochondrial DNA content compared to uninfected males as would be expected under diploidization. Furthermore, diploid sperm should lead to an increase in mortality in the offspring of these males, for which we find no evidence. Second, the presence of *Wolbachia* may result in a reduction in sperm production, leading to the observed pattern. However, this reduction needs to be substantial, because males used in this experiment were virgin and only had one female to mate with. The third hypothesis is that the parthenogenesis-inducing *Wolbachia* strain *wAjap* is able to induce CI when present in males. One way to test for this hypothesis would be to test for the rescue ability of *wAjap* in infected females. Unfortunately, females from thelytokous strains are not receptive to mating (Kremer *et al.* 2009), making this experiment impossible.

The mean difference in sex ratio induced by infected versus uninfected males was very small (9%) and the frequency distributions between the groups overlapped. If this effect is indeed due to CI expression, there are several potential explanations for the low degree of CI induced by the normally parthenogenesis-inducing *wAjap*. We show that the sex ratio produced by females is correlated to the *Wolbachia* titer in their mates. The males with the highest *Wolbachia* titers in our sample induced sex ratios up to 98% males, which approaches the sex ratio bias seen in incom-

patible crosses in other hymenopterans infected with CI-*Wolbachia*. For example, the difference in sex ratio between compatible and incompatible crosses was 35% in *A. tabida* (Dedeine *et al.* 2004) and 40% in *Trichopria cf. drosophilae* (Vavre *et al.* 2002). CI results in all-male broods in *Leptopilina heterotoma* and *Nasonia vitripennis* (Vavre *et al.* 2001, Bordenstein *et al.* 2003, respectively). Thus, *wAjap* may be able to induce full CI, but the overall low degree of CI that we saw may be due to low *Wolbachia* titers in most infected males. However, given that only one mating resulted in nearly complete sex bias, it is too early to rule out alternative explanations. *A. japonica* may be a host in which *Wolbachia* is not able to induce strong CI or *A. japonica* may be a competent host, but *wAjap* may not be able to induce strong CI. Hurst *et al.* (2002) predicted that an ancestral ability to induce CI would degrade when an invading mutant that distorts sex ratio approaches 100% efficiency. As far as is known, the infection with *wAjap* is fixed on the islands where it occurs. *wAjap* normally induces >97% parthenogenesis. In the field, infected males can not encounter uninfected females, since infected and uninfected populations are on different islands, and females from thelytokous populations do not elicit courtship by males. CI is thus not expressed in the field. In addition, any fitness cost imposed by the retention of CI ability will select for its loss. It is possible that the low level of CI induced by *wAjap* is partly due to this ability having degenerated by selection and/or neutral mutation accumulation.

Contrary to the situation observed in *A. japonica*, several male-killing *Wolbachia* induce complete CI when introgressed within a resistant host background. Why is low CI not seen in male-killing *Wolbachia*? The first possible explanation is related to bacterial load. Resistance to male-killing does not rely on the reduction of the *Wolbachia* titer, but to a direct resistance to the male-killing phenotype. On the contrary, we show here that infected males have low *Wolbachia* density in *A. japonica*, and this might be related to the fact that individuals with high density are diploidized and thus converted into females. This difference in the mechanisms leading to male production may structurally impose lower CI in PI-*Wolbachia* compared to male-killing *Wolbachia*. The other possibility is related to the dynamics of the co-evolutionary process between partners. Male-killing imposes strong selection for suppression in its host, because it kills a large proportion of offspring (Hurst *et al.* 2002). Thus, hosts infected by a male-killing *Wolbachia* may evolve resistance before sufficient time has passed to allow degradation of its ancestral CI ability (Hornett *et al.* 2008). In contrast, parthenogenesis is a very efficient mode of reproduction and selection for its suppression may be weak. In particular, as soon as parthenogenesis is fixed, as seems to be the case in *A. japonica*, there is no selection for resistance. Further understanding of the interplay between parthenogenesis- and CI-induction by *Wolbachia* might come from comparative studies. If the CI ability has indeed degraded in *wAjap*, it might be possible to find stronger CI in host species that have only recently become infected with a parthenogenesis-inducing *Wolbachia*.

Acknowledgements

This work was supported by a Veni-grant from the Netherlands Organization for Scientific Research (NWO) awarded to KK.