

ORIGINAL ARTICLE

The genetics and evolution of obligate reproductive parasitism in *Trichogramma pretiosum* infected with parthenogenesis-inducing *Wolbachia*JE Russell¹ and R Stouthamer²¹Department of Biology, University of California, Riverside, Riverside, CA, USA and ²Department of Entomology, University of California, Riverside, CA, USA

Parthenogenesis-inducing (PI) *Wolbachia* belong to a class of intracellular symbionts that distort the offspring sex ratio of their hosts toward a female bias. In many PI *Wolbachia*-infected species sex ratio distortion has reached its ultimate expression-fixation of infection and all-female populations. This is only possible with thelytokous PI symbionts as they provide an alternative form of reproduction and remove the requirement for males and sexual reproduction. Many populations fixed for PI *Wolbachia* infection have lost the ability to reproduce sexually, even when cured of the infection. We examine one such population in the species *Trichogramma pretiosum*. Through a series of backcrossing

experiments with an uninfected *Trichogramma pretiosum* population we were able to show that the genetic basis for the loss of female sexual function could be explained by a dominant nuclear effect. Male sexual function had not been completely lost, though some deterioration of male sexual function was also evident when males from the infected population (created through antibiotic curing of infected females) were mated to uninfected females. We discuss the dynamics of sex ratio selection in PI *Wolbachia*-infected populations and the evolution of non-fertilizing mutations. *Heredity* (2011) **106**, 58–67; doi:10.1038/hdy.2010.48; published online 5 May 2010

Keywords: *Wolbachia*; *Trichogramma*; parthenogenesis; sexual function; sex ratio

Introduction

Symbiosis is a continuum of interaction incorporating all points between parasitism and mutualism. Intracellular symbionts in the bacterial genus *Wolbachia* span this continuum in the multitude of invertebrate hosts they infect. *Wolbachia* are maternally inherited reproductive symbionts from the alpha proteobacteria clade and as reproductive parasites are known to induce reproductive incompatibility, male killing, feminization, and thelytokous parthenogenesis in many arthropod hosts (Rousset *et al.*, 1992; Stouthamer *et al.*, 1993; Turelli, 1994; Hurst *et al.*, 1999). Parasitic *Wolbachia* are generally considered facultative symbionts; meaning host organisms can survive and reproduce when cured. *Wolbachia* function in a more mutualistic manner in nematodes from the family Onchocercidae where experiments have shown they are essential for growth, longevity, and oogenesis (Bandi *et al.*, 1999; Hoerauf *et al.*, 1999). A symbiosis in the parasitoid *Asobara tabida* has been described as an obligatory dependence (Dedeine *et al.*, 2001; Pannebakker *et al.*, 2007), where an essential host reproductive function has been co-opted by *Wolbachia*. Hosts fail to develop or reproduce when cured of mutualistic or obligatory-dependent *Wolbachia* infections. *Wolbachia* in

these species are obligate symbionts providing necessary developmental and reproductive functions.

The form of *Wolbachia* that induces thelytokous parthenogenesis (parthenogenesis-inducing (PI) *Wolbachia*), rather than providing an essential reproductive function, offers an alternative reproductive option: thelytokous parthenogenesis. The PI *Wolbachia* symbiosis is the only described *Wolbachia* phenotype that alters the mode of reproduction. Like other sex ratio distorting *Wolbachia* phenotypes, PI *Wolbachia* in haplodiploid organisms has been considered a facultative symbiont, unnecessary and often detrimental to host fitness (Stouthamer *et al.*, 1990; Stouthamer and Luck., 1993). However, crossing experiments with several PI *Wolbachia*-infected species have shown *Wolbachia* infection in some species is required for female offspring production, making *Wolbachia* an obligate reproductive symbiont (Pijls *et al.*, 1996; Jeong and Stouthamer, 2005; Kremer *et al.*, 2009). Here, we describe the genetics underlying a proposed transition from facultative to obligate symbiont status as a consequence of PI *Wolbachia*'s ability to alter the mode of reproduction.

By inducing thelytokous parthenogenesis in *Wolbachia*-infected females, female offspring production is no longer dependent on mating and egg fertilization. Trichogrammatid wasps, like other haplodiploid organisms, normally produce females by fertilizing eggs; males are derived directly from unfertilized eggs and are haploid. PI *Wolbachia* convert unfertilized haploid eggs into diploid embryos through an aborted first mitotic division, a process called gamete duplication

Correspondence: Dr JE Russell, Department of Biology, University of California, Riverside, 900 University Avenue, Riverside, CA 92521, USA.
E-mail: jeruss002@gmail.com

Received 20 August 2009; revised 6 January 2010; accepted 1 February 2010; published online 5 May 2010

(Stouthamer and Kazmer, 1994), and the resulting homozygous individual develops as an infected functional female. Many hymenopteran populations are fixed for PI *Wolbachia* and considered completely thelytokous (females produce only female offspring). Within and among *Trichogrammatid* wasp species PI *Wolbachia* infection frequencies range from low to complete fixation (Huigens and Stouthamer, 2003).

The complete fixation of PI *Wolbachia* infection in a population is often accompanied by the loss of female sexual function. Several studies have reported that mated females from PI *Wolbachia*-fixed populations fail to fertilize eggs with sperm from fully functional males (Zchori-Fein *et al.*, 1995; Pijls *et al.*, 1996; Arakaki *et al.*, 2001; Jeong and Stouthamer, 2005; Pannebakker *et al.*, 2005; Kremer *et al.*, 2009). In mixed populations with low to intermediate infection frequencies infected females readily fertilize eggs when mated to conspecific males (Stouthamer *et al.*, 1990). The reported data suggest that the population infection frequency selects for the loss of female sexual function.

There are three hypotheses explaining the observed loss of fertilization in PI *Wolbachia*-fixed populations and species. Once a population has reached a high level of PI *Wolbachia* infection the primary mode of reproduction is parthenogenesis and alleles coding for sexual traits are expected to accumulate mutations at a neutral rate (Carson *et al.*, 1982). Both male and female sexual functions are subject to deterioration by genetic drift according to the mutation accumulation hypothesis. The costly female trait hypothesis (Pijls *et al.*, 1996) suggests that once PI *Wolbachia* has spread parthenogenesis through a population, obsolete female sexual traits that are physiologically costly to maintain will be selected against (the loss of costly female sexual traits will result in increased fitness); male sexual traits will deteriorate at a neutral rate through mutation accumulation. The virginity mutation hypothesis (Huigens and Stouthamer, 2003) proposes sex ratio selection (Fisher, 1930; Hamilton, 1967) is responsible for the loss of female sexual function, regardless of physiological cost, and the maintenance of male sexual function as PI *Wolbachia* spreads through populations.

When PI *Wolbachia* enters a population it introduces a deviation from 'optimal' sex ratios (Fisher, 1930; Hamilton, 1967). As the infection spreads and the population sex ratio becomes more female biased the value of outcrossing males increases. Females with reduced fertilization frequency in haplodiploid populations gain the sex ratio benefit of more valuable sons. A female's fitness in such populations is inversely related to fertilization frequency; lower fertilization frequency genotypes outcompete higher fertilization frequency genotypes (Huigens and Stouthamer, 2003).

The virginity mutation hypothesis predicts and unpublished simulation models have confirmed that alleles decreasing fertilization rates ('virginity' alleles) are positively selected in PI *Wolbachia*-infected populations and sweep to fixation. Once a population is fixed for the 'virginity' allele *Wolbachia*-induced thelytokous parthenogenesis becomes the sole means of female production. Therefore, the *Wolbachia* infection spreads to all females and the population becomes functionally parthenogenetic. In a population fixed for *Wolbachia* whose sole means of reproduction is parthenogenesis, alleles coding

for male sexual traits would be subject to mutation accumulation; and superfluous female sexual traits will be eliminated if they are costly to maintain (Pijls *et al.*, 1996). Therefore, both costly female trait and virginity mutation hypotheses are not necessarily mutually exclusive.

It is assumed in the virginity mutation hypothesis that, in normal sexual populations mutations are present at low frequencies that cause females expressing these mutations to fertilize a lower proportion of their eggs. In an uninfected population such mutations may not be selected against, depending on the mating structure of the population. In randomly mating populations, selection against these mutations will not be very strong. However, in *Trichogramma* with its high frequencies of sibmating, we expect strong selection against fertilization rate mutations. Once the infection frequency in a population has reached a substantial level, mutations that reduce the fertilization proportion will be selected and the lower the fertilization rate induced by the mutation the larger the selective advantage. Small influence 'virginity' mutations may act in a quantitative manner, however mutations with major effects on the fertilization rate will spread and bring *Wolbachia* and the mutant allele to fixation faster.

Here, we determine whether in a population of *Trichogramma pretiosum* fixed for the PI-*Wolbachia* infection, females express the non-fertilization phenotype and the genetic basis for this trait. It is important to note here that the use of the word virginity strictly refers to the loss of female sexual function. Sexual function involves many physiological and behavioral traits, of which many may have a polygenic basis (Carson *et al.*, 1982). However, only one of these traits need be affected by a mutant allele to induce the non-fertilization phenotype. Using a crossing protocol we show the genetic basis for such an allele in a population of *Wolbachia*-infected *Trichogramma pretiosum*.

Materials and methods

Insect cultures

Trichogramma pretiosum stocks: *Trichogramma pretiosum*-infected and -uninfected cultures originated in Peru and California, respectively. Infected *Trichogramma pretiosum* cultures were established from collections at high altitude locations in Peru where the *Trichogramma pretiosum* population is fixed for *Wolbachia* infection. Uninfected *Trichogramma pretiosum* from California were collected from *Heliothus virescens* eggs on experimental tomato plants at the University of California South Coast Research and Extension Center in Irvine, California in 2004. All *Trichogramma pretiosum* cultures originated from single females (mated if uninfected) and maintained in the laboratory on *Ephesia kuehniella* eggs (Beneficial Insectary, Redding, CA, USA) at 24 °C, L:D = 16:8 and 50% relative humidity.

Species identification: *Trichogramma pretiosum* species were identified using protocols described in Stouthamer *et al.* (1999). Sequencing of the ITS2 gene region was conducted at the University of California Riverside Genomics Institute Core Instrumentation Facility using the Applied Biosystems 3730 DNA analyzer with

Big-Dye version 3.1 kit (Applied Biosystems, Foster City, CA, USA). Sequences were aligned using BioEdit version 6.0.7 (Hall, 1999) and the identification of the species was confirmed by comparing the DNA sequences to already known ITS2 sequences for this species (Stouthamer *et al.*, 1999).

Wolbachia infection status: The infection status of the experimental lines was tested by virgin reproduction and molecular methods. PI *Wolbachia* infection can be detected by the female-biased offspring sex ratio of virgin females. Virgin experimental lines originating from the Peruvian and Californian cultures produced 97 and 0% female offspring, respectively.

As further proof of infection status, PCR primers specific for the *Wolbachia* B strain were used according to previously described procedures (Werren *et al.*, 1995). All females sampled from the Peruvian culture tested positive for *Wolbachia* infection. All females from the Californian culture tested negative for infection.

Fertilization frequency of infected and uninfected *Trichogramma pretiosum*

As haploid males develop directly from unfertilized eggs and females from fertilized eggs, the fertilization frequency of haplodiploid organisms can be approximated by the observed offspring sex ratio. Therefore, our offspring sex ratio values were determined by calculating the proportion of female offspring (female offspring/total offspring) to reflect female fertilization frequencies. To determine the fertilization frequency of the females of the infected line, we tried to cure this line of their infection. This was done by subjecting 20 isofemale lines from the *Wolbachia* infected culture to the antibiotic rifampicin, which is known to eliminate *Wolbachia* (Stouthamer *et al.*, 1990). Newly emerged infected females were fed a 0.5% g ml⁻¹ antibiotic/honey solution for 1 day. Each day new host eggs were supplied and the previous days host eggs were incubated. All females had access to antibiotic honey solution throughout the 8 days of oviposition. Next generation offspring emerging from the daily hosts were observed for sex ratio and those broods that showed a sex ratio approaching 50% were again given the antibiotic treatment described above. This curing procedure lasted no more than three generations before some lines expired due to an absence of female offspring. By generation 7 all 20 lines had died out. Despite numerous attempts to adjust the antibiotic concentration no sexually reproducing lines from the infected culture established.

Trichogramma wasps infected with PI *Wolbachia* will often fertilize their eggs at frequencies similar to uninfected females (Stouthamer and Kazmer, 1994). The fertilization rate of eggs can be determined genetically by determining the fraction of female offspring that carry a nuclear marker from the father. Two microsatellite markers specifically designed for *Trichogramma* species (TAC 47: forward CTACGGCGACAATTGCCAC, reverse CATCTTGGTTCGAACCGAGCAG; TTG 46: forward GATGTTTACTTCGCAGGCCGC, reverse CTACGGGG CATA CGATATGTG; Huigens, 2003) were used to test paternally inherited alleles in the female offspring from mated infected mothers using the following thermocycle conditions: 94 °C for 3 min, then 35 cycles of 94 °C for

45 s, 50 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 3 min. Fourteen virgin infected females were isolated and given virgin males from the uninfected population. After mating was observed each female was allowed to oviposit. One day after mating, all males were removed and after 6 days of oviposition the females were removed. Both males and females were preserved in 95% EtOH. Between 5 and 8 female offspring from 13 of the 14 infected mated females (one female died without reproducing) were genotyped using the microsatellite markers. To determine the fertilization frequency of the uninfected California culture 65 virgin females were mated to virgin males from the same culture and allowed to oviposit on *E. kuehniella* hosts for 6 days. The number and sex of the offspring were determined after their emergence.

Crossing experiments

A series of crosses aimed at determining the genetic basis of non-fertilization are described in Figure 1. The nomenclature used was meant to reflect the nuclear genetic background and ploidy of the crosses. For instance, females from the California culture are characterized as 'CC', males from the Peruvian culture as 'P'; hybrid females resulting from a cross between California females and Peru males as 'CP', and recombinant males produced by CP females as 'c/p'. Introgression of the nuclear genome from the infected line into the uninfected line was accomplished by mating males from the infected line with females of the uninfected line. Males from the infected line were produced by feeding infected females antibiotics, thus curing eggs of *Wolbachia* and resulting in mostly male offspring. Males and females were isolated in 12 mm × 75 mm glass culture tubes for 24 h, after which males were removed and females were given *E. kuehniella* host eggs *ad libitum* for 6 days.

The logic of the experiments was to (1) introgress the Peruvian (non-fertilization) genome into the California genome, and (2) determine the egg fertilization rate of the introgressed females. The potential methods of inheritance for the non-fertilization trait in the *Trichogramma pretiosum* line from Peru can be broken down into nuclear/cytoplasmic, dominant/recessive, and one-locus/multi-loci models. The cytoplasmic model assumes that cytoplasmically inherited *Wolbachia* directly interacts with the fertilization process. Two lines of evidence suggest that this is not the case in species infected with PI *Wolbachia*: (1) In populations where the PI *Wolbachia* infection has progressed to fixation, females cured of *Wolbachia* infection with antibiotics still fail to fertilize the eggs (Arakaki *et al.*, 2001; Jeong and Stouthamer, 2005; Pannebakker *et al.*, 2005; Pijls *et al.*, 1996; Kremer *et al.*, 2009), and (2) in populations where the PI *Wolbachia* infection has not reached fixation infected females continue to fertilize eggs (Stouthamer *et al.*, 1990). Evidence supporting the nuclear model is found in Jeong and Stouthamer (2005) for *Telonomus nawai*, where nuclear introgression induced the non-fertilization phenotype in an uninfected population. Assuming a nuclear mode of inheritance for the trait in *Trichogramma pretiosum* we can distinguish between dominant and recessive models through a crossing experiment between females from the California uninfected population (CC) and males derived from the

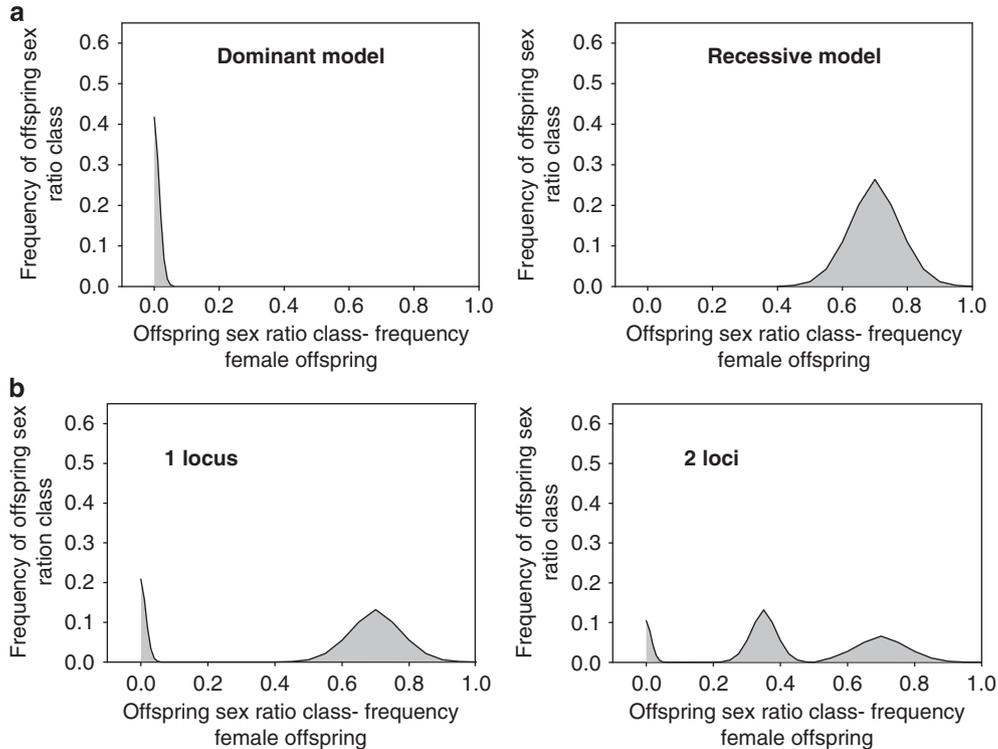


Figure 1 (a) Hybrid females (CP) resulting from the cross between California (CC) females and Peru (P) males are expected to produce male-biased offspring sex ratios (female offspring/total offspring) according to the dominant model of inheritance for the non-fertilization allele, and female-biased sex ratios according to the recessive model of inheritance. (b) Assuming a dominant mode of inheritance, one-locus and two-loci models of inheritance can be distinguished by the modal distribution of offspring sex ratios in the D1 cross (a cross between control California males (C) and daughters of California females (CC) and recombinant males (*c/p*) cross). The one-locus model results in a bimodal distribution and the two-loci model results in a trimodal distribution assuming strictly additive effects of the non-fertilization alleles.

Peruvian-infected population (P) (Figure 1a). If the trait is inherited in a dominant manner we expect that hybrid females (CP), derived from the $CC \times P$ cross, will not fertilize their eggs and will produce male-biased offspring sex ratios. If the trait is inherited in a recessive manner we expect hybrid females to fertilize their eggs at a normal (control) frequency and produce wild-type offspring sex ratios.

If the non-fertilization trait is inherited in a dominant manner, the question of number of dominant alleles with major effect arises. This is tested by using recombinant haploid males (*c/p*) that are mated with California females (CC). If there is only a single locus involved in the trait then recombinant males are either carriers of the non-fertilization trait or have the wild-type allele. Consequently, the females fathered by recombinant males (*Cc/p*) should either express the non-fertilization trait and fertilize their eggs at a low frequency or have normal egg fertilization rates; thus producing a bimodal distribution of offspring sex ratios among the daughters of wild-type California females (CC) mated with recombinant males (*c/p*). We refer to the cross of such daughters with control males (C) as the Dominance 1 (D1) cross (Figure 1b).

The alternative model involving more loci is more complex, but if we assume a simple two-loci model where each locus contributes equally to the low fertilization rate, then we would expect a female that carries a non-fertilization allele at one of the two loci to fertilize her eggs at half the rate of a wild-type female.

Under this model, females fathered by recombinant males would fall into four types: females homozygous for the wild-type alleles at both loci, females homozygous for the wild type at one of the two loci and heterozygous at the other locus, and finally females heterozygous at both loci. Assuming unlinked loci, 25% of the females would be expressing the wild-type phenotype, 50% would express the phenotype where the fertilization rate is half of the wild-type fertilization rate, and 25% of the females would express the non-fertilization phenotype. This should result in a trimodal distribution of offspring sex ratios. The difference between the one and two-loci models is the bimodal distribution expected under the single-locus model for the offspring produced by the females produced in the cross between a CC female and a recombinant male, whereas under the two-loci model a trimodal distribution is expected (Figure 1b).

The expected frequency distributions shown in Figure 1 are intended to illustrate the expected outcome of different inheritance models, dominant versus recessive and single-locus versus two-loci models. These distributions are meant to give a general indication of offspring sex ratio frequencies given either a one-locus or two-loci dominant model for the non-fertilization trait. There are no *a priori* exact frequency distribution expectations other than a female-biased offspring sex ratio for control females, as mentioned in previous analyses for *Trichogramma pretiosum* (Kazmer, 1992; Stouthamer and Kazmer, 1994) and a male-biased sex

ratio distribution for hybrid females carrying a dominant non-fertilization allele.

D experiments

The choice of a dominant model was governed by the observed results in the initial cross between F1 hybrid females and males from both populations (Figure 2b; Table 1). For the D experiments we were able to generate expected distributions based on the results of the crosses involving control and hybrid females given an assumed one-locus dominant model that could be analyzed quantitatively. To test the dominance of the non-fertilization trait the offspring of the $CC \times c/p$ cross (California, uninfected sexual females mated to recombinant males) were crossed and tested for fertilization frequency. This test was conducted twice (D1 experiment, $n=77, 137$) with the results of the second test ($n=137$) used for further analysis. In this cross the resultant females (Cc/p) were effectively 1/2 hybrids because of the recombined paternal genome. If functional virginity is controlled by a single dominant locus that segregates in Mendelian manner then the

offspring sex ratios of the cross involving Cc/p females mated to C males should show a bimodal distribution corresponding to the control ($CC \times C$) and hybrid female ($CP \times C$) offspring sex ratio distributions taken together (Figures 2a and b). Expected distributions for the D1 and D2 male-biased crosses were derived by multiplying the observed offspring sex ratio class frequencies by 0.5 for both the $CC \times C$ and $CP \times C$ crosses, then adding the offspring sex ratio class frequencies from both crosses. The expected distribution for the D2 female-biased cross was taken directly from the observed distribution for the control $CC \times C$ cross.

The offspring of the second D1 experiment were used for validation of a single-locus dominance effect on fertilization (D2 female/male bias; Supplementary Table S1 in Supplementary Material; Figures 3a–f). If the dominant mutant allele for loss of fertilization segregates in Mendelian manner, the allele should be present in half of the female offspring of the females expressing the trait ((CP) genotype females), that is mothers that produce offspring sex ratios in the 0–0.39 female proportion range; the left side of the bimodal distribution, assuming

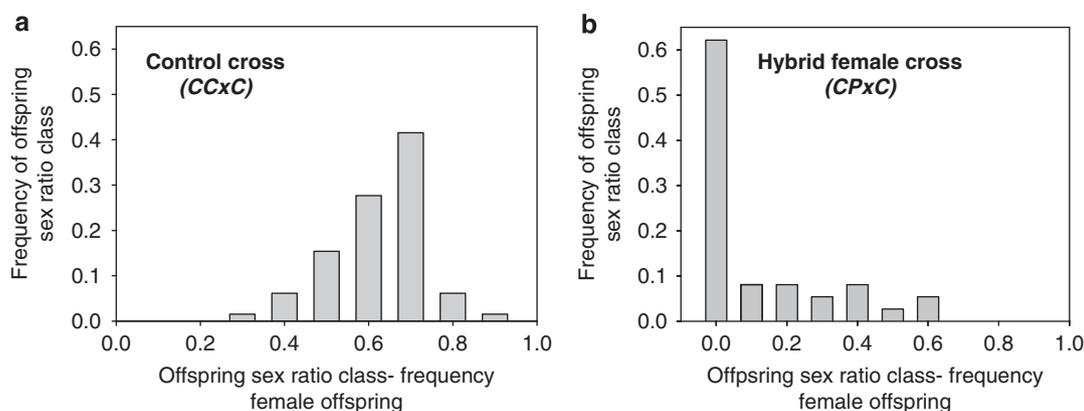


Figure 2 (a, b) Observed distributions of the control ($CC \times C$) and hybrid female ($CP \times C$) crosses are shown. The crosses are designated by the diploid genotype females (CC and CP) and haploid genotypes of males (C). The x axis represents the offspring sex ratio (female offspring/total offspring) classes such that the 0.0 class represents all offspring sex ratio frequency values lower than 0.1, the 0.1 class represents all offspring sex ratio frequency values greater than or equal to 0.1 and lower than 0.2.

Table 1 Mean offspring production values, standard deviation, and sex ratios in crosses to determine the genetics of the lack of fertilization in the completely parthenogenetic females from the Peru population of *Trichogramma pretiosum* are shown

$\text{♀} \times \text{♂}$	N	$\bar{x}_{\text{♀}} \pm \text{s.d.}$	$\bar{x}_{\text{♂}} \pm \text{s.d.}$	$\bar{x}_{\text{offsp.}} \pm \text{s.d.}$	% Female
CCV	40	0 ± 0 ^c	44.9 ± 12.2 ^a	44.9 ± 12.2 ^{a,b}	0 ^f
CC × C	65	29.9 ± 8.5 ^a	14.9 ± 7.3 ^c	44.7 ± 12.3 ^b	67.5 ^b
CC × P	67	18.7 ± 9.9 ^b	19.8 ± 12.6 ^c	38.5 ± 14.1 ^b	49.8 ^c
CC × <i>c/p</i>	30	28.0 ± 9.0 ^a	17.3 ± 6.7 ^c	45.3 ± 8.1 ^{a,b}	61.3 ^b
CPV	14	0 ± 0 ^c	47.4 ± 14.7 ^a	47.4 ± 14.7 ^{a,b}	0 ^f
CP × C	37	5.4 ± 7.5 ^c	32.0 ± 11.4 ^b	37.4 ± 9.3 ^b	14.7 ^e
CP × P	51	1.0 ± 2.7 ^c	31.1 ± 11.1 ^b	32.7 ± 10.0 ^b	3.6 ^f
PPV	42	31.0 ± 10.1 ^a	1.1 ± 1.5 ^d	32.1 ± 10.3 ^b	96.6 ^a
<i>Cc/p</i> × C (D1)	137	24.2 ± 24.8 ^{a,b}	31.1 ± 23.8 ^b	55.3 ± 22.1 ^a	42.6 ^d
<i>Cc/p</i> × <i>c/p</i> (D2 male)	61	21.3 ± 19.9 ^{a,b}	33.3 ± 21.1 ^b	54.5 ± 21.2 ^a	37.7 ^d
CC × <i>c/p</i> (D2 female)	27	34.8 ± 14.5 ^a	18.9 ± 13.6 ^c	53.6 ± 13.6 ^a	65.4 ^b

$\text{♀} \times \text{♂}$, experimental cross-last letter(s) represents the male; C, control California line; CP, hybrid female; *c/p*, recombinant male; *Cc/p*, females resulting from a cross between California females and recombinant males; P, parthenogenetic Peruvian line; V, virgin/unmated female. Female genotypes for the D2 male and D2 female cross genotypes are presumed for the single-locus dominant model of the 'virginity' locus; N, number of replicates; $\bar{x}_{\text{♀}} \pm \text{s.d.}$, mean female offspring; $\bar{x}_{\text{♂}} \pm \text{s.d.}$, mean male offspring; $\bar{x}_{\text{offsp.}} \pm \text{s.d.}$, mean total offspring; % female, offspring sex ratio (female/total offspring); different letter superscripts designate statistically significant differences (ANOVA, $P < 0.0001$, Scheffe's *post hoc* $P < 0.0271$).

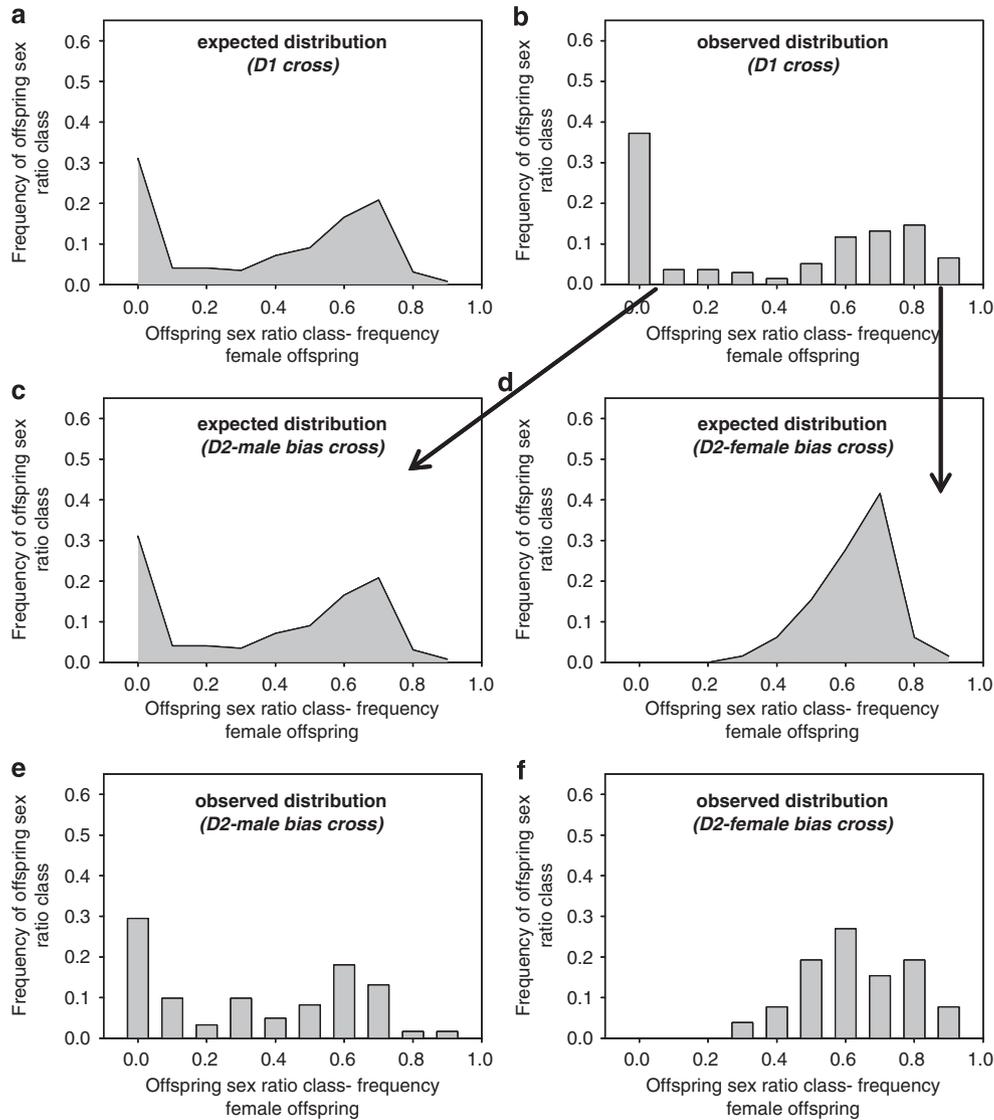


Figure 3 (a–f) Expected and observed distributions of the D1 and D2 crosses assuming a dominant non-fertilization effect. The expected distributions assume a high level of dominance for the non-fertilization allele. Expected distributions for the D1 and D2 male-biased crosses were derived by multiplying the observed offspring sex ratio class frequencies by 0.5 for both the CC × C and CP × C crosses, then adding the offspring sex ratio class frequencies from both crosses. The expected distribution for the D2 female-biased cross was taken directly from the observed distribution for the control CC × C cross. The x axes represent offspring sex ratio classes calculated as female offspring/total offspring and grouped by frequency increments of 0.1.

incomplete dominance. Therefore, female offspring from this side of the bimodal distribution should replicate the bimodal offspring sex ratio distribution (Figure 3c). Female offspring from mothers that do not express the loss of fertilization trait (CC genotypes) should produce offspring sex ratios no different than control females (Figure 3d). A complete list of all experimental crosses is presented in Supplementary Table S2.

Degree of dominance

Using methods described in Lynch and Walsh (1998) hypotheses regarding the degree of dominance for the non-fertilization trait can be illustrated by dominance coefficient values for the California control (sexual) × Peru parthenogenetic hybrid female (CP) fertilization phenotype. Dominance values in the –1 to

0 range represent the impact of the parthenogenetic Peruvian genome, with a –1 value representing complete dominance. Dominance values in the 0–1 range represent the impact of the control Californian genome, with a value of 1 representing complete dominance.

Statistics

Offspring sex ratio, male, female, and total offspring production, and pupal mortality were analyzed using ANOVA, Fisher's Protected Least Significant Difference, and Scheffe's *post hoc* comparison tests. Offspring sex ratio distributions were subjected to Kolmogorov–Smirnov goodness of fit and G tests. Analyses were conducted using StatView (SAS Institute Inc., Cary, NC, USA) and R programming language (Ihata and Gentleman, 1996).

Results

Fertilization frequency of experimental cultures

The offspring sex ratio (proportion females = females/total offspring) of the uninfected Californian (C) culture was used as a measure of fertilization frequency. Matings between CC females and C males (CC × C, $n=65$) produced a mean offspring sex ratio of $0.675 \pm 0.11_{s.d.}$ (67.5% female offspring). The offspring sex ratio distribution for the C culture shows that over 70% of all females produce offspring sex ratios in the 0.6 and 0.7 classes (Figure 2a). The lowest sex ratio class was 0.3 (30–39% female offspring); the highest was 0.9 (90–99% female offspring).

The offspring sex ratio of the infected Peruvian (P) culture could not be used to assess the fertilization frequency of PP females because of our inability to establish a cured culture, despite the presence of males in antibiotic-treated cultures and observed mating. Therefore, microsatellites were used to determine whether infected PP females fertilized any eggs when mated to C males. A total of 94 female offspring representing between 5 and 8 offspring from 13 mated PP females showed no successful fertilization of eggs. Microsatellite analysis plus the inability to establish a cured culture, despite repeated attempts, showed that females from the Peruvian parthenogenetic population did not successfully fertilize any eggs.

Introgression

Total offspring production values for uninfected CC females mated to males from the Peruvian (P) and Californian (C) populations were 38.5 ± 14.1 and 44.7 ± 12.3 , respectively (Table 1). Mean fertilization frequency for CC females mated to P males (CC × P cross) as measured by the offspring sex ratio was 0.50 ± 0.19 (50% female offspring). The variance of the CC × P cross offspring sex ratios ($\sigma^2=0.038$) was more than twice the variance found in the control CC × C cross ($\sigma^2=0.012$).

The hybrid females (CP), resulting from the CC × P cross, backcrossed to males from both parental backgrounds produced offspring sex ratios considerably lower than control females (CC) (Table 1). Hybrid females mated to control males (CP × C cross) produce male-biased offspring sex ratios (Figure 2b).

Among the various matings mean offspring sex ratios varied in the following order: (CC × C, CC × c/p, D2 female) > (CC × P) > (D1, D2 male) > (CP × C) > (CP × P) (Table 1).

Offspring production values were higher for the D-test crosses when compared with all other crosses, except CC × c/p (Table 1). Hybrid females mated to P males and PPV females produced fewer offspring than all other crosses. The reduction in average offspring between control CC females and hybrid (CP) females is a product of fewer females produced by the hybrid females. All hybrid females produced more male offspring than all control females except virgin CC females (Scheffe's $P<0.0001$). All hybrid females produced statistically similar numbers of female offspring.

D experiments

Roughly half of the offspring sex ratio distribution found in the test for dominance (D1 experiment, $n=137$) was

absent in the control cross (Figures 2a and 3b). Offspring sex ratio values from the D1 cross in the 0–0.39 range (higher male production) accounted for 50.1% of the frequency distribution. For comparison, in the control cross the offspring sex ratios in this range only accounted for 1.5% of the frequency distribution (Figure 2a), and 83.8% of the observations among hybrid females in the CP × C cross (Figure 2b) were in this range. The offspring sex ratio distribution from the second D1 cross ($N=137$) was statistically no different from the first D1 cross (Kolmogorov–Smirnov, $P=0.2956$; Supplementary Table S3). Both D1 cross distributions were significantly different (individually and pooled, G-test, $P<0.023$) than the expected distribution (Figure 3a; Supplementary Table S3).

Sibling-mated females selected from the respective modes of the bimodal distribution seen in the second D1 experiment made up the D2 male bias (females selected from the 0–0.39 offspring sex ratio classes, $n=61$) and the D2 female bias (females selected from the 0.4–0.99 offspring sex ratio classes, $n=27$) crosses. The D2 male bias cross offspring sex ratio distribution (Figure 3e) is not statistically different from the first D1 experiment (Kolmogorov–Smirnov, $P=0.8767$; Supplementary Table S4), but showed a statistical difference from the second D1 experiment, from which it was taken, (Kolmogorov–Smirnov, $P=0.0425$; Supplementary Table S4) and the expected D1 distribution ($G=19.653$, 9 d.f., G-test, $P<0.02$; Supplementary Table S4). There is no statistical difference between the D2 female bias (Figure 3f) and the CC × C offspring sex ratio distributions (Kolmogorov–Smirnov, $P=0.3159$; Supplementary Table S4). There were no significant differences in offspring production among the D crosses (ANOVA, $P=0.9171$; Supplementary Table S5).

Degree of dominance

A purely additive effect of introgression of the genome from the parthenogenetic (infected) population on the control sexual (uninfected) genome would result in a midpoint fertilization percentage in the F1 hybrids of 33.75% (Figure 4). Instead, we found a fertilization percentage of 14.7% when hybrid females were mated to control males and 3.6% when mated to males antibiotic-derived from parthenogenetic females. This represented a 78 and 93% reduction in fertilization when compared with control females. A dominance coefficient of -0.64 indicated a high degree of dominance for the parthenogenetic background in terms of the non-fertilization trait in *Trichogramma pretiosum*.

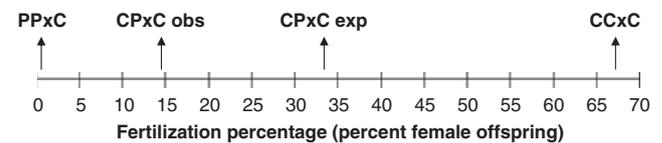


Figure 4 The genotypic values for fertilization percentage are shown for the experimental lines used (PP × C, Peru, parthenogenetic female crossed with a control male; CC × C, California, control female crossed with a control male; CP × C obs, observed hybrid female crossed with a control male; CP × C exp, expected hybrid female crossed with a control male). The actual values are PP × C = 0, CC × C = 67.5%, CP × C exp = 33.75%, and CP × C obs = 14.7%.

Discussion

Our investigation into the non-fertilization trait in *Trichogramma pretiosum* began with an assumption of genetic recessivity, similar to that found in *Telenomus nawai* (Jeong and Stouthamer, 2005), and a protocol that required at least two generations of backcrossing. The observed dominant effect on fertilization in first generation hybrid females was confirmed by the following D1 and D2 crosses. The results of the D experiments support the dominant single-locus model explaining the loss of female sexual function. Rather than the reproductive incompatibility observed in *Trichogramma deion* where female offspring simply died and no change occurred in male offspring production (Stouthamer *et al.*, 1996), hybrid females in our study produce only male offspring with no significant change in total offspring production; a result that is concordant with the virginity mutation hypothesis—a significant reduction in fertilization. However, alternative explanations for the change in female fertilization behavior and offspring sex ratios could be selection for the loss of costly female traits in an infected parthenogenetic population (Pijls *et al.*, 1996), or mutation accumulation in pathways associated with egg fertilization. Our results do not directly distinguish between the possible mechanisms.

The development and full functionality of recombinant males in the CP × C and CC × c/p crosses does not support hybrid breakdown because of incompatible genetic interactions. The offspring sex ratio range 0–0.39, which is absent in the control CC × C cross but present in the D1, D2 male bias cross, and hybrid female (CP) crosses, appears to result from a dominant effect of the P genome. The predicted bimodal offspring sex ratio distribution given a dominant genetic effect was observed (Figures 1–3), though the predicted and observed bimodal distributions were statistically different for the D-test crosses.

The observed reduced offspring production of the mated hybrid females together with reduced female production (Table 1) point to a possible interaction between the C and P genomes that have some deleterious effect on female development. However, this cannot completely explain the differences in offspring sex ratios and egg fertilization between control and hybrid females. Even when the female offspring, assumed lost through developmental mortality, are replaced there is still a large difference that results in a reversal of offspring sex allocation between control and hybrid females (67.5% female offspring for the control versus 31.9% female offspring for hybrid).

A reversal of offspring sex allocation when PI *Wolbachia*-infected and -uninfected populations are introgressed was shown for *Telenomus nawai* (Jeong and Stouthamer, 2005). However, the inheritance of the non-fertilization trait in *Telenomus nawai* showed a recessive pattern that could be explained as a single gene of major effect with modifiers or two linked loci. Our results for *Trichogramma pretiosum* also suggest a major effect gene associated with fertilization (functional virginity), but expressed in a dominant manner. The lack of statistical fit in the D1 and D2 male-biased dominance tests also may be a consequence of genetic modifiers. In highly inbred populations such as *Trichogramma pretiosum* (Kazmer, 1992) the difference in selection pressures for

dominant and recessive low frequency fertilization alleles is significantly decreased. The effect of inbreeding results in levels of allelic homozygosity and phenotypic expression that differ little between recessive and dominantly expressed alleles (Hedrick and Parker, 1997). Therefore, the observed dominant ‘virginity’ effect of the Peruvian parthenogenetic genome is not unexpected.

The dominant effect on female sexual function we observe in the hybrid females of this study may indeed be a result of a selective sweep in the parthenogenetic population for the removal of costly female sexual traits (Pijls *et al.*, 1996). Mutation accumulation in sexual traits in a parthenogenetic population would likely result in some loss of function for both male and female reproductive traits (Carson *et al.*, 1982). However, the observed pattern in PI *Wolbachia*-infected populations suggest female-limited reproductive traits are more likely to decay before male reproductive function is lost (Zchori-Fein *et al.*, 1995; Pijls *et al.*, 1996; Arakaki *et al.*, 2001; Jeong and Stouthamer, 2005; Pannebakker *et al.*, 2005; Kremer *et al.*, 2009). If the loss of female sexual function is a product of selection for mutations in costly female reproductive traits then we have to assume the genes that code for these traits are positively selected in a sexual context and negatively selected in a parthenogenetic context; that is, the traits carry a fitness advantage in a sexual population and a fitness cost in a parthenogenetic population. This could be considered a type of antagonistic pleiotropy associated with the mode of reproduction rather than reproduction itself (Hedrick, 1999). The question becomes which female sexual traits are intrinsically costly?

The virginity mutation hypothesis is based on a selective advantage for the loss of female sexual function traits regardless of associated physiological fitness benefits. Furthermore, the virginity mutation hypothesis, which is based on the fitness advantage of rare males in a population with a sweeping PI *Wolbachia* infection, requires the maintenance of male sexual function (Huigens and Stouthamer, 2003). Any gene allowing females in an infected population to produce more male offspring that can take advantage of surplus mating opportunities provided by unmated females will be selected accordingly. The two scenarios, selection for mutations in costly female sexual traits and selection for ‘virginity’ mutations, are not mutually exclusive. The difference between the two scenarios is a selective advantage for the loss of female sexual function and maintenance of male sexual function in the initial stages of *Wolbachia* infection according to the virginity mutation hypothesis, and the loss of female sexual function by selection and male sexual function by genetic drift according to the costly female trait hypothesis. The virginity mutation hypothesis gives an adaptive explanation for the maintenance of male sexual function. The costly female trait hypothesis explains the maintenance of male sexual function as a function of drift having less influence over evolutionary forces, resulting in selection for the loss of costly female traits preceding the loss of male sexual traits by drift. The loss of female sexual function in either context is not the product of neutral or indirect selection processes (Fong *et al.*, 1995; Maughan *et al.*, 2007), but the result of selection imposed by a bacterial infection that initially induces facultative

thelytokous parthenogenesis; only later evolving into an obligate parthenogenetic symbiont.

In the populations studied where PI *Wolbachia* has swept to fixation it appears the sexual functionality of females and antibiotic-induced males conform to the predictions of the virginity mutation hypothesis (Pijls *et al.*, 1996; Arakaki *et al.*, 2000; Weeks and Breeuwer, 2001; Jeong and Stouthamer, 2005; Pannebakker *et al.*, 2005; Kremer *et al.*, 2009), namely, that females are sexually non-functional and males are sexually functional. Once the *Wolbachia* infection and virginity mutation are fixed male sexual function is subject to drift, mutation accumulation, and ultimate deterioration (Muller, 1932; Carson *et al.*, 1982). A high degree of male sexual function variance in a PI *Wolbachia*-fixed population of *Leptopilina clavipes* was responsible for the variation seen in offspring sex ratios (Pannebakker *et al.*, 2005). Similar results are reported here with sex ratio variance doubling in crosses between control females (CC) and Peruvian males derived from the parthenogenetic line (P). In *Muscidifurax uniraptor*, a species completely fixed for PI *Wolbachia*, male sexual function is completely lost and female sexual function has been eliminated both behaviorally and morphologically—suggesting a long history of PI *Wolbachia* infection (Gottlieb and Zchori-Fein, 2001). Our results also suggest male sexual function, though not completely lost, has deteriorated (Table 1, compare CC × C with CC × P, and CP × C with CP × P). Alternatively, deleterious genomic interactions may be responsible for the observed results. If male sexual function variance in *Trichogramma pretiosum* is a product of incompatible genomic interactions we might expect to see some deleterious sexual function in the recombinant males. However, this is not the case. The offspring and offspring sex ratio values for recombinant males mated to control females were statistically similar to control males (Table 1).

Similarities between the *A. tabida* and *Trichogramma pretiosum* symbioses are informative. Both systems have evolved reproductive dependence on *Wolbachia*. However, the two trajectories are distinct. In *A. tabida*, *Wolbachia* takes over a pre-existing host function oogenesis (Dedeine *et al.*, 2001; Pannebakker *et al.*, 2007; Vavre *et al.*, 2008). In *Trichogramma pretiosum* *Wolbachia* provides a novel function, thelytokous parthenogenesis through gamete duplication (Stouthamer and Kazmer, 1994; Stouthamer *et al.*, 1999). Both scenarios lead to the obligate dependence on *Wolbachia* for reproductive function. These similarities and differences highlight the dexterity of *Wolbachia* when it comes to achieving obligate symbiont status.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Len Nunney, Roger Butlin, and three anonymous reviewers for helpful comments on the paper. This work was supported by funds from the US National Science Foundation Grant EF 0328363 to RS.

References

- Arakaki N, Miyoshi T, Noda H (2001). *Wolbachia*-mediated parthenogenesis in the predatory thrips *Frankliniopsis vespiformis* (Thysanoptera: Insecta). *Proc R Soc Lond B Biol Sci* **268**: 1011–1016.
- Arakaki N, Noda H, Yamagishi K (2000). *Wolbachia*-induced parthenogenesis in the egg parasitoid *Telenomus nawai*. *Entomol Exp Appl* **96**: 177–184.
- Bandi C, McCall JW, Genchi C, Corona S, Venco L, Sacchi L (1999). Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. *Int J Parasitol* **29**: 357–364.
- Carson HL, Chang LS, Lyttle TW (1982). Decay of female sexual behavior under parthenogenesis. *Science* **218**: 68–70.
- Dedeine F, Vavre F, Fleury F, Loppin B, Hochberg ME, Boulétreau M (2001). Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc Natl Acad Sci USA* **98**: 6247–6252.
- Fisher RA (1930). *The Genetical Theory of Natural Selection*. Oxford University Press: Oxford.
- Fong DW, Kane TC, Culver DC (1995). Vestigialization and loss of nonfunctional characters. *Annu Rev Ecol Syst* **26**: 249–268.
- Gottlieb Y, Zchori-Fein E (2001). Irreversible thelytokous reproduction in *Muscidifurax uniraptor*. *Entomol Exp Appl* **100**: 271–278.
- Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**: 95–98.
- Hamilton WD (1967). Extraordinary sex ratios. *Science* **156**: 477–488.
- Hedrick PW (1999). Antagonistic pleiotropy and genetic polymorphism: a perspective. *Heredity* **82**: 126–133.
- Hedrick PW, Parker JD (1997). Evolutionary genetics and genetic variation of haplodiploids and X-linked genes. *Annu Rev Ecol Syst* **28**: 55–83.
- Hoerauf A, Nissen-Paehle K, Schmetz C, Henkle-Duehresen K, Blaxter ML, Buettner DW *et al.* (1999). Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. *J Clin Invest* **103**: 11–18.
- Huigens ME (2003). On the evolution of *Wolbachia*-induced parthenogenesis in *Trichogramma* wasps. PhD dissertation, Wageningen University, the Netherlands.
- Huigens ME, Stouthamer R (2003). Parthenogenesis associated with *Wolbachia*. In: Bourtzis K, Miller TA (eds). *Insect Symbiosis*. CRC Press: Boca Raton, pp 247–266.
- Hurst GDD, Jiggins FM, von der Schulenburg JHG, Bertrand D, West SA, Goriacheva II *et al.* (1999). Male-killing *Wolbachia* in two species of insect. *Proc R Soc Lond B Biol Sci* **266**: 735–740.
- Ihata R, Gentleman R (1996). R: a language for data analysis and graphics. *J Comput Graph Stat* **5**: 299–314.
- Jeong G, Stouthamer R (2005). Genetics of female functional virginity in the Parthenogenesis-*Wolbachia* infected parasitoid wasp *Telenomus nawai* (Hymenoptera: Scelionidae). *Heredity* **94**: 402–407.
- Kremer N, Charif D, Henri H, Bataille M, Prevost G, Kraaijeveld K *et al.* (2009). A new case of *Wolbachia* dependence in the genus *Asobara*: evidence for parthenogenesis induction in *Asobara japonica*. *Heredity* **103**: 248–256.
- Kazmer DJ (1992). The evolution of reproductive strategies in *Trichogramma* and its relationship to biological control. PhD dissertation, University of California, Riverside.
- Lynch M, Walsh B (1998). *Genetics and Analysis of Quantitative Traits*. Sinauer Associates Inc.: Sunderland, MA, pp 69–71.
- Maughan H, Masei J, Birky CW, Nicholson WL (2007). The roles of mutation accumulation and selection in loss of sporulation in experimental populations of *Bacillus subtilis*. *Genetics* **177**: 937–948.
- Muller HJ (1932). Some genetic aspects of sex. *Am Nat* **66**: 118–138.

- Pannebakker BA, Loppin B, Elemans CPH, Humblot L, Vavre F (2007). Parasitic inhibition of cell death facilitates symbiosis. *Proc Natl Acad Sci USA* **104**: 213–215.
- Pannebakker BA, Schidlo NS, Boskamp GJF, Dekker L, Van Dooren TJM, Beukeboom LW *et al.* (2005). Sexual functionality of *Leptopilina clavipes* (Hymenoptera: Figitidae) after reversing *Wolbachia*-induced parthenogenesis. *J Evol Biol* **18**: 1019–1028.
- Pijls JWAM, Van Steenberg HJ, Van Alphen JJM (1996). Asexuality cured: the relations and differences between sexual and asexual *Apoanagyrus diversicornis*. *Heredity* **76**: 506–513.
- Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M (1992). *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc R Soc Lond B Biol Sci* **250**: 91–98.
- Stouthamer R, Breeuwer JAJ, Luck RF, Werren JH (1993). Molecular-identification of microorganisms associated with parthenogenesis. *Nature* **361**: 66–68.
- Stouthamer R, Hu JG, van Kan FJPM, Platner GR, Pinto JD (1999). The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. *Biocontrol* **43**: 421–440.
- Stouthamer R, Kazmer DJ (1994). Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity* **73**: 317–327.
- Stouthamer R, Luck RF (1993). Influence of microbe-associated parthenogenesis on the fecundity of *Trichogramma deion* and *T. pretiosum*. *Entomol Exp Appl* **67**: 183–192.
- Stouthamer R, Luck RF, Hamilton WD (1990). Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera, Trichogrammatidae) to revert to sex. *Proc Natl Acad Sci USA* **87**: 2424–2427.
- Stouthamer R, Luck RF, Pinto JD, Platner GR, Stephens B (1996). Non-reciprocal cross-incompatibility in *Trichogramma deion*. *Entomol Exp Appl* **80**: 481–489.
- Turelli M (1994). Evolution of incompatibility-inducing microbes and their hosts. *Evolution* **48**: 1500–1513.
- Vavre F, Kremer N, Pannebakker BA, Loppin B, Mavingui P (2008). Is symbiosis evolution influenced by the pleiotropic role of programmed cell death in immunity and development? In: Bourtzis K, Miller TA (eds). *Insect Symbiosis*, vol. 3. CRC Press: Boca Raton. pp 57–75.
- Weeks AR, Breeuwer JAJ (2001). *Wolbachia*-induced parthenogenesis in a genus of phytophagous mites. *Proc R Soc Lond B Biol Sci* **268**: 2245–2251.
- Werren JH, Windsor D, Guo L (1995). Distribution of *Wolbachia* among neotropical arthropods. *Proc R Soc Lond B Biol Sci* **262**: 197–204.
- Zchori-Fein E, Faktor O, Zeidan M, Gottlieb Y, Czosnek H, Rosen D (1995). Parthenogenesis-inducing microorganisms in *Aphytis* (Hymenoptera: Aphelinidae). *Ins Mol Biol* **4**: 173–178.

Supplementary Information accompanies the paper on Heredity website (<http://www.nature.com/hdy>)