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ORIGINAL ARTICLE

Occasional males in parthenogenetic populations of *Asobara japonica* (Hymenoptera: Braconidae): low *Wolbachia* titer or incomplete coadaptation?

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Wolbachia are endosymbiotic bacteria known to manipulate the reproduction of their hosts. Some populations of the parasitoid wasp *Asobara japonica* are infected with *Wolbachia* and reproduce parthenogenetically, while other populations are not infected and reproduce sexually. *Wolbachia*-infected *A. japonica* females regularly produce small numbers of male offspring. Because all females in the field are infected and infected females are not capable of sexual reproduction, male production seems to be maladaptive. We investigated why these females nevertheless produce males. We tested three hypotheses: high rearing temperatures could result in higher offspring sex ratios (more males), low *Wolbachia* titer of the mother could lead to higher offspring sex ratios and/or the *Wolbachia* infection is of relatively recent origin and not enough time has passed to allow complete coadaptation between *Wolbachia* and host. In all, 33% of the *Wolbachia*-infected females produced males and 56% of these males were also infected with *Wolbachia*. Neither offspring sex ratio nor male infection frequency was significantly affected by rearing temperature or *Wolbachia* concentration of the mother. The mitochondrial DNA sequence of one of the uninfected populations was identical to that of two of the infected populations. Therefore, the initial *Wolbachia* infection of *A. japonica* must have occurred recently. Mitochondrial sequence variation among the infected populations suggests that the spread of *Wolbachia* through the host populations involved horizontal transmission. We conclude that the occasional male production by *Wolbachia*-infected females is most likely a maladaptive side effect of incomplete coevolution between symbiont and host in this relatively young infection.
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INTRODUCTION

Wolbachia are maternally inherited, intracellular, symbiotic bacteria belonging to the order Rickettsiales within the α-Proteobacteria. It has been estimated that *Wolbachia* infect about 66% of all insect species, and either some or all individuals per species (Hilgenboecker *et al.*, 2008). To enhance its own transmission, *Wolbachia* can induce various alterations of the reproduction of its host, such as cytoplasmic incompatibility, feminization, male killing and parthenogenesis induction (PI). *Wolbachia*-induced parthenogenesis is most commonly found in haplodiploid organisms, such as Hymenoptera (Werren, 1997; Stouthamer *et al.*, 1999; Werren *et al.*, 2008). In uninfected haplodiploid organisms, fertilized eggs develop into diploid daughters and unfertilized eggs develop into haploid sons (arrhenotoky). In haplodiploids, PI *Wolbachia* cause diploidization of the haploid eggs by alteration of meiotic and/or mitotic processes (Huigens and Stouthamer, 2003; Pannebakker *et al.*, 2004) resulting in the production of daughters from unfertilized eggs (thelytoky). In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the whole host population consists of females (Huigens and Stouthamer, 2003). Males tend to be absent or rare in such populations. Here, we identify an exception to this pattern and examine its cause.

Asobara japonica (Hymenoptera: Braconidae) is a larval–pupal parasitoid of drosophilid flies and naturally occurs in Japan. Populations of *A. japonica* on the main islands of Japan exhibit highly female-biased sex ratios (92.7–99.2% females), whereas population sex ratios on the smaller southern islands are not biased (Mitsui *et al.*, 2007). The populations on the main islands are infected with parthenogenesis-inducing *Wolbachia*, while the populations on the smaller southern islands are not (Kremer *et al.*, 2009). Also, during routine culturing in our lab, *Wolbachia*-infected *A. japonica* females regularly produce small numbers of male offspring and in rare cases even male-biased sex ratios. The production of males in parthenogenetic populations of *A. japonica* seems to be maladaptive, because parthenogenetic females are not capable of sexual reproduction (Kremer *et al.*, 2009).

The occasional male production suggests that transmission of *Wolbachia* from mother to daughter is not always 100% efficient. In several hosts, infected females loose their *Wolbachia* when exposed to high temperatures (for example Pijls *et al.*, 1996; Clancy and Hoffmann, 1998; Pintureau *et al.*, 1999; Hurst *et al.*, 2000). Eggs laid at high temperatures would then contain low *Wolbachia* concentrations, which may cause the effect of *Wolbachia* to be reduced or lost (Clancy

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and Hoffmann, 1998; Hurst *et al*., 2000). In PI *Wolbachia*-infected females this would lead to male offspring production. A similar effect would be predicted if *Wolbachia* concentrations are reduced for reasons other than temperature. In contrast, Mouton *et al*. (2006) found increased *Wolbachia* densities in the parasitoid wasp *Leptopilina heterotoma* when reared at high temperatures, but this did not influence the effect of *Wolbachia* on its host.

An alternative explanation for inefficient transmission of *Wolbachia* might be that the *Wolbachia* infection is relatively young. While vertical transmission is the main transmission mode of *Wolbachia* within established hosts, horizontal transmission plays a major role in the spread of *Wolbachia* in(to) novel hosts (Hurst *et al*., 1992; O'Neill *et al*., 1992; Rousset *et al*., 1992; Werren *et al*., 1995; Vavre *et al*., 1999; Huigens *et al*., 2000, 2004; Kraaijeveld *et al*., 2011a). This predicts that upon invasion of a new host population, *Wolbachia* would initially be selected for efficient horizontal transmission, as well as efficient vertical transmission. This would be followed by adaptation to vertical transmission only once most of the host population is infected. For example, the parasitoid wasp *Leptopilina boulardi* is infected with a symbiont that manipulates the superparasitism behavior of its host in order to enhance its own horizontal transmission (Varaldi *et al*., 2003). Successful experimental horizontal transmission of *Wolbachia* often leads to unstable infections in the new host and reduced or altered expression of the reproductive manipulation (Grenier *et al*., 1998; Heath *et al*., 1999; Huigens *et al*., 2004; Jaenike, 2007). An explanation for such poor vertical transmission might be residual incompatibilities or asynchronies between *Wolbachia* and the new host (Heath *et al*., 1999).

In this paper, we investigate why PI *Wolbachia*-infected *A. japonica* females produce males. First, we quantified how often and in what numbers male offspring are produced by infected females and whether male production is affected by rearing temperature. We predicted that higher rearing temperatures would result in higher offspring sex ratios (more males). Second, we examined whether male offspring production is influenced by *Wolbachia* titer of the mother. Quantitative PCR (qPCR) was used to measure *Wolbachia* concentrations of *A. japonica* females. We predicted that lower *Wolbachia* concentrations would lead to higher offspring sex ratios. Last, we used mitochondrial DNA (mtDNA) sequences to date the initial infection of *A. japonica* with *Wolbachia*. We hypothesized that the *Wolbachia* infection in *A. japonica* is relatively young.

As far as we know, this is the first example from the field of a possible relation between incomplete *Wolbachia*–host coadaptation and a relatively recent *Wolbachia* infection.

MATERIALS AND METHODS

A. japonica strains

Five *Wolbachia*-infected thelytokous strains of the parasitoid wasp *A. japonica* (Hymenoptera: Braconidae) were used in all experiments: Sapporo, Hirosaki, Sendai, Tokyo and Kagoshima. In addition, two uninfected arrhenotokous strains were used for the mtDNA analysis: Amami and Iriomote. The strains were kindly provided by M.T. Kimura from cultures derived from field samples, collected along the entire length of Japan, described in Mitsui *et al*. (2007) and Murata *et al*. (2009). Maps of the sampled locations can be found in both papers. These strains were subsequently maintained in our lab under a partial inbreeding regime. Each generation, three females per strain were allowed to parasitize about 100–200 2-day-old (first or second instar) larvae of *Drosophila melanogaster* in glass jars with a medium of agar covered by a layer of 2 ml baker's yeast suspension and kept at 25 °C, light:dark 16:8, 65% relative humidity.

Temperature experiment

To test whether temperature affected male production, we examined the offspring sex ratio of *Wolbachia*-infected thelytokous strains of *A. japonica* at two temperatures. One-week-old females from five thelytokous strains (Sapporo, Hirosaki, Sendai, Tokyo and Kagoshima) were placed individually in a glass jar with a medium of agar covered by a layer of 2 ml baker's yeast suspension, in which they were allowed to parasitize about 100–200 2-day-old (first or second instar) larvae of *D. melanogaster*. As thelytokous *A. japonica* females are not capable of sexual reproduction (Kremer *et al*., 2009), we assumed that all females used in the sex ratio experiment were virgins. For each strain, half of the females were kept at 25 °C, light:dark 16:8, 65% relative humidity, while the other half were kept at 20 °C, light:dark 16:8, 65% relative humidity. We chose the normal rearing temperature as the highest temperature, because we previously observed that males are produced at that temperature. After 10–13 days, mothers were removed from the jars. The number of male and female offspring was counted several times a week for the next 7 weeks. To count the offspring, wasps were anaesthetized with CO_2. Females could be distinguished from males by their ovipositor, which permanently and prominently protrudes from the posterior abdomen. Mothers and offspring were stored separately in 70% ethanol in 1.5 ml eppendorf tubes until DNA extraction.

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

All male offspring were tested for *Wolbachia* infection by amplifying the *Wolbachia*-specific *wsp* and *ftsZ* genes, using the primers *wsp*-81F/*wsp*-691R (Braig *et al*., 1998; Zhou *et al*., 1998) and *ftsZ*-F/*ftsZ*-R (Holden *et al*., 1993; Sinkins *et al*., 1995), respectively. PCRs were performed in a total volume of 20 µl, containing 1× PCR-buffer (Qiagen), 62.5 µM dNTPs, 1 unit Taq polymerase, 250 nM forward primer, 250 nM reverse primer and 2 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 s 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.

qPCR analysis

To test whether offspring sex ratios produced by *Wolbachia*-infected females were affected by *Wolbachia* titer, we measured *Wolbachia* concentration of the mothers from the temperature experiment using qPCR. We also tested whether the *Wolbachia* concentration of the mother affected the percentage of *Wolbachia*-infected males among her offspring.

qPCRs were performed for the *Wolbachia*-specific *wsp* and *gatB* genes, with the nuclear *ITS2* gene as a control. To optimize the qPCRs, we designed new primers for all three genes. Primer sequences for *wsp* and *gatB* were based on the sequences for *wAjap* described in Kraaijeveld *et al*. (2011b), while the primer sequences for *ITS2* were based on the sequence for *A. japonica* described in Kremer *et al*. (2009). The following primers were used: *wsp*-wAjap-F: 5′-GAG GCA AAA TTT ACG CCA GA-3′ and *wsp*-wAjap-R: 5′-AAC TAG CCC TGA AAT TGC TGT TA-3′, producing a 60-bp amplicon; *gatB*-wAjap-F: 5′-GAA GCA AAG AGG ATG CAA GC-3′ and *gatB*-wAjap-R: 5′-TCC TGG CTT ACC TCA ACA GG-3′, producing a 73-bp amplicon; *ITS2*-Ajap-F: 5′-GGC AAG CAC AAT CAA GGT CT-3′ and *ITS2*-Ajap-R: 5′-ACA AAA ACA AAT TTT GCG GC-3′, producing a 93-bp amplicon. Each qPCR was performed in a total volume of 10 µl, containing 1× SybrGreen Mastermix (Roche, Penzberg, Germany), 300 nM forward primer, 300 nM reverse primer

and 2 µl DNA template. A LightCycler 480 Real-Time PCR System (Roche) was used for all qPCRs. qPCR conditions for all genes were as follows: 10 min at 95 °C, then 45 cycles of 10 s at 95 °C, 30 s at 60 °C and 20 s at 72 °C, and finally 5 min at 72 °C. Each qPCR was performed in triplicate.

The resulting C_t values were used to calculate the relative quantity of the focal gene (relative gene quantity=mean PCR efficiency $^{(\text{mean overall } C_t \text{ value} - \text{mean sample } C_t \text{ value})}$). To correct for the total amount of DNA, we calculated the ratio between the *Wolbachia* gene and the control gene (ratio=relative quantity *Wolbachia* gene/relative quantity control gene).

mtDNA analysis

To estimate the age of the *Wolbachia* infection, we sequenced a part of the mtDNA of *A. japonica* from both infected and uninfected strains. We sequenced part of the *NADH 1 dehydrogenase* (*ND1*) gene, using the primers *ND1*-F (Smith and Kambhampati, 1999) and *ND1*-R (Smith *et al.*, 1999), producing a 447-bp amplicon, and the *cytochrome oxidase 1* (*CO1*) gene, using the primers *CO1*-1775F/*CO1*-2413R and *CO1*-2222F/*CO1*-2773R (Scheffer and Grissell, 2003), together producing a 997-bp amplicon. PCRs were performed in a total volume of 25 µl, containing 1× PCR-buffer (Qiagen), 750 µM (extra) $MgCl_2$, 200 µM dNTPs, 1.25 units Taq polymerase, 320 nM forward primer, 320 nM reverse primer and 2.5 µl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research) was used for all PCRs. PCR conditions for both genes were as follows: 5 min at 94 °C, then 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, and finally 10 min at 72 °C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Sequencing was performed by Macrogen Inc. (Seoul, Korea). Sequences were checked with Sequencher software (version 4.2; Gene Codes, Ann Arbor, MI, USA) and aligned with BioEdit software (version 7.0.9; Hall, 1999).

Haplotype diversity and nucleotide diversity were calculated using DnaSP software (version 5.10; Librado and Rozas, 2009). The haplotype diversity (H_d), the nucleotide diversity (π), the average number of synonymous substitutions per synonymous site between populations (k_s) and the average number of nucleotide substitutions per site between populations (*Dxy*) were determined. Median joining haplotype networks were drawn using Network software (version 4.6.0.0; Bandelt *et al.*, 1999).

To calculate the divergence time between strains, we used the estimates for the *CO1* gene mutation rate in the parasitoid wasp *Nasonia* (Hymenoptera: Pteromalidae), described in Raychoudhury *et al.* (2010). However, mutation rates can vary considerably between species. Therefore, the calculated divergence times are only very rough estimates and must be interpreted with caution. The mitochondrial mutation rate in *Nasonia* was estimated to be 3.5–13 times higher than in *D. melanogaster* (6.2×10^{-8} mutations per site per generation; Haag-Liautard *et al.*, 2008), that is 2.2×10^{-7}–8.1×10^{-7} mutations per site per generation (Raychoudhury *et al.*, 2010). The divergence time (in generations) between two populations can be calculated by dividing k_s (the average number of synonymous substitutions per synonymous site between populations) by this mutation rate (the number of mutations per site per generation).

Statistical analysis

Statistical analyses were performed in R software (version 2.12. 1; R Developmental Core Team, 2010). Generalized linear models with a binomial error distribution and an empirically estimated scale parameter were used to test for differences in sex ratio and male infection frequency. In the sex ratio model, the number of males was used as the response variable and clutch size as the binomial denominator. In the male infection frequency model, the number of infected males was used as the response variable and the total number of males as the binomial denominator. Significance of explanatory variables (*Wolbachia* concentration of the mother, rearing temperature and *A. japonica* strain) was tested by dropping (interactions between) explanatory variables from the model and comparing the resulting change in deviance using an F-test. As the *Wolbachia* concentration of the mother was measured twice, using two different genes for *Wolbachia*, the correlation between the two estimates was calculated using the Pearson's product moment correlation method.

RESULTS

Temperature experiment

In total, 100 individual females were used: 20 females from each of the five thelytokous strains, of which 10 were kept at 25 °C and the remaining 10 at 20 °C. All females produced offspring (ranging from 5 to 133 individuals). In all, 67 of these females produced only daughters, while 33 females produced both daughters and sons (ranging from 1 to 69 sons per clutch). The mean sex ratio was 2%, ranging from 0 to 60% per clutch. Most of the male producing females produced small numbers of males, with sex ratios ranging from 1 to 11%. Only two females produced higher sex ratios (Sapporo at 25 °C: clutch size=121, 57% males and Tokyo at 20 °C: clutch size=45, 60% males). There were no significant differences in sex ratio between rearing temperatures, strains, or their interaction (Figure 1, Supplementary Table 1; overall: $F_{9,90}=1.77$, $P=0.09$; interaction: $F_{4,90}=1.18$, $P=0.33$; temperature: $F_{1,98}=1.08$, $P=0.30$; strain: $F_{4,95}=1.46$, $P=0.22$).

Wolbachia detection

The two *Wolbachia*-specific genes used in the qPCRs were amplified in all samples. Therefore, all mothers were infected with *Wolbachia*.

In total, 177 sons were produced by 33 thelytokous females. Of these, 100 (56%, produced by 31 females) were infected with *Wolbachia*. The male infection frequency per clutch ranged from 0 to 100% (mean 88%). There was a significant interaction effect between rearing temperatures and strains on male infection frequency (Figure 2; overall: $F_{8,24}=5.99$, $P<0.001$; interaction: $F_{3,24}=4.05$, $P=0.02$). However, this effect appeared to be spurious. When we removed two outlying data points with extreme sex ratios, there were no significant differences in male infection frequency between rearing temperatures, strains or their interaction (Figure 2, Supplementary Table 1; overall: $F_{8,22}=1.41$, $P=0.25$; interaction: $F_{3,22}=2.16$, $P=0.12$; temperature: $F_{1,29}=0.58$, $P=0.45$; strain: $F_{4,26}=0.71$, $P=0.59$).

qPCR analysis

The *Wolbachia* concentration of 64 females from the temperature experiment was measured. Half of these females produced both daughters and sons, while the other half produced only daughters (*gatB*: $n_{\text{with sons}}=32$, $n_{\text{without sons}}=32$; *wsp*: $n_{\text{with sons}}=30$, $n_{\text{without sons}}=31$). The two estimates for *Wolbachia* concentration (*gatB*/*ITS2* and *wsp*/*ITS2*)

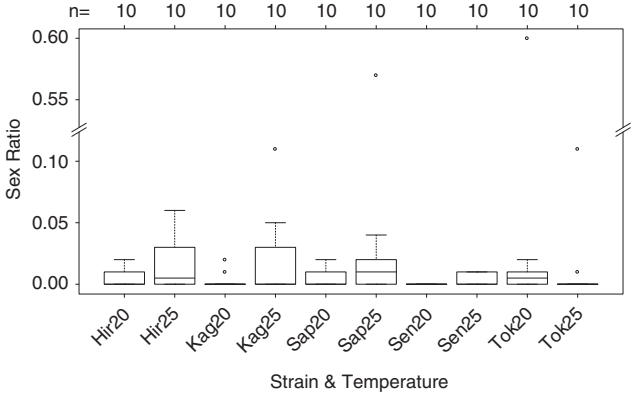

Figure 1 Sex ratio (proportion males) per strain and rearing temperature (20 °C and 25 °C). Number of clutches (*n*=sample size) are indicated above the graph. The horizontal dark lines represent the median sex ratios, the bottom and top of the boxes indicate the 25th and 75th percentiles, the whiskers show up to 1.5 times the interquartile range and the dots represent outliers. Hir, Hirosaki; Kag, Kagoshima; Sap, Sapporo; Sen, Sendai; Tok, Tokyo.

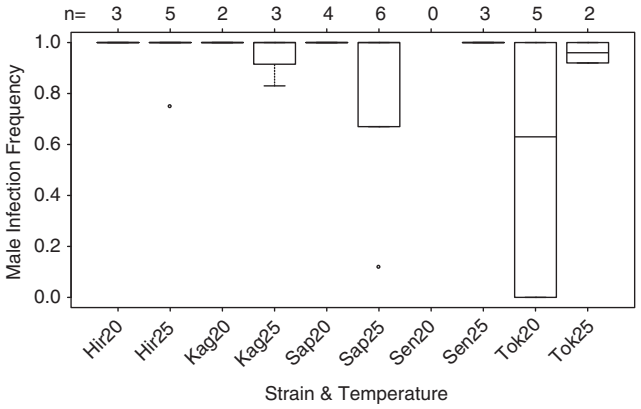

Figure 2 Male infection frequency (proportion infected males) per strain and rearing temperature (20 °C and 25 °C). Number of clutches (*n*=sample size) are indicated above the graph. The horizontal dark lines represent the median male infection frequencies, the bottom and top of the boxes indicate the 25th and 75th percentiles, the whiskers show up to 1.5 times the interquartile range and the dots represent outliers. Hir, Hirosaki; Kag, Kagoshima; Sap, Sapporo; Sen, Sendai; Tok, Tokyo.

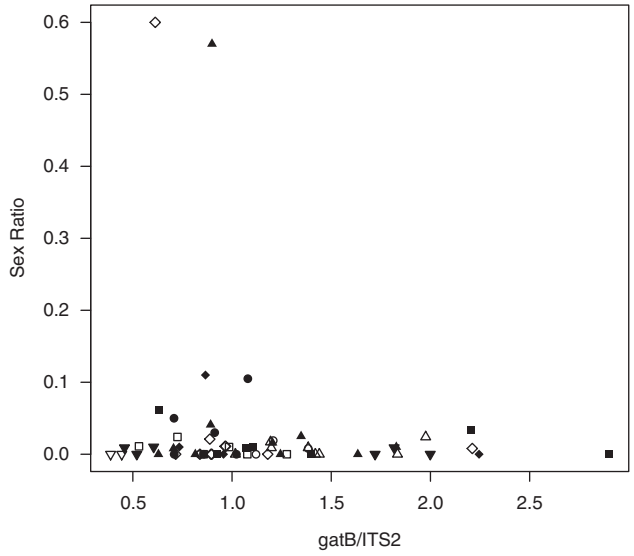

Figure 3 Relation between sex ratio (proportion males) and *Wolbachia* concentration (measured as the ratio between the *Wolbachia*-specific gene *gatB* and the control gene *ITS2*) per rearing temperature (white symbols: 20 °C, black symbols: 25 °C) and strain (squares: Hirosaki, circles: Kagoshima, upward triangles: Sapporo, downward triangles: Sendai, diamonds: Tokyo).

correlated significantly with each other (r=0.92, t=17.90, df=59, $P<0.0001$).

There were no significant relations between sex ratio and *Wolbachia* concentration of the mother, rearing temperatures, strains or their interactions (*gatB*: Figure 3; overall: $F_{7,56}$=0.08, P=0.99; *Wolbachia*: $F_{1,62}$=1.20, P=0.28; temperature: $F_{1,62}$=0.75, P=0.39; strain: $F_{1,62}$=0.15, P=0.70; none of the interactions between explanatory variables were significant; *wsp*: overall: $F_{7,53}$=0.30, P=0.95; *Wolbachia*: $F_{1,59}$=0.32, P=0.58; temperature: $F_{1,59}$=0.47, P=0.50; strain: $F_{1,59}$=0.04, P=0.85; none of the interactions between explanatory variables were significant).

Also, there were no significant relations between male infection frequency and *Wolbachia* concentration of the mother, rearing

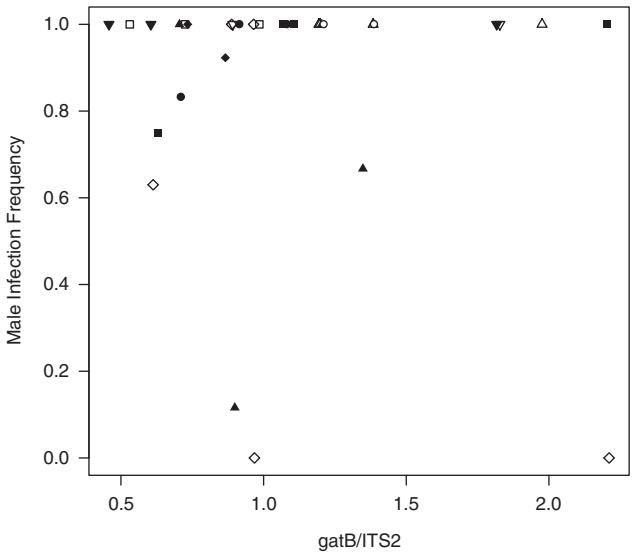

Figure 4 Relation between male infection frequency (proportion infected males) and *Wolbachia* concentration (measured as the ratio between the *Wolbachia*-specific gene *gatB* and the control gene *ITS2*) per rearing temperature (white symbols: 20 °C, black symbols: 25 °C) and strain (squares: Hirosaki, circles: Kagoshima, upward triangles: Sapporo, downward triangles: Sendai, diamonds: Tokyo).

temperatures, strains or their interactions (*gatB*: Figure 4; overall: $F_{7,24}$=1.05, P=0.42; *Wolbachia*: $F_{1,30}$=1.17, P=0.29; temperature: $F_{1,30}$=2.20, P=0.15; strain: $F_{1,30}$=0.03, P=0.86; none of the interactions between explanatory variables were significant; *wsp*: overall: $F_{7,22}$=1.24, P=0.33; *Wolbachia*: $F_{1,28}$=0.01, P=0.91; temperature: $F_{1,28}$=3.73, P=0.06; strain: $F_{1,28}$=0.53, P=0.47; none of the interactions between explanatory variables were significant).

mtDNA analysis

We sequenced part of the mtDNA of seven females, representing five thelytokous and two arrhenotokous strains of *A. japonica*. The sequences have been submitted to the GenBank database (*CO1* accession numbers: JF430425–JF430431; *ND1* accession numbers: JF430432–JF430438).

We found five unique mitochondrial haplotypes (H_d=0.8571). Two thelytokous strains (Hirosaki and Sendai) and the arrhenotokous strain Amami exhibited the same DNA sequence and thus shared the same haplotype, while the other four strains (Sapporo, Tokyo, Kagoshima and Iriomote) all exhibited unique haplotypes (Figure 5). These five haplotypes contained 55 polymorphic sites (π=0.0111), of which 50 were synonymous and five were non-synonymous mutations. The arrhenotokous strain Iriomote differed with 51–53 mutations from all the other haplotypes (k_s=0.1741; *Dxy*=0.0360). Within these other four haplotypes there were zero to three mutations between strains and four polymorphic sites (π=0.0011; three synonymous and one non-synonymous mutations).

The five haplotypes could be distinguished within the *CO1* gene sequence (H_d=0.8571). Within the *CO1* gene, the five haplotypes contained 40 polymorphic sites (π=0.0118; 37 synonymous and 3 non-synonymous mutations). The arrhenotokous strain Iriomote differed with 36–38 mutations from all the other haplotypes (k_s=0.1788; *Dxy*=0.0371). Within these other four haplotypes there were zero to three mutations between strains and four polymorphic sites (π=0.0016; three synonymous and one non-synonymous

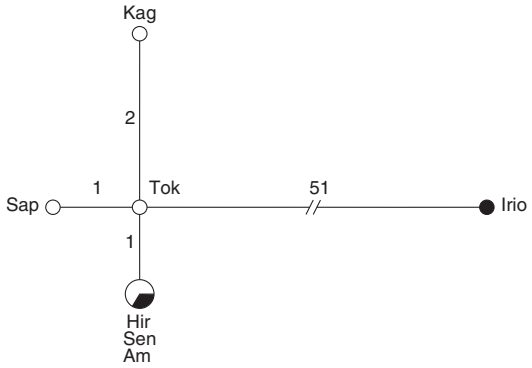

Figure 5 Haplotype network based on 997 bp of the mitochondrial *CO1* gene and 447 bp of the mitochondrial *ND1* gene of *A. japonica*. Each circle represents one haplotype. The size of the circle represents the haplotype frequency. White circles indicate haplotypes found in thelytokous strains, black circles indicate haplotypes found in arrhenotokous strains, mixed black and white circles indicate haplotypes shared by thelytokous and arrhenotokous strains. Strain names are given for the haplotype in which they were found. Lines show mutational routes between haplotypes. The length of the line and the numbers on the line indicate the number of mutations between haplotypes. Am, Amami; Hir, Hirosaki; Irio, Iriomote; Kag, Kagoshima; Sap, Sapporo; Sen, Sendai; Tok, Tokyo.

mutations). Only two haplotypes were found for the *ND1* gene sequence (H_d=0.2857). All thelytokous strains (Sapporo, Hirosaki, Sendai, Tokyo and Kagoshima) and the arrhenotokous strain Amami exhibited the same *ND1* haplotype, while the second *ND1* haplotype was only represented by the arrhenotokous strain Iriomote. The two *ND1* haplotypes differed by 15 polymorphic sites (π=0.0096; 13 synonymous and 2 non-synonymous mutations; k_s=0.1625; *Dxy*=0.0336).

The mean divergence time, based only on the *CO1* gene, between the arrhenotokous strain Iriomote and the other six strains (k_s=0.1788) was estimated to be between 8.2×10^5 and 2.2×10^5 generations ago. Because the arrhenotokous strain from Amami exhibited the same mitochondrial haplotype as two thelytokous strains, we concluded that these strains have not (yet) diverged in their mtDNA.

DISCUSSION

PI *Wolbachia*-infected *A. japonica* females regularly produced small numbers of male offspring and rarely male-biased offspring sex ratios. Slightly more than half of these males were infected with *Wolbachia*. Neither offspring sex ratio nor male infection frequency was affected by strain, rearing temperature or *Wolbachia* concentration of the mother.

Within seven strains of *A. japonica*, we found five unique mitochondrial haplotypes. Four of these haplotypes, represented by six strains, were closely related, while the uninfected arrhenotokous strain from Iriomote was very different from the rest. The uninfected arrhenotokous strain from Amami exhibited the same haplotype as two thelytokous strains from Hirosaki and Sendai. More or less the same pattern, based on 645 bp of the mitochondrial *CO1* gene, was found by Murata *et al.* (2009).

Fertile crosses between males and females from Iriomote and Amami, and between (natural and cured) males from Kagoshima and females from Amami indicate that individuals from these strains belong to the same species (Murata *et al.*, 2009; Kraaijeveld *et al.*, 2011b). However, Murata *et al.* (2009) also found indications for weak asymmetrical sexual isolation, suggesting that Iriomote and Amami have been geographically isolated for a long time. Based on the mDNA sequences, we estimated that the divergence time between Iriomote and the other strains was between 8.2×10^5 and 2.2×10^5 generations ago. However, given that these estimates are extrapolated from mutation rates in a different species (*Nasonia*), they should be interpreted with caution.

The uninfected arrhenotokous strain from Amami exhibited the same mitochondrial haplotype as two thelytokous strains. There was also very little mitochondrial variation between Amami and the other three thelytokous strains. Moreover, there is no variation between the *Wolbachia* strains in the five thelytokous *A. japonica* strains (Kraaijeveld *et al.*, 2011b). This suggests that the *Wolbachia* infection in *A. japonica* is relatively young. An alternative possibility might be that the strain from Amami was infected with *Wolbachia* before, but has lost its infection. However, this seems unlikely because thelytokous females that have been cured from their *Wolbachia* infection with antibiotics are not capable of sexual reproduction (Kremer *et al.*, 2009).

New bacterial symbiont infections can spread rapidly in host populations. Invasions of *Wolbachia* in *Drosophila simulans* in California and *Rickettsia* in *Bemisia tabaci* in Arizona have been reported in which the infection frequency increased from 0% to near fixation in $<$100 generations (Turelli and Hoffmann, 1991; Himler *et al.*, 2011). The mitochondrial variation among the five thelytokous strains suggests that multiple infection events have occurred and that the *Wolbachia* infection has spread (partly) via horizontal transmission. The five infected thelytokous strains were collected from the two Japanese main islands, with 1580 km distance between the northernmost location Sapporo and the southernmost location Kagoshima (for maps of the locations see Mitsui *et al.*, 2007 and Murata *et al.*, 2009). The distance between the two main Japanese islands is very small (20 km) and the *Wolbachia* infection probably spread easily between the two islands. However, the distance between the island of Amami and the main islands of Japan may be too large (290 km) for the *Wolbachia* infection to invade Amami. No geographical gradient could be distinguished from the mitochondrial data. Although Kagoshima and Amami are geographically closest to each other (370 km), based on their mtDNA they are the least closely related within the 'thelytokous' population. Also, Hirosaki and Sendai are geographically distant from Amami (1680 km and 1520 km, respectively), but based on their mtDNA they are closely related.

The occasional male production by PI *Wolbachia*-infected *A. japonica* females is most likely due to a relatively young *Wolbachia* infection. There may have been too little time to allow complete coevolution between *Wolbachia* and *A. japonica*. Incomplete coadaptation may be caused by remaining incompatibilities or asynchronies between *Wolbachia* and its host (Heath *et al.*, 1999). The incomplete adaptation of *Wolbachia* to its host *A. japonica* may lead to incomplete diploidization of the haploid eggs, so that part of the eggs remains haploid and develop into sons. As thelytokous *A. japonica* females are not capable of sexual reproduction (Kremer *et al.*, 2009), these males will have zero fitness.

The inability of thelytokous *A. japonica* females to reproduce sexually could be due to selection against the maintenance of costly sexual traits, or due to accumulation of neutral mutations (Pijls *et al.*, 1996). However, this interpretation seems at odds with the recent origin of the *Wolbachia* infection in this species. The spread of parthenogenesis-inducing *Wolbachia* may be facilitated by the concomitant spread of 'functional virginity mutations' (Stouthamer *et al.*, 2010). Mutations that prevent females from fertilizing their eggs will have a selective advantage in the presence of PI *Wolbachia*-infected

females because they induce the bearer to produce more sons which will have many mating opportunities. Virginity mutations may thus explain both the rapid spread of PI *Wolbachia* through the population of *A. japonica* and the inability of *A. japonica* females to reproduce sexually.

We conclude that the occasional male production by PI *Wolbachia*-infected *A. japonica* females is not due to high rearing temperatures or low *Wolbachia* concentrations of the mother, but most likely is a maladaptive side effect of the relatively young age of the *Wolbachia* infection. *Wolbachia* possibly is not (yet) fully adapted to its host *A. japonica*.

DATA ARCHIVING

Data of the temperature experiment, *Wolbachia* detection and qPCR experiment have been deposited at Dryad: doi: 10. 5061/dryad.5qm51. Sequence data have been deposited at GenBank: *CO1* accession numbers: JF430425–JF430431; *ND1* accession numbers: JF430432–JF430438.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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