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ARTICLE

A *Wolbachia* infection from *Drosophila* that causes cytoplasmic incompatibility despite low prevalence and densities in males

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Abstract

Wolbachia bacteria are common insect endosymbionts transmitted maternally and capable of spreading through insect populations by cytoplasmic incompatibility (CI) when infected males cause embryo death after mating with uninfected females. Selection in the *Wolbachia* endosymbiont occurs on female hosts and is expected to favour strong maternal transmission to female offspring, even at the cost of reduced CI. With maternal leakage, nuclear genes are expected to be selected to suppress cytoplasmic incompatibility caused by males while also reducing any deleterious effects associated with the infection. Here we describe a new type of *Wolbachia* strain from *Drosophila pseudotakahashii* likely to have arisen from evolutionary processes on host and/or *Wolbachia* genomes. This strain is often absent from adult male offspring, but always transmitted to females. It leads to males with low or non-detectable *Wolbachia* that nevertheless show CI. When detected in adult males, the infection has a low density relative to that in females, a phenomenon not previously seen in *Wolbachia* infections of *Drosophila*. This *Wolbachia* strain is common in natural populations, and shows reduced CI when older (infected) males are crossed. These patterns highlight that endosymbionts can have strong sex-specific effects and that high frequency *Wolbachia* strains persist through effects on female reproduction. Female-limited *Wolbachia* infections may be of applied interest if the low level of *Wolbachia* in males reduces deleterious fitness effects on the host.

Introduction

Wolbachia are common endosymbionts of insects with diverse effects on insect reproduction. Cytoplasmic incompatibility (CI: reduced egg hatch when uninfected females mate with infected males) is the most common phenotype associated with this endosymbiont (Hoffmann and Turelli 1997). There are also *Wolbachia* strains with other phenotypic effects on host reproduction, including male killing (Hurst and Jiggins 2000) and feminization (Kern et al. 2015). Although *Wolbachia* are often described as "manipulating" host reproduction to aid their own spread and persistence, *Wolbachia* in some populations may also have little impact on host reproduction (Hoffmann et al. 1996). Such *Wolbachia* persist by generating other fitness advantages for their hosts, perhaps involving nutrition or protection from viruses (Teixeira et al. 2008; Brownlie et al. 2009), although the particular advantages are often unknown (Hamm et al. 2014; Kriesner et al. 2013).

Evolutionary changes in the nuclear genome of hosts or the genome of *Wolbachia* strains will drive shifts in phenotypic effects on hosts, including both fitness effects and reproductive manipulations, as well as the efficiency of maternal transmission across generations (Bull and Turelli 2013; Prout 1994; Turelli 1994). This raises the issue of which types of changes in CI, fitness, and transmission efficiency might be expected to occur across time, and which are due to changes in host and/or *Wolbachia*

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genomes. Microinjection experiments and backcrosses can help to sort out which genomes are involved (Boyle et al. 1993; Braig et al. 1994). Theoretical models (Prout 1994; Turelli 1994) suggest that *Wolbachia* should be selected on the basis of maternal transmission success, given that a high rate of transmission can ensure that *Wolbachia* persist in populations even in the absence of reproductive effects. For this reason, *Wolbachia* genes that favour transmission could be selected even under decreasing levels of incompatibility (Tortosa et al. 2010). If transmission is imperfect, selection also acts on host genes that decrease the impact of *Wolbachia* on host reproduction. This reflects the fact that females that lose *Wolbachia* will be sterilized by males carrying *Wolbachia* unless they carry genes that overcome the incompatibility. Selection pressures acting on the sexes are quite different, because inheritance of *Wolbachia* normally involves female hosts and paternal transmission appears rare (Hoffmann and Turelli 1988; Turelli et al. 2018); any fitness effects of *Wolbachia* that are male-specific and associated with the bacterium would not be passed on. Selection on *Wolbachia*, therefore, should proceed via the female (Frank 1997; Turelli 1994). For this reason, feminization caused by *Wolbachia* is favoured by selection, and male-killing is also favoured if there is an advantage to females developing at a relatively lower density, for example, with reduced sib competition or cannibalism, as well as for the avoidance of inbreeding (Hurst et al. 2002).

Despite the sex-specific nature of selection effects generated by *Wolbachia*, there is, so far, limited evidence for sex-specific differences in the density of *Wolbachia* in insects. In some mosquito studies, lower densities have been found in males, particularly in *Aedes albopictus* (Dutton and Sinkins 2004; Tortosa et al. 2010) and *Culex pipiens* (Berticat et al. 2002). In other cases, densities are similar between the sexes, as in *Aedes aegypti* (Ross et al. 2017). Where differences have been detected, as in the examples cited above, they typically involve 2–15 fold differences in density, although in one species of planthopper, a more substantial difference between the sexes has been noted and this has been associated with a low level of CI (Noda et al. 2001). There are still relatively few *Wolbachia* infections for which phenotypic effects have been investigated in detail, particularly across both sexes and with field-derived lines, raising the issue of whether *Wolbachia* strains that are strongly sex-limited in density or even occurrence might yet exist on a broader scale.

Here we describe a *Wolbachia* infection in a *Drosophila* species, *D. pseudotakahashii*, that seems to have sex-specific effects associated with differences in *Wolbachia* density. This strain, *w*Pse, has previously been identified in *D. pseudotakahashii* (14022-0301.01, Tucson Stock Centre, origin Atherton Tableland, Australia) (Mateos et al. 2006) using *wsp* primers, but no phenotypic or molecular studies on the strain have been done. *Drosophila pseudotakahashii* falls in the *takahashii* species subgroup, which is in the *D. melanogaster* species group. We show that the *w*Pse strain is detected in females at a high frequency, but that it appears at a much lower frequency in males. Moreover, there is strong CI in crosses involving uninfected females, even when the males from infected mothers have low-density *Wolbachia* or it is not detected. We show that maternal transmission of the strain to females is high and that there are no obvious host fitness effects associated with this infection.

Methods

D. pseudotakahashii field collections and laboratory lines

Samples of female *D. pseudotakahashii* (Appendix 1) were obtained from two locations in New South Wales, three locations in south eastern Queensland, and six locations in northern Queensland. Of these samples, all females except some from New South Wales were used to initiate isofemale lines. Species identification was based on male offspring and involved examination of the configuration of the sex combs on tarsomeres I and II of the male foreleg and the dark abdominal banding on the posterior tergites (see Bock. 1976). Lines were screened for *Wolbachia* infection by PCR and RT-PCR as outlined below using a single female and multiple males where possible.

Several laboratory lines of *D. pseudotakahashii* were used in crosses and experiments (Appendix 1). One line (designated *Moor+* with the + symbol indicating an infected line) was established by pooling flies from three infected isofemale lines collected from Moorland, New South Wales. Four other infected isofemale lines from northern Queensland were used in crosses and experiments and originated from Smithfield and Townsville with designations *Smith+*, *Town1+*, *Town2+* and *Town3+*. Females from all lines were infected by *Wolbachia* based on PCR characterization, however males were not consistently infected (see below).

Because there were no naturally uninfected lines (see below), uninfected lines were generated from three of the isofemale lines (*Moor+, Smith+, Town3+*) by treatment with 0.03% tetracycline (Sigma, Castle Hill, NSW, Australia) in cornmeal media for one to two generations as outlined in Hoffmann et al. (1986). Derived *Wolbachia*-negative lines are designated with a "-" symbol (i.e., *Moor−* and *Town3−*). Curing of *Wolbachia* infection was verified via PCR (see below). Lines were maintained in the laboratory on cornmeal media at 19 °C with a 12:12 L:D

cycle and treated lines were allowed to recover in the absence of tetracycline for at least two generations before being used in experiments. Note that the uninfected status of these lines was confirmed when flies were used in experimental crosses.

Wolbachia infection detection

A preliminary screen for *Wolbachia* infection was conducted for all field isofemale lines and preserved samples. DNA extractions were performed using a 5% Chelex® (Bio-Rad Laboratories, Gladesville, NSW, Australia; Cat. No. 142-1253; w/v in distilled water) based plate method on single individuals as outlined by Richardson et al. (2016), with extractions diluted to 1 in 10 for all screening work. Samples were screened for *Wolbachia* via PCR with the *gatB* primers for the Multilocus Sequence Typing System (MLST) for *Wolbachia* (Baldo et al. 2006) or via RT-PCR using the *wsp_validation* primers (Kriesner et al. 2013; Lee et al. 2012). PCR conditions and visualisation techniques are outlined in Richardson et al. (2016).

To investigate the *Wolbachia* infection in more detail, we used the forward and reverse *coxA*, *hcpA*, *ftsZ*, *fpbA* and *gatB* MLST primers (Baldo et al. 2006) and *wsp_validation* primers (Kriesner et al. 2013; Lee et al. 2012). While whole genome sequencing would have been ideal (Bleidorn and Gerth. 2017; Turelli et al. 2018), it is more expensive, and MLST is an established system that also allows links to be made to previous work. The MLST and *wsp_validation* primers were used to screen additional PCR amplifications for two individuals from the isofemale lines from Moorland and Smithfield as well as Lake Placid (Appendix 1). Conditions were as outlined in Richardson et al. (2016). PCR products were sent to Macrogen (Korea) for purification and Sanger sequencing. Sequencing chromatograms were examined and processed using Finch TV v1.4.0 (Geospiza, Seattle, WA) and MEGA version 6 (Tamura et al. 2013). Allelic profiles were compared to those in the MLST database at http://pubmlst.org/wolbachia/ (Jolley and Maiden 2010).

For routine scoring of *Wolbachia* in the flies, we used a genotyping assay (Kriesner et al. 2013; Lee et al. 2012) which determines *Wolbachia* infection from the melting temperature (T_m) of the *wsp* validation PCR amplicons. High-resolution melt analysis on the Roche LightCycler® 480 system produced a T_m range from approximately 81.5 to 81.9 °C for *D. pseudotakahashii*. The same system also determines the cycle number at detection threshold (Cp) which can be used for relative quantification; if we assume that the efficiency of the reaction is perfect (i.e., $R = 2^{-\Delta\Delta Cp}$), and identical gDNA template concentration, the difference in Cp values between samples can give an estimate of the relative concentration of *Wolbachia* DNA when computed as $2^{(Cp1\ -Cp2)}$, assuming the housekeeping gene has similar copy numbers of DNA in both samples. We do not consider Cp value as a tool for definitive quantification, but it provides a general guide such that lower Cp values correlate with stronger *Wolbachia* infection. To verify that high Cp values indicated true *Wolbachia* infection, we Sanger sequenced 14 samples with Cp values that ranged from 24.99 to 34.14 with the *wsp* validation primers as outlined previously. For comparative purposes, we also measured Cp values of other strains of *Wolbachia* in males and females of *D. pandora* (*w*Ri-like infection, *pl*+ line), *D. melanogaster* (*w*Mel infection, HAW+ line) and *D. simulans* (*w*Ri, Burnley line and *w*Au, Coffs line) (for strain descriptions see Kriesner et al. 2013, 2016; Richardson et al. 2016). Note that these are strain rather than population samples.

To create control primers to verify DNA extraction, *Drosophila* universal primers were designed, targeting the ribosomal protein gene *L40* (*RpL40*). *RpL40* coding sequences of 12 *Drosophila* species were retrieved from Flybase (flybase.org) and aligned using Sequencher 5.4 (Gene Codes Corporation). Based on the nucleotide alignment of these 12 *RpL40* orthologues, primers (Dros_RpL40_F: 5′-AACTGCCGCAAGAAGAAGTG-3′; Dros_RpL40_R: 5′-CTACTTCAACTTCTTCTTGGG-3′) were placed at two conserved regions, amplifying the last 64 bases of the *RpL40* gene (Figure S1).

To verify the relationship between Cp value and *Wolbachia* density, selected *Drosophila* samples were screened for *Wolbachia* using a QX100™ Droplet Digital™ PCR (ddPCR™) system (Bio-Rad Laboratories Pty., Ltd. Hercules, CA, USA) with a hydrolysis probe assay (PrimePCR™, Bio-Rad Laboratories, Inc. Cat. No. 10031261) to obtain absolute quantification of *Wolbachia* copies/µL of DNA. Primers and probe were designed from the *Wolbachia* 16S ribosomal RNA gene (GenBank accession no. AF093510) to amplify a 62 bp product (Rao et al. 2006). The assay was converted to a ddPCR™ assay by labelling the 5′ end of the probe with the fluorophore FAM (6-carboxyfluorescein) and a Black Hole Quencher (BHQ) at the 3′ end. Sequences used were forward primer, 5′-CCAGCAGCCGCGGTAAT-3′; reverse primer, 5′ CGCCCTTTACGCCCAAT-3′; probe, 5′-CGGAGAGGGCTAGCGTTATTCGGAATT3′ and were synthesized by Bio-Rad Laboratories, Inc.

Genomic DNA (2 µL) (Chelex®-extracted and not diluted or digested) was added to a PCR master mix containing 12.5 µL 2x ddPCR™ Supermix for probes (no dUTP) (Bio-Rad Laboratories, Inc. Cat. No.186-3024), 1.25 µL PrimePCR™ assay (Wol16S) and 9.25 µL PCR-grade water to a total volume of 25 µL. 20 µL of the master mix were added to a DG8™ cassette (Bio-Rad Laboratories, Inc. Cat. No. 1864008) followed by 70 µL of Droplet Generation Oil

for Probes (Bio-Rad Laboratories, Inc. Cat. No. 1863005) and droplets were generated in a QX100™ Droplet Generator (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. After generation, droplets were transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany) which was heat sealed with pierceable foil (Bio-Rad Laboratories, Inc. Cat. No. 1814040) and then subjected to PCR in a C1000 Touch™ thermal cycler (Bio-Rad Laboratories, Inc. Cat. No. 1851197). The PCR cycling conditions were: 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 60 °C for 1 min, 98 °C for 10 min with a 12 °C hold on completion. Ramp rate throughout the PCR program was 2.0 °C/s. The data were analysed using Quantasoft™ Analysis Pro Software v 1.0.

Wolbachia tree-building

To assess similarity of the *D. pseudotakahashii Wolbachia* infection to other known *Wolbachia* strains in *Drosophila* species, we built a Bayesian species tree. We obtained sequence data from *Drosophila*-infecting *Wolbachia* (which are all in *Wolbachia* supergroup A) and representatives of the other *Wolbachia* supergroups. Taxa were selected that had sequences available for the five MLST markers (Baldo et al. 2006) but not *wsp* which is known to show recombination (Baldo et al. 2005). The majority of sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank), and several unpublished sequences for *Folsomia candida* were kindly provided by Laura Baldo from the University of Barcelona and John H. Werren from the University of Rochester. These are summarised in Table S1. Sequence alignment was performed with Geneious v6.1 http://geneious.com (Kearse et al. 2012) and alignments were trimmed to remove primers and ensure consistent coverage across taxa. Nucleotide evolution and codon position partitioning models were selected for each gene region in turn by Bayesian Information Criterion (BIC) value in PartitionFinder v1.1.1 (Lanfear et al. 2012).

We performed an initial round of tree-building for each locus individually in BEAST v2.4.3. 10 M generations were run with all free priors left to vary widely. These runs were used to choose more stringent priors for the proportion of invariant sites, gamma category shape, and other nucleotide evolution model parameters (see Table S2) by examining the trace in Tracer v1.6.0 (Rambaut et al. 2014) and setting bounds to capture roughly 0.95 CI.

These estimated priors were then used in a *BEAST run (Bouckaert et al. 2014; Heled and Drummond 2010) combining all gene regions. This approach estimates a species tree along with individual gene trees for each region included. Molecular clocks and gene trees were unlinked between markers, but linked across partitions within each marker. This allowed for the possibility of horizontal gene transfer or gene conversion and avoided problems that were caused by topology differences in individual genes. Strict molecular clocks were used with gamma priors (shape = 2, scale = 2); ploidy was set to 'Y or mitochondrial' for each marker; the species tree population size was set as linear, the population mean size was estimated, and the species tree was modelled with a Yule model with birth rate distribution 1/X. All SubtreeSlide operator sizes were set to 0.02.

Five independent runs were initiated and run for 50 M generations each, sampling every 5000 generations. After confirming all effective sample size (ESS) values were >200 for the combined runs (checked in Tracer), and that independent runs converged on species tree topology (checked in Densitree v2.0), we combined the five runs with LogCombiner (Bouckaert et al. 2014), removing 20% of each run as burn-in. We then calculated the maximum clade credible tree in Treeannotator v2.4.3 (Bouckaert et al. 2014), using median branch lengths. This tree was plotted with the ape package v3.5 (Paradis et al. 2004) in R v3.3.1.

We also generated bootstrapped (1000 replications) maximum-likelihood trees using *MEGA* version 5 (Tamura et al. 2011) and default settings for individual genes as well as combined genes except for *wsp* because of reported problems with intra-genic recombination (Baldo and Werren 2007).

Phenotype characterization

The infection phenotypes were characterized in the '*Moor* +', '*Smith+', 'Town1+'* and *'Town2+'* infected *D. pseudotakahashii* lines by conducting a series of experiments investigating CI, fitness and maternal transmission of *Wolbachia*. Experiments were conducted at 19 °C with a 12:12 L:D cycle.

CI and fecundity

Incompatibility and fecundity associated with the *Moor+* line were assessed in an experiment in July 2014 in which the *Moor+* and *Moor−* lines were reciprocally crossed in addition to control crosses within lines (Cross 1. ♀- × ♂ +, 2. ♀+ × ♂-, 3. ♀+ × ♂ +, 4. ♀- × ♂-) to control for nuclear effects. Virgins were collected within 6 h of emergence and holding vials were checked for absence of progeny after 10 days to confirm virgin status. Fifteen replicates of each cross were set up when virgins were five days old; mating was observed and males were removed and stored in ethanol. Females were provided with spoons containing cornmeal media and a brush of yeast paste to encourage egg laying. Spoons were scored for egg number and replaced every 24 h for the following nine days. A duration of nine days was chosen because initial egg production was delayed, and nine days was the point at which most females

had laid sufficient eggs, but had not yet stopped producing viable eggs (males had been removed immediately after mating). Spoons were scored for fecundity immediately upon collection. Those collected from days 6 to 9 (when egg laying was more consistent) were assessed for CI after a further 24 h by scoring hatched and unhatched eggs and then transferring the spoons to 6 ml of cornmeal media to provide food for growing larvae. The egg laying rate for this species is low relative to species like *D. melanogaster*, although not necessarily other endemic species. We therefore used spoons where eight or more eggs over three days had been laid to assess CI. Progeny took approximately 18 days to develop, emerging adults were stored in 100% ethanol and sexed.

CI was also tested by crossing the infected *Town1+* and *Town2+* lines with a tetracycline cured line (*Town3-*) with the same crosses as outlined above. Prior screening indicated variable infection levels in males from these lines (see below), so all males involved in the crosses were screened. Ten to 16 replicates were set up for all crosses when males and females were 4–7 days post eclosion. Eggs were collected and scored in 24-h increments until a minimum of ten eggs had been collected or until 13 days after mating. Because of particularly low egg numbers produced in this experiment, we included all replicates with five or more eggs in analyses. Where pairs did not mate, or laid fewer than five eggs, they were excluded.

The effect of male age on CI was assessed following previous protocols for *D. simulans* (Hoffmann et al. 1994; Hoffmann et al. 1986); we crossed *Wolbachia* infected virgin males of various ages from the *Smith+* line with uninfected females from the *Moor−* line (×15 replicates). Males were 3, 5, 8, 11 and 14 days old and females were 5 days old. *Wolbachia*-infected males and females of the same ages (×3 replicates) were crossed as a control. Mating, egg collection, egg scoring and progeny collection were carried out as described previously (Hoffmann et al. 1994). Replicates that did not mate, or that laid fewer than five eggs, were excluded from further analyses.

Incompatibility relationships between lines

Given the different rates of detection of *Wolbachia* in males from the *Smith+* line compared to those from the *Moor+* line (see below), compatibility between these lines was explored in an experiment aimed at separating potential *Wolbachia* strain effects from nuclear host effects where the *Wolbachia* background came from one line and the nuclear background was 75% from one line and 25% from the other line (i.e., four combinations of background were produced, as given in Table 4). Initially, reciprocal crosses between the two lines were set up at 19 °C with 31–32 replicates per cross type (Cross1 (C1) = ♀*Moor+* × ♂*Smith+*, Cross 2 (C2) = ♀*Smith 2+* × ♂*Moor+*). Crosses consisted of pairwise matings on cornmeal media which were maintained for ten days before males and females were placed into ethanol for later PCR screening. Male progeny from these reciprocal crosses collected as virgins were backcrossed with females from the two parental lines (Backcross: 1 (B1) = ♀*Moor* + × ♂C1; 2 (B2) - ♀*Moor+* × ♂C2; 3 (B3) - ♀*Smith* + × ♂C1; 4 (B4) = ♀*Smith+* × ♂C2) to produce males with the four nuclear/*Wolbachia* background combinations. Where possible, 2–3 replicate backcrosses were set up per reciprocal cross line, however emergence times and progeny numbers meant this was not possible for all lines. Males and females used in backcrosses were retained in ethanol for screening *Wolbachia* later. To assess the extent to which CI was retained in males after backcrossing had been completed, twenty male progeny per backcross were collected. Where possible, two males were collected for a representative ten lines per backcross, however where this was not possible, single males were collected from as many lines as possible. To score CI, males were crossed 3 days after eclosion with virgin females from the uninfected *Moor* − line. Eggs were collected, and hatch rates scored for 7 days as above. Males were screened for *Wolbachia* using the *wsp* validation primers and LightCycler® assay outlined above.

Maternal transmission to adults

Wolbachia transmission in the *Moor+* and *Smith+* lines to adults of the next generation was assessed following a three-step protocol: (1) eggs were collected from an individual female from each line; (2) 19 and 28 sub-lines (*Smith* + and *Moor+* lines respectively) were created by crossing the resulting female progeny (all of which were infected) with uninfected males from the *Moor−* line; and (3) 2–3 male progeny from each sub-line were crossed with an equivalent number of uninfected females from the *Moor−* line. Individuals for steps 2 and 3 of this experiment were collected as virgins and crossed at age 6 days ♂/ 7–8 days ♀ for step 2 and 4-5 days ♂/ 6 days ♀ for step 3. Eggs were collected on spoons over 24 h and scored for hatched and unhatched eggs after a further 24 h. Progeny were collected into 100% ethanol and sexed. For analysis, we only used crosses producing 8 or more eggs. Males involved in the step 3 crosses and their mothers were screened for *Wolbachia* using the *wsp-validation* primers and LightCycler® assay outlined previously.

Statistical analysis

To compare infection frequencies among samples, we ran contingency analysis and tested significance with the likelihood ratio (G) statistic unless an Exact test could be used.

Table 1 Percentage of individuals tested that were infected with *Wolbachia* and mean *wsp* Cp value ± SD for F1/F2 males and females of *D. pseudotakahashii* compared to other *Drosophila* species

Species/infection	*N*		% *Wolbachia* infected		Mean *wsp* Cp value ± SD	
	Male	Female	Male	Female	Male[a]	Female
D. pseudotakahashii						
*w*Pse						
Northern Queensland	62	26	35.5	100	35.25 ± 2.70	27.44 ± 2.70
South eastern Queensland	122	128	91.8	100	31.69 ± 3.13	23.41 ± 1.60
New South Wales	83	24	85.5	100	32.58 ± 3.19	24.44 ± 2.47
D. melanogaster						
*w*Mel (HAW lline)	4	4	100	100	24.63 ± 0.42	23.88 ± 0.09
D. pandora						
*w*Ri	4	4	100	100	23.81 ± 0.56	24.18 ± 0.18
D. simulans						
*w*Au (Coffs Harbour line)	4	4	100	100	22.51 ± 0.49	23.29 ± 1.53
*w*Ri (Burnley line)	4	4	100	100	21.63 ± 0.15	21.55 ± 0.52

A single female for each line was tested, while multiple males were used in some cases. Lower *wsp* Cp values correlate with higher *Wolbachia* infection density

N is the number of samples tested

[a]Only infected males were used for determining Cp values using the *wsp* validation primers

Exact tests were also used to compare the incidence of infected and uninfected males that produced hatch rates of 0 or >0 in crosses. For the quantitative measures (hatch rate, fecundity) we used each pairwise cross as a replicate rather than individual eggs or offspring. While this meant that we sometimes computed hatch rates based on low replicate numbers, it was considered an appropriately conservative approach particularly given variability in infection status among the males. Data often tended to deviate from normality and we therefore compared hatch rate and fecundity across crosses with Kruskal-Wallis (non-parametric) tests. Analyses were run in IBM® SPSS® Statistics version 25.0.

Results

Wolbachia infection frequency

Females from all lines ($N = 178$) of *D. pseudotakahashii* across the regions sampled were infected with *Wolbachia* and most were of a similar infection density to other *Drosophila* species (Table 1). In contrast, many *D. pseudotakahashii* males appeared to be either infected at a lower level (indicated by high *wsp* Cp values), or uninfected altogether, despite the control gene *RpL40* amplifying strongly and consistently in these samples across the sexes in all lines (indicated by low average Cp values and narrow Cp ranges). For the Townsville lines for example, the mean Cp value for *RpL40* was 25.36 ± 0.52 in females and 26.16 ± 0.59 in males. At the F1/F2 stage, a sex difference in *Wolbachia* infection frequency was particularly evident for the lines from northern Queensland where males had a much lower infection frequency compared to the lines from south-eastern Queensland and New South Wales populations (contingency test, $G = 34.43$, $df = 2$, $P < 0.001$). When *Wolbachia* was detected in the males, the relative strength of infection (i.e., *wsp* Cp value) was similar between populations and much lower in males than females (Table 1). Assuming perfect amplification efficiency, the difference in *wsp* Cp values between males and females provides an estimate of the relative density ($R = 2^{(Cp1 - Cp2)}$, see 'methods'). Based on an average *wsp* Cp of 33.237 for males and 25.097 for females, males were estimated to have on average 0.3% of the concentration of *Wolbachia* of females.

In this experiment, high Cp values obtained using primers that amplify *wsp* (*Wolbachia* surface protein gene) are associated with a low number of copies/μL of *Wolbachia* amplified with primers that target 16S rRNA. For the nine samples screened using ddPCR™, Cp values ranged from 22 to 40 and the number of copies in the original DNA extraction ranged from 9.5 to 78,033.6 per microlitre with 95% confidence intervals from the Poisson distribution spanning 4.3 to 81,764.8 across all samples. The relationship between Cp value and copies/μL closely fits an exponential function ($y = 1E + 10e^{-0.539x}$) ($R^2 = 0.985$). Samples which had no Cp value assigned showed either no copies or a very low number per microlitre (2.9–6.2 with 95% confidence intervals spanning 0.7 to 13.5). Therefore, the use of Cp value as an indication of *Wolbachia* density is justified.

Table 2 Percent *Wolbachia* infection and mean *wsp* Cp value ± *SD* for F1 and F8 males from Townsville lines of *D. pseudotakahashii*

	F1			F8		
Line	*N*	% Infected	Mean *wsp* Cp ± *SD*	*N*	% Infected	Mean *wsp* Cp ± *SD*
Town 1+	8	0		8	100	29.83 ± 3.62
Town 2+	8	0		8	75	30.86 ± 2.68
Town 3+	8	0				
PH34	8	0				
PH29	8	25	36.39 ± 0.72			
PH41	8	25	34.28 ± 0.01			
PH36	8	37.5	37.92 ± 0.57			
KH50	8	50	32.29 ± 0.41	9	100	31.08 ± 4.01
PH26	8	62.5	36.26 ± 1.68			
PH27	8	75	32.43 ± 2.63			
PH28	8	100	36.62 ± 1.98	8	100	27.33 ± 1.41

N is number of males tested

We found that infections were more frequently detected in males after lines had been held for a time in the laboratory and *wsp* Cp values were also lower (reflecting a higher density) (Table 2). For example, *Wolbachia* was detected in the *Town2+* line from Townsville at a frequency of 75% after eight generations in the laboratory (Table 2), but was not detectable in the four males tested for this line at the F1 stage (comparison of frequency in F1 versus lab population, contingency (Exact) test, $P = 0.061$). Similarly, males collected from Nowra were initially detected as being 50% ($N = 20$) infected, but this increased to 86% ($N = 70$) in the F1 generation. This difference is significant by a contingency test ($G = 10.21$, df = 1, $P = 0.001$). Females were always detected as being 100% infected in field and laboratory samples. Regardless of time in the laboratory, the *Wolbachia* infection strength in male *D. pseudotakahashii* remained much lower than that of females. For example, after 29 generations in the lab, the mean *wsp* Cp value of 70 males tested from the three lines from Smithfield was 30.13 ($SD = 3.31$), compared to a mean *wsp* Cp value of 22.00 ($SD = 1.54$) for 97 females tested from the same lines (males again estimated to have a *Wolbachia* concentration of 0.3% that of females assuming perfect amplification efficiency).

To characterise the *Wolbachia* infection further, nucleotide sequences from multiple locations were obtained for the five MLST loci and *wsp* (Genbank accession numbers MF348256-MF348261). The *Wolbachia* sequences were identical for all source locations and the *wsp* sequence was the same as that identified as *w*Pse by Mateos et al. (2006). For this reason, only one *D. pseudotakahashii Wolbachia* representative was included in the species tree (see below). *Wolbachia* infection was also verified for samples with Cp values ranging from 24.99 to 34.14 using the *wsp_validation* primers, by sequencing the PCR products.

Wolbachia relationships

In the Bayesian species tree, the *Wolbachia* isolate from *D. pseudotakahashii* fell into Supergroup A (Fig. 1) and *D. bifasciata* was indicated as being the nearest relative. A similar result was obtained with the maximum likelihood analysis (Appendix 2) which led to the same arrangement across the supergroups. Proximity of *D. pseudotakahashii Wolbachia* to *D. bifasciata* was supported by Bayesian analysis of several individual genes including *coxA*, *fbpA*, and *hcpA* as well as *wsp* (Appendix 2), but this relationship would need to be clarified with additional genes and based ideally on the entire *Wolbachia* genome sequence. The relationships between strains evident for the individual genes in the Bayesian analyses were similar to those obtained in the maximum likelihood analyses (Appendix 2).

Phenotypic characterization

Cytoplasmic incompatibility

CI of the *Moor* lines of *D. pseudotakahashii* was assessed by crossing within and between infected and uninfected lines. Crosses differed significantly in egg hatch rate (Kruskal-Wallis test, $X^2 = 21.11$, df = 3, $P < 0.001$): only one cross between infected *Moor+* males and uninfected *Moor−* females produced any hatched eggs (11% hatching) in contrast to the expected compatible crosses, indicating high levels of CI but some heterogeneity in hatch rates (Table 3). The successful cross between infected *Moor+* males and uninfected *Moor−* females produced one male and one female offspring. Cross results also indicated high levels of CI for the *Town+* lines, despite weaker *Wolbachia* infections than in the *Moor+* strain from NSW (Table 3),

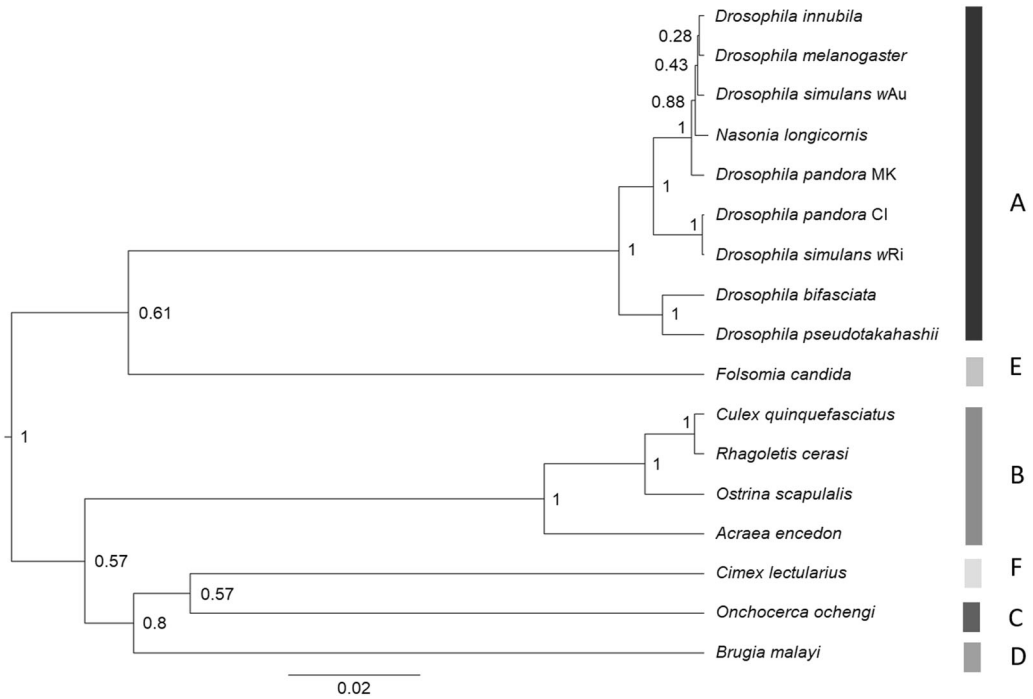

Fig. 1 Maximum clade credibility tree generated from BEAST v2.4.3 (Bouckaert et al. 2014) on concatenated gene set excluding *wsp*. Gamma time-reversible nucleotide evolution model was used with 4 gamma categories. All other priors were left as per the default settings. Ten million generations were run, sampling every 1000 generations. The maximum clade credible tree was then calculated in TreeAnnotator v2.4.3 (Bouckaert et al. 2014) after removing the first 20% of the run as burn-in, producing median node heights. The posterior probability value is shown at each node. The known supergroups are labelled (coloured bars and upper-case letters) (colour figure online)

and differences between crosses were again significant (Kruskal-Wallis test, $X^2 = 39.51$, df = 3, $P < 0.001$)

We also crossed *Moor*+ and *Smith*+ lines reciprocally and found no obvious CI between these strains (data not shown, hatch rates >80%). To investigate potential differences between these lines in the ability of males to produce CI associated with nuclear or *Wolbachia* effects that might lead to differences in Cp values and male infection incidence, we created males with different combinations of *Moor*+ and *Smith*+ *Wolbachia* and nuclear backgrounds (Table 4) and tested males for CI. The male progeny continued to show strong CI with uninfected females from the *Moor*− line regardless of their background (Table 4) and the difference between hatch rates across the four crosses was not significant (Kruskal-Wallis, $X^2 = 2.9895$, df = 3, $P = 0.394$). Note that four males were negative for the *Wolbachia* infection (Table 4), and many of the males were not strongly infected (high *wsp* Cp values). For the four crosses with apparently uninfected males, the hatch rate was zero even though the males were known to have mated. For the same two crosses where males did have detectable *Wolbachia* ($N = 32$), we had hatch rates of 0 in all but five of these. This difference in incidence between 0 hatch rates and some eggs hatching is not significantly different between males with and without detectable *Wolbachia* by an Exact test ($P = 0.645$). This indicates that CI is expressed in male offspring from infected females regardless of whether the adult males have or lack the infection at the time of crossing.

CI and male age

The effect of male age on CI was tested by crossing *Smith*+ infected males of 3, 5, 8, 11 and 14 days old with *Moor*− uninfected females and scoring egg hatch rate. Egg hatch rate increased significantly as males aged indicating that CI was decreasing with time (Kruskal-Wallis test, $X^2 = 14.99$, df = 4, $P = 0.005$); while no eggs hatched for 3-day-old males, the average hatch rate was 31% when males were 14 days old (Table 5). The *wsp* Cp value did not differ significantly between the male age groups (Kruskal-Wallis test, $X^2 = 6.61$, df = 4, $P = 0.16$)

Fecundity

Fecundity assessed for crosses between the *Moor*+ and *Moor*− lines was similar for the control and reciprocal crosses with a mean of around 25 eggs over the 9 days although the incompatible cross had a relatively low fecundity (Table 6). Egg production did not differ significantly across the crosses (Kruskal-Wallis test, $X^2 = 7.289$, df = 3, $P = 0.063$). While there was no large cost for

Table 3 Results of crosses between and within a) *Moor+* and *Moor−* lines and b) *Town+* and *Town−* lines

Cross	*N*	Mean % hatch	Range % hatch	Range of egg # scored	% Male Progeny ± *SD*	Male parent *wsp* Cp ± *SD*
(a) *Moor* line crosses						
♀- × ♂+	8	3.5	0.0–11.1	8–19	50 ($N = 1$)	28.97 ± 2.79
♀+ × ♂-	11	87.2	70.0–100	8–32	49.0 ± 21.0	
♀+ × ♂+	10	95.9	70.0–100	8–28	42.6 ± 17.4	28.36 ± 1.84
♀- × ♂-	12	65.2	43.7–100	9–25	64.6 ± 21.8	
(b) *Town* line crosses						
♀- × ♂+	19	8.6	0.0–36.6	5–42	41 ($N = 1$)	32.95 ± 2.91
♀+ × ♂-	20	91.1	50.0–100	5–44	44.9 ± 15.3	
♀+ × ♂+	19	86.5	9.7–100	5–35	43.7 ± 18.6	34.28 ± 3.11
♀- × ♂-	5	98.1	95.4–100	22–31	43.0 ± 28.3	

N is the number of replicates after excluding those that did not mate

infected females, the low and variable egg production of *D. pseudotakahashii* mean that there is not a lot of power to detect effects on fecundity. For instance, with a mean of 24 eggs and standard deviation of 10, it is only possible to detect a difference of 11 eggs between two groups with 80% power given a sample size of 13.

Maternal transmission

We assessed maternal transmission of *Wolbachia* in *D. pseudotakahashii* from mothers to adult daughters by testing the infection status of daughters in the 19 *Smith+* and 28 *Moor+* lines set up in this experiment. All 42 of the 47 daughters able to be tested were infected with *Wolbachia* (mean *wsp* Cp value = 23.58 ± 0.56), suggesting 100% maternal transmission (binomial confidence intervals, 92.4, 100%).

We assessed maternal transmission of *Wolbachia* to adult sons phenotypically by generating 1–3 sublines from an individual female, crossing their male progeny with uninfected females and assessing the hatch rates of their eggs. We also assessed maternal transmission at a molecular level by screening adult males from crosses using the *wsp_validation* primers. Uninfected males and high hatch rates were expected where adult transmission was imperfect, and infected males and low hatch rates were expected in most crosses when maternal transmission was high, consistent with the high CI that occurs when an infected male mates with an uninfected female.

Phenotypic assessment suggested that maternal transmission was high and near 100% for the *Moor+* and *Smith+* lines. Of 70 crosses involving the *Moor+* line (26 sublines × 2–3 replicates), only five replicates had any hatched eggs (3.7,10% hatch), to produce an overall hatch rate of 0.25% (95% confidence interval 0.05, 0.73%). Of 37 crosses involving the *Smith+* line (17 sub-lines × 1–3 replicates), 12 replicates had hatched eggs (4.5, 63.6% hatch) with an overall hatch rate of 8.3% (95% confidence intervals, 6.0, 10.4%). These results are summarized in Fig. 2 which highlights the marked difference in distribution of hatch rates in crosses involving the two strains.

Molecular screening suggested a more moderate maternal transmission of *Wolbachia* to adult males of 88% (95% confidence intervals 81.1, 95.6%) for the *Moor+* males and 59% (confidence intervals 39.1, 75.5%) for the *Smith+* males, despite 100% maternal infection. Most crosses with hatched eggs were associated with uninfected males (Fig. 2) suggesting that imperfect maternal transmission to adults was involved (we found only three infected males of the 16 involved in crosses in which any progeny emerged, and those were infected at a low level with *wsp* Cp values ranging from 33.45–40). Males with moderate to weak infections (suggested by Cp values) appeared to produce mixed results with complete CI observed for some individuals, but incomplete CI for others. Despite being uninfected, six males were associated with 0% hatch rates, consistent with our earlier observations. Four of these were from the *Moor+* line, with three apparently uninfected males from this line that had hatch rates >0; this differed (Exact test, $P = 0.006$) from the 59 infected males producing 0 hatch rates compared to two infected males with hatched eggs. The equivalent numbers for *Smith+* were two uninfected males producing no hatched eggs (versus 10 with hatched eggs) and comparable figures of 18 (2) for infected males (Exact test, $P < 0.001$). Note that control crosses with Moor− females run around this time (and throughout the experimental period) always led to vials with hatched eggs. The *RpL40* housekeeping gene indicated that the extractions were sound, so we attribute these results to two possible causes: either *Wolbachia* infection is present but below the detection limit of our current methods; or CI is induced early in male development, prior to mating, and the *Wolbachia* infection is lost from adult males.

Table 4 Average % hatch for CI crosses involving males with different combinations of *Smith* and *Moor* genetic backgrounds and different *Wolbachia* backgrounds from the maternal line

Wolbachia from maternal line	Male genetic background	*N*	Average % hatch	Range % hatch	Mean *wsp* Cp ± SD of infected males	% Males uninfected
Moor	75%*Moor*, 25%*Smith*	17	0	0	30.70 ± 2.00	0
Smith	75%*Moor*, 25%*Smith*	18	0.8	0–9.1	29.12 ± 1.83	5.6
Moor	25%*Moor*, 75%*Smith*	19	1.4	0–22.2	30.30 ± 1.90	0
Smith	25%*Moor*, 75%*Smith*	18	0.7	0–5.7	31.98 ± 2.18	16.7

wsp Cp values and infection levels relate to males involved in the CI crosses

N is the number of replicates after excluding those with fewer than eight eggs

Table 5 Results of crosses between *Smith*+ males and *Moor*− females showing effect of male age on average % egg hatch

Male age (days)	*N*	Average % hatch	Range % hatch	Male parent *wsp* Cp ± *SD*
3	8	0	0	31.80 ± 1.97
5	8	14	0–50	31.93 ± 3.03
8	12	10.7	0–42.9	30.24 ± 4.45
11	6	15.1	0–50	31.39 ± 2.12
14	7	30.7	4.8–64.7	29.47 ± 2.15

Mean male parent *wsp* Cp values are also reported

N is the number of replicates after excluding those which did not mate, or which had fewer than five eggs. Male *wsp* Cp values were based on all males crossed, regardless of hatch rates or mating

Table 6 Fecundity of *D. pseudotakahashii* crosses between '*Moor*+' and '*Moor*−' lines scored over nine days

Cross	*N*	Mean Fecundity ± *SD*
♀- × ♂+	11	15.64 ± 12.19
♀+ × ♂-	15	25.87 ± 10.20
♀+ × ♂+	10	24.50 ± 8.11
♀- × ♂-	15	24.93 ± 11.84

N is the number of replicates after excluding those which did not mate

Discussion

The infection we describe here is the first example of a *Wolbachia* strain in *Drosophila* that has become partly sex-limited. We found that the infection appears to be at a high frequency in field females. However, when flies were tested after being cultured for one or two generations under laboratory conditions, the infection was often not detectable in adult males based on PCR and RT-PCR assays. Where it was detected, the densities of *Wolbachia* in adult males were low. We also showed that transmission appears near-perfect to female offspring, whereas transmission to adult male offspring is variable. These effects depend to some extent on the strain of *Drosophila* used in crosses. The results indicate that either transmission to male embryos is low or male embryo and/or immature development leads to a loss of *Wolbachia*.

The mechanism behind this difference in *Wolbachia* density and transmission between the sexes is unclear. *Wolbachia* density is thought to be controlled partly by the *Wolbachia* genome and partly by the host nuclear genome (Boyle et al. 1993; Mouton et al. 2003; Kondo et al. 2005), and density (as well as tissue tropism) is thought to, in turn, control some phenotypes affected by *Wolbachia*. These phenotypes include virus blocking (Teixeira et al. 2008; Martinez et al. 2014) and the magnitude of deleterious fitness effects (Hoffmann et al. 2015). For strains that cause CI, there is often an association between density of *Wolbachia* in a strain and the level of CI (Bourtzis et al. 1996). Consistent with this pattern, we found that CI was lower on average when males lacked detectable *Wolbachia*. However, in the case of *D. pseudotakahashii* and unlike in previous work, adult male offspring lacking detectable *Wolbachia* are still capable of inducing strong CI when mated with uninfected females. When exactly in their lifetime male hosts lose detectable infection levels needs further investigation but may provide insights into mechanisms underlying CI in this system.

Under assumptions of no fitness costs in females and 100% maternal transmission to daughters, with strong CI despite the loss of infection in males, we anticipate that this *Wolbachia* infection should spread to fixation in natural populations of *D. pseudotakahashii*. This hypothesis is consistent with the limited data we have collected so far, but more information is needed, particularly under different environmental conditions known to influence *Wolbachia* fitness effects on hosts including heat stress (Ross et al. 2017). The decrease in CI in older males and increased detection of infected males in established laboratory stocks also seen in other *Drosophila-Wolbachia* systems (Hoffmann et al. 1986) suggests that this infection and its phenotypic effects are likely influenced by a range of internal and external factors. *Drosophila pseudotakahashii* is a tropical/subtropical species which is attracted to fruit bait, but its ecology is not well understood (Bock 1976) and it will be critical to expand information about this species

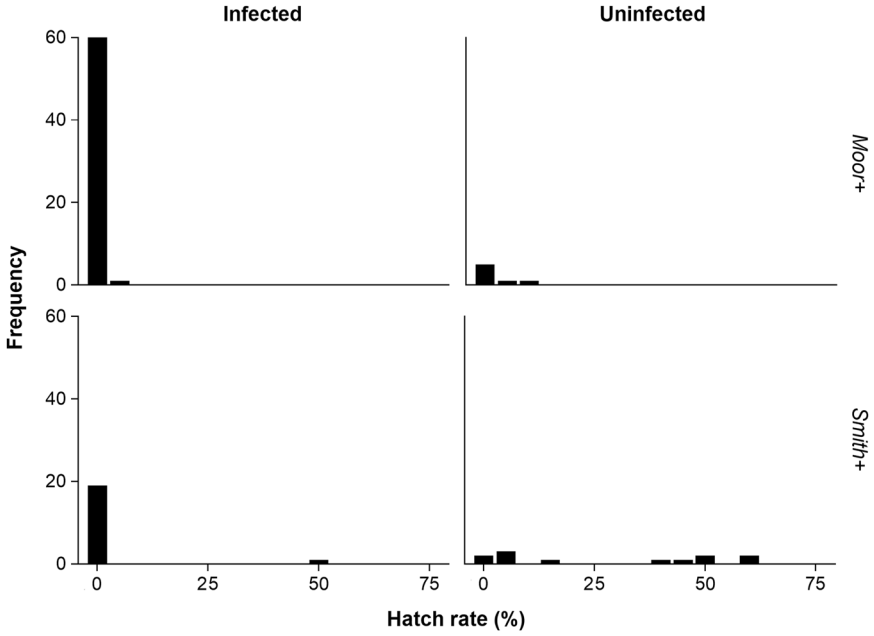

Fig. 2 Maternal transmission of *Wolbachia* effects. Histograms showing number of males from two strains (*Moor*+, *Smith*+) producing different hatch rates when crossed with uninfected females. All males were taken from lines where females were infected, however some adult males screened as uninfected and these are presented separately

within its natural environment to understand *Wolbachia* strain dynamics.

The *Wolbachia* strain we have isolated falls within a clade that includes *D. bifasciata*, an infection that is associated with stable and non-reversible male killing (Longdon et al. 2012) whereas for the *D. pseudotakahashii* infection, there is no evidence of an effect on sex ratio. Related infections in this clade (and others in the *takahashii* group) have been detected with molecular probes (Mateos et al. 2006) but have yet to be investigated; these infections could provide an opportunity for using comparative genomics to investigate the molecular basis of *Wolbachia*-induced phenotypes. Such comparisons have recently shown that loci associated with CI (Bonneau et al. 2018; LePage et al. 2017) are likely to be under strong selection in *w*Ri *Wolbachia* infections that have spread rapidly across species and resulted in divergent (CI and non-CI) phenotypes (Turelli et al. 2018).

The presence of CI in males with low *Wolbachia* load raises issues around the mechanisms responsible for CI. Recent data from *Drosophila* and mosquitoes point to CI being associated with a toxin-antitoxin mechanism encoded by prophage genes of *Wolbachia* (Beckmann et al. 2017; Bonneau et al. 2018; LePage et al. 2017) with two genes inducing CI and one of these in the maternal lineage rescuing CI (Shropshire et al. 2018). Supporting evidence includes the fact that expression of these genes changes with male age in a way that is correlated with changes in CI with male age (LePage et al. 2017). In *D. pseudotakahashii* data we also see a decrease in CI with male age, but *Wolbachia* are absent or at a very low density in adults. If prophage genes are involved in CI in *D. pseudotakahashii*, perhaps the prophage persist independently of *Wolbachia* in males, and/or there is maternal transmission of CI effectors. On the other hand, CI in this system could also be associated with a completely different mechanism given that multiple factors can influence CI in species (Ju et al. 2017).

Although the *w*Pse infection is currently at a high frequency in populations, partial loss from males may affect the dynamics of CI in populations and even equilibrium frequencies of *Wolbachia*. If male fitness is compromised by *Wolbachia*, there may be an ongoing shift towards loss of the infection in male offspring and an associated reduction in CI. This would make it harder for such *Wolbachia* to invade new uninfected populations. *Wolbachia*-based selection normally acts on females rather than males (Frank 1997) and reproductive effects mediated through males might be incidental to other effects of *Wolbachia* and female transmission fidelity which is expected to be under strong selection (Turelli 1994). Nevertheless, infections such as *wPse* may be of interest from an applied perspective. Females maintain a high density of the infection and may block virus transmission after transfer to novel hosts. While any loss of CI due to low densities of the infection in males may decrease the rate at which the infection invades populations (Hoffmann et al. 1990), invasion is still likely given that substantial CI seems to persist even when the infection is at a low density. Moreover, male fitness costs may be low for *w*Pse, increasing their competitiveness with uninfected males from natural populations. Of course, it is unclear whether the sex-specific densities will be maintained when *w*Pse is transferred to other species, which will depend on whether the phenotype described here has evolved as a consequence of genomic changes in *Wolbachia* or in the host genome.

Data Archiving

Sequences have been deposited in GenBank; accession numbers MF348256 - MF348261. Experimental data are available via Figshare at https://doi.org/10.26188/5b5a630cce4ba

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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