Heredity (2008), 1–10

www.nature.com/hdy

ORIGINAL ARTICLE

Wolbachia plays no role in the one-way reproductive incompatibility between the hybridizing field crickets *Gryllus firmus* and *G. pennsylvanicus*

LS Maroja[1], ME Clark[2] and RG Harrison[1]
[1]*Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, USA and* [2]*Department of Biology, University of Rochester, Rochester, NY, USA*

Wolbachia are cytoplasmically inherited α-proteobacteria that can cause cytoplasmic incompatibility (CI) in insects. This incompatibility between sperm and egg is evident when uninfected females mate with infected males. *Wolbachia*-driven reproductive incompatibilities are of special interest because they may play a role in speciation. However, the presence of *Wolbachia* does not always imply incompatibility. The field crickets *Gryllus firmus* and *G. pennsylvanicus* exhibit a very clear unidirectional incompatibility and have been cited as a possible example of *Wolbachia*-induced CI. Here, we conduct curing experiments, intra- and interspecific crosses, cytological examination of *Wolbachia* in testes and *Wolbachia* quantifications through real-time PCR. All of our data strongly suggest that *Wolbachia* are not involved in the reproductive incompatibility between *G. firmus* and *G. pennsylvanicus*.
Heredity advance online publiction, 6 August 2008; doi:10.1038/hdy.2008.75

Keywords: real-time PCR; reproductive isolation; cytoplasmic incompatibility; antibiotic treatment

Introduction

Wolbachia are cytoplasmically inherited α-proteobacteria that can infect reproductive tissues of insects and cause reproductive alterations, including parthenogenesis (Stouthamer *et al*., 1993), feminization of males (Rousset *et al*., 1992), male killing (Hurst *et al*., 1999) and cytoplasmic incompatibility (CI) (Breeuwer *et al*., 1992; O'Neill *et al*., 1992). Surveys have found that 16–76% of insects sampled are infected with *Wolbachia* (Werren *et al*., 1995; West *et al*., 1998; Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000; Hilgenboecker *et al*., 2008). A recent meta-analysis adjusting for infection frequency within species predicts that around 66% of insects are infected with *Wolbachia* (Hilgenboecker *et al*., 2008). CI is a sperm–egg incompatibility, manifested when uninfected females mate with infected males. Shortly after fertilization, asynchrony in male and female pronuclei development leads to a series of mitotic defects and ultimately to embryonic death (Yen and Barr, 1971; Callaini *et al*., 1996; Lassy and Karr, 1996; Stouthamer *et al*., 1999; Tram and Sullivan, 2002). Although the molecular mechanism of CI is still poorly understood, it appears that *Wolbachia* present inside the testes 'modify' the sperm, which must then be 'rescued' in the egg by the same *Wolbachia* strain, if successful embryonic development is to occur (Werren, 1997a). *Wolbachia* strains can be classified based on their ability to modify sperm (mod$^+$ or mod$^-$) and rescue in eggs (resc$^+$ and resc$^-$) (Werren, 1997a).

The effect of CI is unidirectional (usually involving crosses between infected and uninfected individuals) or bidirectional (involving crosses between individuals with different *Wolbachia* types) (Barr, 1980; Breeuwer and Werren, 1990; O'Neill and Karr, 1990). *Wolbachia*-driven reproductive incompatibilities are of special interest because they may play a role in speciation by facilitating the evolution of reproductive isolation between incipient species (Werren, 1997b; Telschow *et al*., 2005a, b; Jaenike *et al*., 2006).

The presence of *Wolbachia* does not always imply incompatibility. *Wolbachia* infections exist with no obvious phenotypic effects (Hoffmann *et al*., 1996). Even in cases where incompatibility is observed, *Wolbachia* may not be the causal agent (Weeks *et al*., 2002). Nuclear genes can also be involved, and in some cases both nuclear-induced and *Wolbachia*-induced incompatibilities are known to play a role (Breeuwer and Werren, 1995; Navajas *et al*., 2000; Vala *et al*., 2000). Proving that *Wolbachia* is a causal agent requires curing experiments (treatment with antibiotics) and a rigorous series of crosses between infected and uninfected individuals with the same genetic background.

The hybridizing field crickets *Gryllus firmus* and *G. pennsylvanicus* exhibit a one-way reproductive incompatibility; female *G. firmus* produce no progeny when mated with male *G. pennsylvanicus*, but the reciprocal cross produces viable and fertile offspring (Harrison,

Correspondence: Dr RG Harrison, Department of Ecology and Evolutionary Biology, Cornell University, Corson Hall, Ithaca, NY 14853, USA.
E-mail: rgh4@cornell.edu
Received 19 April 2008; revised 24 June 2008; accepted 5 July 2008

1983). The bimodal mosaic hybrid zone formed by these very closely related species is a well-studied model system in speciation research (Harrison, 1983, 1985; Harrison and Rand, 1989; Willett *et al*., 1997; Ross and Harrison, 2002, 2006). Understanding the causes of the one-way incompatibility between *G. firmus* and *G. pennsylvanicus* is important for understanding the evolution of barriers to gene exchange.

Because *Gryllus* species harbor *Wolbachia*, it has been proposed that the bacterial infections are the cause of the one-way reproductive incompatibility (Giordano *et al*., 1997). Giordano *et al*. (1997) argued that *G. pennsylvanicus* was infected, whereas *G. firmus* was not, consistent with the pattern expected for *Wolbachia*-induced CI. However, due to incorrect assignment of crickets to species and lack of a perfect correlation between species and infection status, this conclusion was later rejected (Mandel *et al*., 2001). Mandel *et al*. (2001) showed that many *G. firmus* are infected, harboring what they termed the *w*G2 *Wolbachia* strain. Most *G. pennsylvanicus* harbor the *w*G1 strain, but some individuals were doubly infected (*w*G1 and *w*G2), and a few carried only the *w*G2 strain. Extrapolating from these results, Mandel *et al*. (2001) suggested that about 13% of the heterospecific crosses should produce offspring; yet in dozens of crosses observed, not a single one yielded any progeny (Harrison, 1983; RG Harrison, unpublished data). They concluded that *Wolbachia* is unlikely to play a role in the one-way reproductive incompatibility between *G. firmus* and *G. pennsylvanicus*.

Here, we present additional data that argue against a role for *Wolbachia* in reproductive isolation between the cricket species. We conduct curing experiments and intra- and interspecific crosses, use microscopy to examine the presence/absence of *Wolbachia* in reproductive tissues and quantify *Wolbachia* loads in *G. firmus* and *G. pennsylvanicus* using real-time PCR. If *Wolbachia* is responsible for the observed reproductive incompatibility ('the *Wolbachia* hypothesis'), then uninfected (cured) male *G. pennsylvanicus* should be able to sire hybrid progeny when mated with *G. firmus* females. Furthermore, under the '*Wolbachia* hypothesis,' *Wolbachia* should be present somewhere in testes (Boyle *et al*., 1993; Bressac and Rousset, 1993; Giordano *et al*., 1995; Bourtzis *et al*., 1996; Poinsot *et al*., 1998; Clark *et al*., 2002, 2003, 2008; Riparbelli *et al*., 2007), and hybrid eggs should be fertilized but later fail to develop (Callaini *et al*., 1996, 1997; Lassy and Karr, 1996; Tram and Sullivan, 2002; Duron and Weill, 2006). None of these predictions were supported, and we thus reiterate Mandel's *et al*. (2001) conclusion that *Wolbachia* infections are not the cause of the one-way reproductive incompatibility between *G. firmus* and *G. pennsylvanicus*.

Materials and methods

Cricket rearing

We collected late instar *G. firmus* nymphs in Guilford, CT, USA (41°15′; −72°42′) and *G. pennsylvanicus* nymphs in Ithaca, NY, USA (42°24′; −76°31′). Both species were collected during August–September 2004. We sorted the crickets by species and maintained five plastic cages (30 cm × 16 cm × 9 cm) for each species. Each cage contained five males and five females (total of 50 crickets for each species). Crickets were provided with *ad libitum* food (Purina Cat Chow, Nestle Purina, St Louis, MO, USA), a water vial, cardboard for shelter and a Petri dish of sterilized soil as oviposition substrate. The cages were kept at 25 °C, 12 h–12 h light–dark. Oviposition dishes containing eggs were incubated for a maximum of 40 days at 25 °C and then placed in a refrigerator at 4 °C for 102 days to insure synchronous hatch of nymphs (Harrison, 1985).

Hatching started on 15 February 2005, 21 days after eggs were removed from the refrigerator. We divided the offspring from each species into two groups: an antibiotic feeding treatment and an untreated control. Crickets from each group/species were reared in a separate plastic group cage (65 cm × 45 cm × 40 cm) with *ad libitum* food (Purina Cat Chow) and oviposition dishes. The antibiotic treatment group received 0.25% tetracycline HCl (Sigma, St Louis, MO, USA) in the water (changed three times per week) throughout development, whereas the untreated group received pure water. Individuals within each group were allowed to mate freely. Oviposition dishes were treated as described above and hatching began on 2 January 2006. Treatment was continued as in the previous generation. In March 2006, virgin late instar crickets were separated to single-sex plastic cages (30 cm × 16 cm × 9 cm), receiving the same treatment as before, until assigned to an experimental cross.

Experimental crosses

Our experimental design for crosses was the most complete possible given the poor performance of our *G. pennsylvanicus* colonies (only four untreated and six treated males were ultimately available for crossing). Each *G. pennsylvanicus* male was therefore mated with multiple *G. firmus* females. To discover whether *Wolbachia* causes intraspecific CI in *G. firmus*, we also conducted crosses between treated and untreated *G. firmus*.

We abbreviate cross types using three letters (for example, H/tu): The first letter indicates whether the cross is heterospecific or conspecific (H or C), the second letter indicates the male group (treated, t or untreated, u) and the third letter indicates the female group (t or u). Experimental females are always *G. firmus*, thus an H/tt cross involves a treated *G. pennsylvanicus* male and a treated *G. firmus* female, whereas a C/tt cross involves a treated *G. firmus* male and female.

For the heterospecific crosses (H), a treated (t) or untreated (u) *G. pennsylvanicus* male was put with two treated (t) or untreated (u) *G. firmus* females for 3 days. Females were then removed to individual plastic cages and provided with *ad libitum* food, water and an oviposition dish. The male was placed in an individual cage and reused in subsequent matings. Twenty-six *G. firmus* females were crossed to six treated *G. pennsylvanicus* males: eight females were treated (H/tt) and 18 females were untreated (H/tu). Twenty-six *G. firmus* females were crossed with four untreated males: 10 females were treated (H/ut) and 16 females were untreated (H/uu). All crosses resulted in spermatophore transfer to the female.

For the conspecific (C) crosses, a treated (t) or untreated (u) *G. firmus* male was paired with a treated (t) or untreated (u) *G. firmus* female for 3 days. The male

was then frozen at −80 °C for DNA extraction, and the female was placed in an individual plastic cage as described above. We performed 32 crosses using 16 treated and 16 untreated males: 5 males of each group mated with a treated female (C/tt and C/ut) and 11 males of each group mated with an untreated female (C/tu and C/uu). Again, all females were observed with a spermatophore.

All females used in the crosses were 7–10 days old. Female post-mating lifespan was estimated as days from mating until death. Lifetime fecundity was assessed by counting all eggs laid by each female. Eggs were separated from the oviposition substrate using a series of sieves and counted under a stereoscopic microscope.

Fecundity (number of eggs) and fertility (proportion of eggs hatching) for conspecific and heterospecific crosses were analyzed separately. Female lifespan data were analyzed combining data from conspecific and heterospecific crosses.

Data on conspecific fertility were fitted to a general linear model (GLM), weighting for fecundity. Conspecific fecundity and female lifespan data were fitted to a GLM with negative binomial errors. Residuals of all the performed GLMs were analyzed by visual inspection and no significant deviations from normality were observed. No outliers were found using Cook's statistics values. The effects of all dependent variables on the response variables were tested using log-likelihood ratio tests comparing the deviance of a model including and excluding the factor being tested. All analyses were performed with R. 2.6.1 (R Development Core Team, 2006). All results are shown as mean ± s.d., *n*.

Real-time PCR

To determine the *Wolbachia* load of each cricket, we used TaqMan real-time PCR. This approach measures the relative number of template molecules for a *Wolbachia*-specific gene compared with template number for a cricket-specific gene (elongation factor, EF1α). We extracted DNA from whole abdomen of treated and untreated crickets using DNeasy tissue kit (Qiagen, Valencia, CA, USA). All DNA extractions were diluted to 10 μg/μl.

Primers and TaqMan fluorescence-labeled probes for real-time PCR assays were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA). These primers specifically amplify *Gryllus* EF1α (GenBank accession numbers, DQ630925 and DQ630927) and *Wolbachia* ftsZ (GenBank accession numbers, U28195 and U83100, which correspond to the two types of *Wolbachia* found in *G. firmus* and *G. pennsylvanicus*; see Mandel *et al*. 2001). Primers and probes are shown in Table 1.

For real-time PCR assays, 2 μl of the extracted genomic DNA template (20 μg) was combined with 900 μM of each oligonucleotide primer and 250 μM of the TET-(EF1α) or 6FAM-(ftsZ) and TAMRA-labeled probe in 25 μl of total reaction volume using TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan PCR reactions were mixed in 96-well MicroAmp optical plates (Applied Biosystems). The PCR samples were subjected to 45 cycles of amplification in an ABI 7500 real-time PCR System (Applied Biosystems) under the following conditions: 50 °C for 2 min (uracil *N*-deglycosylase digest), 95 °C for 10 min (AmpliTaq Gold pre-activation) and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence data were analyzed using the Applied Biosystems software. The standard curve was always prepared using a dilution series (up to 10^{-5}) for the same DNA sample; water (in place of DNA) was used as blank. Each individual sample was assayed at least twice, but because differences in DNA concentration between replicates were always $<0.01\times$ (after standardization—see below), we report only the average value for each individual.

Our real-time DNA measurements are relative not absolute; therefore, *Wolbachia* loads reported here are only meaningful for samples in this study. To calculate relative values, we assigned an arbitrary DNA quantity for each of the dilutions in the standard curve, from 10^5 in the $1\times$ dilution to 1 in the $10^5\times$ dilution. On the basis of the C_t (threshold cycle) of each sample and the standard curve, we obtained estimates of EF1α and ftsZ amounts for each individual. We then divided the amount of ftsZ DNA by the amount of EF1α DNA, to generate a normalized value. Finally, a randomly selected untreated individual was chosen to represent a standard $1\times$ *Wolbachia* load, and all other values were adjusted in relation to this standard.

We quantified *Wolbachia* load from a total of 53 crickets. These included parents from all conspecific crosses that failed to produce offspring (C/tt, $n=3$; C/tu, $n=3$; C/ut, $n=2$) as well as from all crosses that produced offspring but were not expected to do so under the hypothesis of *Wolbachia*-induced CI (untreated male and treated female, C/ut, $n=3$). In addition, we quantified all *G. pennsylvanicus* males ($n=5$ for treated and $n=4$ for untreated) and *G. pennsylvanicus* females ($n=3$ for treated and $n=3$ for untreated). We also included the following randomly chosen individuals: five *G. firmus* treated males, five *G. firmus* untreated

Table 1 Primers and probes for TaqMan real-time PCR for *Gryllus* and *Wolbachia* genes

Species	*Primer or probe*	*Sequence (5′–3′)*	*Position*
Gryllus…	EF1α_F	CTGACCTCCGCAGCAACA	569–587
	EF1α_R	TTGCCAGTGGTCGAACACA	612–630
	EF1α probe (TET)	TGGCCAGGCATTCCCTCAGT	591–610
Wolbachia…	ftsZ_F	TGAAGAAGTGGATGAAAATGCAAA	729–752
	ftsZ_R	GCCAGTTGCAAGAACAGAAACTC	800–822
	ftsZ probe (6FAM)	ACTTTTGATCAGGCGATGGAGGGAAGA	769–795

The nucleotide positions are those reported in GenBank (accession number DQ630925 for EF1α and U28195 for ftsZ).

males, five *G. firmus* treated females and five *G. firmus* untreated females. *Wolbachia* loads in one male from an infertile cross (C/tu) and two males from C/ut crosses, as well as one male *G. pennsylvanicus*, could not be measured due to poor quality or unavailable DNA.

We assayed an additional 10 wild-caught *G. pennsylvanicus* individuals from Ithaca, NY, USA (five males and five females) to compare *Wolbachia* loads of captive and wild individuals. These crickets were captured as late instar nymphs in August 2007.

To test for differences in *Wolbachia* loads between treatments, sexes and species, we fitted our data to GLMs with γ errors using R. 2.6.1 (R Development Core Team, 2006) as explained above (experimental crosses section).

RFLP analysis

Mandel *et al*. (2001) reported two different *Wolbachia* strains, *w*G1 (accession number U83100) and *w*G2 (accession number U28195), common to *G. firmus* and *G. pennsylvanicus*. We used an RFLP analysis to determine the strain of *Wolbachia* for each individual assayed with RT-PCR. Universal primers to both strains, ftsZ12F (5′-AAAAATTCAACTTGGTATCAA-3′) and ftsZ812R (5′-AGAACAGAAACTCTAACTCTTCC-3′), were used to amplify a short fragment of *Wolbachia*-specific ftsZ. These amplifications were carried out in 10 µl PCR reactions and contained 3 mM $MgCl_2$, 0.2 mM dNTPs, 50 mM KCl, 20 mM Tris (pH 8.4), 2.5 µg of each primer, 1 U of platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 1 µl DNA (20–30 µg). Conditions for thermal cycling were 2 min at 95 °C followed by 35 cycles of 50 s at 94 °C, 60 s at 55 °C and 90 s at 72 °C. The resulting 800-bp ftsZ fragment is differentially cut by the restriction enzyme *Alu*I. We fully digested 3–5 µl of PCR product with *Alu*I and ran the digests on 2% agarose gels, using a doubly infected individual as a control on each gel. We could unambiguously assign *Wolbachia* strain(s) for all assayed infected individuals.

Microscopy

Gryllus testes and ovaries were dissected from adults in a small Petri dish with Tris-buffered saline Tween-20 (TBST) (50 mM Tris, 150 mM NaCl, 0.1% Tween, 0.05% NaN_3, pH 7.5). Whole testes and ovaries were removed and transferred to a depression slide with TBST. Individual follicles were removed from testes, and ovarioles were separated and transferred to 3.7% formaldehyde in TBST for 15–30 min followed by three washes in TBST. Tissues were blocked in TBST with 1% bovine serum albumin for 10 min. *Wolbachia* was visualized using an anti-human hsp60 mouse monoclonal antibody (Sigma), which recognizes *Wolbachia* (Hoerauf *et al*., 2000; McGraw *et al*., 2002). Tissues were incubated in the primary antibody solution (1:500 in TBST, 1% bovine serum albumin, 2 mg/ml RNaseA and 1:500 of the anti-wsp antibody) for 1 h at room temperature, followed by three washes with TBST. This was followed by 1 h at room temperature in 1:500 Alexa-Fluor 488 anti-mouse antibody (Molecular Probes), followed by three washes in TBST. DNA was then stained with either 1 µg/ml DAPI (Invitrogen) for 5 min or with 5 µg/ml propidium iodide (Invitrogen) for 20 min, followed by a brief wash in TBST before mounting in ProLong Gold antifade mounting media (Invitrogen). Images were obtained using a Zeiss Axio-Imager Z1 microscope. We observed both captive and wild-caught untreated crickets as well as first and second generation antibiotic-treated crickets.

Egg analysis

Anecdotal evidence indicates that eggs from hybrid crosses (*G. firmus* female × *G. pennsylvanicus*) are smaller and resemble unfertilized eggs. By measuring eggs to the nearest 0.01 mm under a dissecting scope (× 15 magnification), we obtained data on length and width of 10 unfertilized *G. firmus* eggs (virgin females), 10 eggs from crosses within *G. firmus* and 10 eggs from heterospecific crosses. All eggs were from untreated individuals and were measured during the diapause period (after 1 month at 4 °C).

We also extracted DNA from individual eggs (six unfertilized, six pure *G. firmus* eggs and six eggs from crosses between *G. firmus* females and *G. pennsylvanicus* males) using a forensic QIAamp DNA micro kit (final dilution in 20 µl). All eggs were from untreated females, and DNA was extracted when eggs were in diapause (after 1 month at 4 °C). After estimating the amount of DNA in each sample, we used cricket-specific microsatellites to test for the presence of maternal and paternal alleles. We also used *Wolbachia* ftsZ primers and cricket-specific EF1α primers (as a positive control). PCRs (10 µl volume) contained 3 mM $MgCl_2$, 0.2 mM dNTPs, 50 mM KCl, 20 mM Tris (pH 8.4), 2.5 µg of each primer, 1 U of platinum *Taq* DNA polymerase (Invitrogen) and 2 µl DNA (20–30 µg). PCR amplifications were performed using a thermal cycler (OmniGene, Hybaid) under the following conditions: 40 cycles of 50 s at 94 °C, 60 s at 52 °C and 90 s at 72 °C.

Results

Experimental crosses

In heterospecific crosses (*G. pennsylvanicus* male × *G. firmus* female), antibiotic treatment had no effect on whether offspring were produced. All of the heterospecific crosses, except for one, failed to produce offspring, including those expected to produce offspring under the assumption that CI-inducing *Wolbachia* had been eliminated (Table 2). The single hybrid produced (the first ever reported in thousands of hybrid cross observations) came from an H/uu cross, in which both parents were infected as shown by RT-PCR analysis (see below). The fecundity of heterospecific crosses was low (67.9 ± 92.2 eggs, $n = 52$).

On average, conspecific crosses had higher fecundity (373 ± 212 eggs, $n = 32$) and fertility (68.9 ± 67.9 offspring, $n = 32$) than heterospecific crosses ($F(1, 84) = 76$, $P = 0.0001$ and $F(1, 84) = 51.6$, $P < 0.0001$, respectively), although some females from conspecific crosses (primarily from the treated group) failed to produce any offspring (Table 2). The conspecific crosses showed no significant male treatment × female treatment interaction in fecundity, and female treatment alone had a marginally nonsignificant effect ($F(29, 1) = 69$, $P = 0.09$)—treated females appear to deposit fewer eggs than untreated females (272 ± 244 eggs, $n = 10$ vs 419 ± 183 eggs, $n = 22$, respectively). Antibiotic treatment had no significant effect on the percentage of eggs hatching. If *Wolbachia*

Table 2 Results of experimental crosses

	Cross type	*Fertility exp?*	*Mean eggs*	*s.d. eggs*	*Mean offsprings*	*s.d. offsprings*	(n) *total*	(n) *fecund*	(n) *fertile*
Conspecific	C/uu	Yes	468.8	159.2	80.2	56.4	11	11	11
	C/tu	Yes	369.4	199.1	69.4	76.5	11	11	8
	C/tt	Yes	293.2	319.1	42.6	58.4	5	5	2
	C/ut	No	250.2	176.4	69.2	92.6	5	5	3
Heterospecific	H/uu	No	86.2	115.2	0.06[a]	0.25	16	15	1
	H/tu	Yes	64.4	78.1	0	0	18	16	0
	H/tt	Yes	66.5	77.3	0	0	8	8	0
	H/ut	No	46.4	93.5	0	0	10	8	0

[a]One hybrid offspring was produced in this cross type. Both parents were infected (RT-PCR and RFLP results).
'Fertility exp?' indicates whether fertility is expected under the *Wolbachia* hypothesis. Results are fecundity (mean eggs and standard deviation), fertility (mean number of offspring and standard deviation), total number of females in each cross type (*n* total), number of fecund females (*n* fecund) and number of fertile females (*n* fertile). For cross-type abbreviations, see Materials and methods.

caused a reproductive incompatibility within species, the C/ut cross should fail to produce offspring (assuming that females were cured); yet this cross was equally fertile.

Antibiotic treatment decreased female post-mating lifespan ($F(80,1)=140.5$, $P<0.0001$); treated females lived for 29.4 ± 14.6 days ($n=28$), whereas untreated females lived for 43.7 ± 14.5 days ($n=56$). Unexpectedly, mating with conspecifics or heterospecifics also affected female lifespan ($F(82,1)=167.6$, $P<0.0001$). Females mated to conspecifics lived longer than females mated to heterospecifics (45.8 ± 14.6 days, $n=32$ vs 35.7 ± 15.4 days, $n=52$, respectively).

Table 3 *Wolbachia* loads (relative to a standard individual) in *Gryllus firmus* and *G. pennsylvanicus* (mean, standard deviation and number of samples) for treated (t) and untreated (u) males and females

Species	*Group*	*Male*			*Female*		
		Mean	*s.d.*	n	*Mean*	*s.d.*	n
G. firmus	t	4.2	±7.3	9	35.8	±57.6	13
	u	22.9	±38.0	8	39.6	±26.5	8
G. pennsylvanicus	t	0.3	±0.2	5	0.8	±0.2	3
	u	0.4	±0.2	4	0.8	±1.1	3
	Wild	5.8	±5.9	5	7.5	±9.6	5

Real-time PCR

Individual *Wolbachia* loads ranged from 0× to 172×. *G. pennsylvanicus* had a significantly lower *Wolbachia* load than *G. firmus* ($F(1,51)=44.6$, $P<0.001$) and females had significantly higher loads than males in both species ($F(1,50)=45.19$, $P<0.03$). In addition, males responded better to treatment than females (significant sex × treatment interaction ($F(1,46)=5.37$, $P<0.03$; Table 3). Furthermore, laboratory-reared *G. pennsylvanicus* had significantly lower *Wolbachia* loads than their wild counterparts ($F(1,23)=45.51$, $P<0.001$; Table 3). Because of the high variance in load between treatments/individuals/species and the presence of cured individuals, it is very unlikely that we were quantifying *Wolbachia* DNA that had been transferred to the host genome.

In spite of two generations of antibiotic treatment, only 9 of the 53 assayed individuals showed no evidence of *Wolbachia* infection; 8 of these crickets were treated and 1 was an untreated *G. pennsylvanicus* female. Of the nine cured individuals, two were *G. pennsylvanicus* (one male and one female) and seven were *G. firmus* (three males and four females). The cured *G. pennsylvanicus* male was mated with four *G. firmus* females, none of whom produced any offspring. Only one of the cured *G. firmus* female was in a C/ut cross; she mated with a heavily infected male (46× load) and produced 30 offspring (19% hatching success). The other three cured *G. firmus* females were in the C/tt group and these females failed to produce offspring. Two of these cured females were paired with two of the cured males (0× load) and deposited only 1–2 eggs. The third cured female mated with an infected male (0.3× load) and laid 301 eggs, all of which failed to hatch. These three C/tt cured females had very reduced post-mating lifespan: 10 and 11 days for females mated with cured males and 27 days for the females mated with the infected males (compared to 46 days average for females mated with conspecifics). The third cured *G. firmus* male mated with an untreated female (C/tu) and produced only 12 offspring (3.8% hatching success). The only hybrid offspring (identity confirmed with microsatellites) came from an H/uu cross in which both male and female were infected (0.3× and 16×, respectively).

RFLP analysis

We could assign a *Wolbachia* strain to all infected individuals. All experimental *G. pennsylvanicus* had the *w*G1 strain ($n=13$) and all experimental *G. firmus* had the *w*G2 strain ($n=31$). However, 5 out of 10 wild-caught *G. pennsylvanicus* individuals had the *w*G2 type and only 3 had the *w*G1 strain (2 females were uninfected). There were no doubly infected individuals in either experimental or wild groups. An additional sample of 14 *G. pennsylvanicus* from three 'pure' populations in New York and Pennsylvania included 9 infected with *w*G1, 4 infected with *w*G2 and 1 doubly infected.

In the wild-caught Ithaca *G. pennsylvanicus*, there seems to be a difference in RT-PCR load between *w*G1 and *w*G2 *Wolbachia* strains. The average load for *w*G1 was 1.8× (±1.2, $n=3$) and the average for *w*G2 was 12.2× (±7.0, $n=5$). Because of the very small sample size we did not conduct any statistical tests.

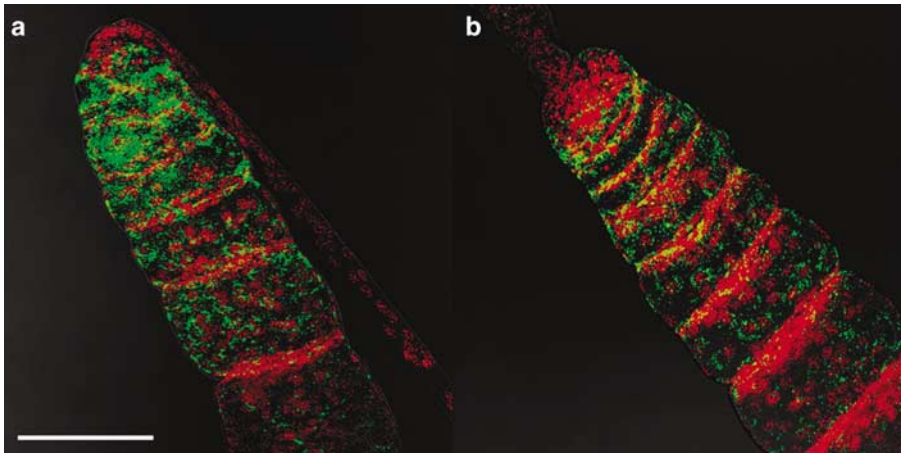

Figure 1 *Wolbachia* within developing ovaries of (**a**) *Gryllus firmus* and (**b**) *G. pennsylvanicus*. Host nuclei are stained with propidium iodide (red) and *Wolbachia* labeled with anti-hsp60 (green/yellow). Scale bar = 100 μm.

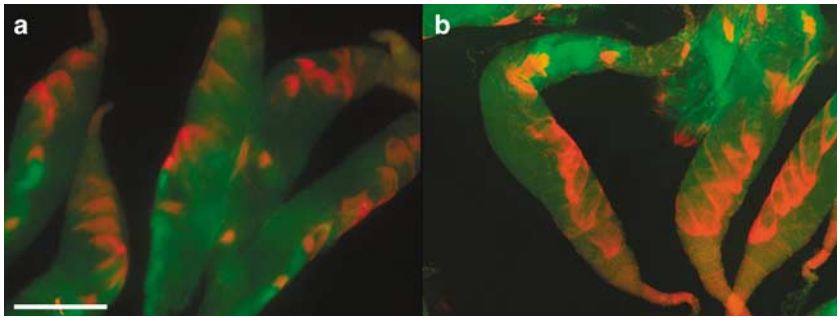

Figure 2 *Wolbachia* are absent from testis follicles in both *Gryllus firmus* (**a**) and *G. pennsylvanicus* (**b**). Spermatid nuclei (red) are seen within developing spermatocysts. All Alexa Fluor 488 staining (green) represents either background staining, or cross-reactivity with spermatid tails. Scale bar = 500 μm.

Microscopy

Wolbachia was easily visualized in ovaries from both *G. firmus* and *G. pennsylvanicus* (Figure 1). The overall *Wolbachia* load within ovaries is consistent with the RT-PCR results, with higher *Wolbachia* densities within the ovaries of *G. firmus* than *G. pennsylvanicus* females. Within testes, *Wolbachia* was typically absent from both species (Figure 2) for treated, untreated and wild individuals. *Wolbachia* were never seen within the developing spermatocytes, spermatids or surrounding cyst cells. Very rarely, *Wolbachia* could be seen within a single somatic cell (not shown) in the outer follicle epithelium, but this was atypical.

Egg analyses

We used a one-way analysis of variance to test for differences between the mean length and width of unfertilized, hybrid cross and pure fertilized *G. firmus* eggs (see Figure 3). We found no significant difference in mean egg length (unfertilized = 3.11 mm, hybrid cross = 3.08 mm and fertilized *G. firmus* = 3.13 mm; $F(2, 27) = 0.37$, $P > 0.5$), but a significant difference in the mean egg width (unfertilized = 0.631 mm, hybrid cross = 0.641 mm, and fertilized *G. firmus* = 0.953 mm; $F(2, 27) = 119.7$, $P < 0.001$). The *post hoc* Tukey test showed

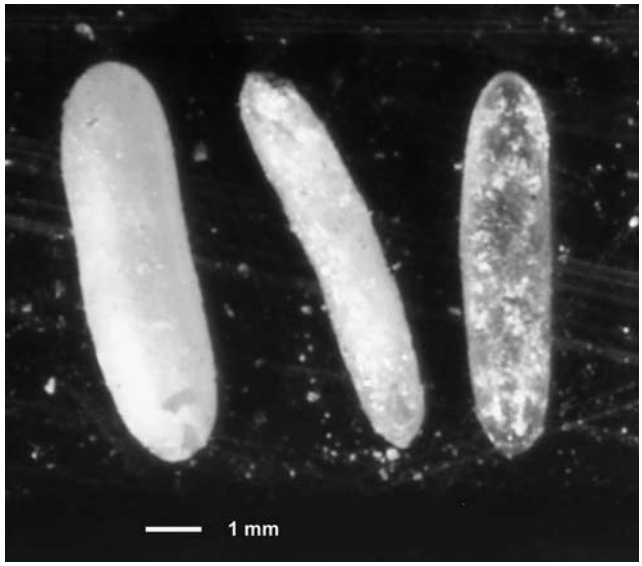

Figure 3 Eggs from *Gryllus firmus* females. The first is a typical fertilized *G. firmus* egg from a conspecific cross, the second is an unfertilized egg and the third is an egg from a hybrid cross. Hybrid cross eggs and unfertilized eggs are always narrower than a fertilized *G. firmus* egg. Color variation is commonly observed in unfertilized and hybrid cross eggs.

that this difference was between fertilized *G. firmus* eggs and the other two categories. There were no differences between hybrid cross and unfertilized eggs ((U = H) ≠ Gf).

All egg DNA extractions had measurable amounts of nucleic acids (mean unfertilized = 11 μg/μl, mean hybrid = 12 μg/μl and mean fertilized *G. firmus* = 18 μg/μl); however, these measurements probably reflect carrier RNA added during the extractions. We were able to amplify *Gryllus*-specific microsatellites and *Wolbachia* ftsZ from all six eggs from crosses between *G. firmus* males and females. We also amplified *Wolbachia* ftsZ from three of six unfertilized eggs and four of six hybrid cross eggs; however, we were unable to amplify cricket-specific genes (microsatellites or *EF1α*) from any of the hybrid cross or unfertilized eggs.

Discussion

Overall, we found no evidence that *Wolbachia* infections play a role in the *G. firmus*/*G. pennsylvanicus* one-way reproductive incompatibility. The original observation (Giordano *et al*., 1997) that *G. pennsylvanicus* is infected and *G. firmus* is not infected is not supported by our data. However, our results do agree with the data of Mandel *et al*. (2001), showing that most individuals in northern populations of both species are infected, that both species harbor strains *w*G1 and *w*G2, but with somewhat different frequencies. Our results show that a completely cured *G. pennsylvanicus* male did not produce offspring when mated with *G. firmus* females, as would be expected if *Wolbachia* was the cause of reproductive incompatibility. Furthermore, we did not find *Wolbachia* inside the testes of adult males, although bacteria were present in other tissues. Finally, there is no evidence that eggs from hybrid crosses are fertilized and die later in development as would be expected if *Wolbachia* was responsible for the reproductive incompatibility. *Wolbachia* also does not seem to cause intraspecific CI in crickets; crosses expected to be incompatible (C/ut—untreated males and treated females) produced as many offspring as controls, and a completely cured female was able to produce offspring with a highly infected male. Taken together, these observations provide strong evidence against the hypothesis of *Wolbachia*-induced CI in the field cricket hybrid zone.

Interspecific one-way reproductive incompatibility

Bacterial density is associated with the prevalence of *Wolbachia*-induced phenotypes, including the expression of CI. Treatments that reduce bacterial densities usually lead to decreases in *Wolbachia*-induced phenotypes (Breeuwer and Werren, 1993; Hurst *et al*., 2000; Zchori-Fein *et al*., 2000). Thus, if *Wolbachia* was the cause of the observed reproductive incompatibility, we would expect a decrease in CI with antibiotic treatment. Although treatment was not 100% effective, males had substantially reduced *Wolbachia* loads, and one *G. pennsylvanicus* male was completely cured (other treated *G. pennsylvanicus* males had loads $<0.5\times$; Table 3). Despite the overall decrease in *Wolbachia* loads (especially in relation to field-collected individuals) and the successful cure, no hybrid crosses produced offspring (except for a single hybrid produced from an infected male and female). Moreover, given that higher bacterial densities are associated with increase in incompatibility and possibly higher efficacy in sperm modification and egg rescue (Boyle *et al*., 1993; Breeuwer and Werren, 1993; Bressac and Rousset, 1993; Poinsot *et al*., 1998; Stouthamer *et al*., 1999), if *Wolbachia* were the cause of reproductive incompatibility, we would expect higher bacterial loads in *G. pennsylvanicus* (as it should be the species with modified sperm). In contrast to those expectations, *G. firmus* had bacterial loads about $50\times$ those in *G. pennsylvanicus*.

In *Drosophila* with CI, both bacterial density within testes (Boyle *et al*., 1993; Bressac and Rousset, 1993; Giordano *et al*., 1995; Bourtzis *et al*., 1996; Poinsot *et al*., 1998; Riparbelli *et al*., 2007) and total amount of infected germ cell cysts (Clark *et al*., 2002, 2003) have been implicated in CI expression. In other hosts, *Wolbachia* modify sperm without being present within an individual spermatocyte or spermatid during development, but instead are present in other cells within testes, suggesting that *Wolbachia* can either act across cells or act prior to spermatogenesis (Clark *et al*. 2008). Within the testes, CI-inducing (mod$^+$) *Wolbachia* modify sperm, such that normal embryonic development can occur only if the modification is rescued by an infected (resc$^+$) egg (Werren, 1997a). *Wolbachia* strains that neither modify nor rescue sperm (mod$^-$ and resc$^-$) also exist; these strains have no effect on host reproduction (Hoffmann *et al*., 1996; Veneti *et al*., 2003; Marshall, 2004). We did not observe *Wolbachia* inside testes or in the surrounding tissues (Figure 2). This absence of *Wolbachia* inside the testes of both *Gryllus* species suggests that these *Wolbachia* strains are unable to modify sperm and cause CI in these hosts.

CI is expected between hybridizing species when one species is infected with CI-causing *Wolbachia* strain and the other species is uninfected or lacks any of the *Wolbachia* types found in the other species. In our experimental crosses, we did not find any doubly infected individuals; all *G. firmus* were infected with the *w*G2 strain and all *G. pennsylvanicus* carried the *w*G1 strain. However, wild-caught *G. pennsylvanicus* carry either *w*G1 or *w*G2, both strains or are uninfected. On the basis of our observed ratios of *w*G1-infected, *w*G2-infected and doubly infected, if *Wolbachia* were the primary cause of the incompatibility between *G. firmus* and *G. pennsylvanicus*, then a large fraction of heterospecific crosses should produce offspring. Contrary to this expectation, in hundreds of heterospecific crosses (Harrison, 1983; Maroja, 2008; RG Harrison, unpublished) only one hybrid has ever been produced (reported in this study). This hybrid came from a cross between untreated crickets, in which the *G. pennsylvanicus* male carried *w*G1 ($0.3\times$ load) and *G. firmus* female carried *w*G2 ($16\times$ load). The extreme rarity of hybrids from *G. firmus* females suggests the existence of very strong barriers to fertilization. This is corroborated by the observation that mitochondrial DNA introgression across the cricket hybrid zone is always from *G. pennsylvanicus* into *G. firmus* (Harrison *et al*., 1987; Harrison and Bogdanowicz, 1997; Ross and Harrison, 2002) as would be expected if the only F1 hybrids produced were offspring of *G. pennsylvanicus* females.

Wolbachia-induced CI usually leads to early embryonic death or haploid development (Callaini *et al*., 1996, 1997; Lassy and Karr, 1996; Tram and Sullivan, 2002; Duron and Weill, 2006). However, cricket eggs from hybrid crosses resemble unfertilized eggs, both in size (Figure 3)

and in the failure to provide suitable DNA templates for amplification of *Gryllus*-specific microsatellites, although still providing templates for amplification of *Wolbachia*-specific genes. If there were early embryos in the eggs from heterospecific crosses, then the amount of DNA should be sufficient to allow amplification of *Gryllus*-specific microsatellites. It is possible that the DNA has degraded subsequent to the death of the embryo; however, in that case we would not expect to be able to amplify *Wolbachia* DNA.

Wolbachia in conspecific crosses and antibiotic treatment effects

Wolbachia does not appear to cause intraspecific CI in *G. firmus*. *Wolbachia* is not found in testes of *G. firmus* males (Figure 2) and is thus unlikely to modify sperm. Furthermore, our crossing data suggest that the observed infertility (or low fecundity) of some male–female pairs is a result of the antibiotic treatment itself, rather than due to presence or absence of *Wolbachia*. If *Wolbachia* caused conspecific CI, and if curing were complete, our C/ut crosses ($n = 5$) should be infertile as an uninfected egg would not be able to rescue modified sperm. The only completely cured female was mated with a highly infected male ($46\times$ load) and had normal fertility. Two C/ut crosses also showed normal fertility (females had reduced *Wolbachia* loads of $5\times$ and $3\times$). Antibiotic treatment significantly reduces female fecundity independent of the status of the male and probably also explains the infertility of the other two C/ut crosses, as well as other infertile crosses (all of which involved treated individuals). In addition, antibiotic treatment decreased female post-mating lifespan. Taken together, these observations suggest direct negative effects of antibiotic treatment, effects that confound interpretation of curing experiments in these insects. Alternatively, *Wolbachia* could be a mutualist (Hoerauf *et al*., 1999; Dedeine *et al*., 2001; Weeks *et al*., 2007) and, in this case, the adverse effects on treated females would be due to a decrease in *Wolbachia* loads.

Curiously, we also found that mating with conspecific males vs heterospecific males affected female lifespan; females mated with conspecifics lived longer than females mated with heterospecifics. This unexpected result could be related to the benefits field cricket females appear to gain from conspecific matings, or an additional unappreciated cost to heterospecific matings (Simmons, 1988; Burpee and Sakaluk, 1993; Wagner *et al*., 2001; Sakaluk *et al*., 2002; Ivy and Sakaluk, 2005). Both costs and benefits might be exaggerated in captive/treated populations. Alternatively, observations of female lifespan differences might be a negative consequence of reduced oviposition rate and accumulation of eggs in females mated with heterospecific males.

Wolbachia load

Total *Wolbachia* loads were consistently much lower in males than in females in both species. This may reflect high *Wolbachia* densities in the female germline and may explain the persistence of *Wolbachia* in the absence of CI. High *Wolbachia* densities in the female germline likely ensure high rates of *Wolbachia* transmission. Interestingly, both wild-caught male and female *G. pennsylvanicus* had much higher *Wolbachia* loads than crickets reared in the lab. The basis of this difference is unclear. Previous reports from *Drosophila* suggest that the phenotypic effects of *Wolbachia* are greater under ideal laboratory conditions compared with either stressed laboratory conditions or wild-caught flies (Ikeda, 1970; Hurst *et al*., 2001). If lab conditions are non-optimal for *G. pennsylvanicus*, *Wolbachia* loads may be higher in individuals living in more optimal (wild) conditions compared with the suboptimal (laboratory) conditions.

The data presented here suggest that *Wolbachia* does not cause CI in *G. pennsylvanicus* and does not currently play a role in the incompatibility between *G. pennsylvanicus* and *G. firmus*. Although *Wolbachia* infections have now been described in hundreds of arthropod species, the phenotypic effects of *Wolbachia* infection have been experimentally examined in only a few model organisms. These are restricted to species easily reared in a laboratory environment. A more thorough understanding of effects of *Wolbachia* on hosts will require examination of a wider range of hosts that may not be as amendable to a laboratory environment as traditional model organisms.

Conclusions

G. firmus and *G. pennsylvanicus* are an important model system in the study of speciation, and understanding the basis of their reproductive isolation is important for understanding the evolution of barriers to gene exchange. The importance of 'infectious speciation' (Coyne, 1992) caused by *Wolbachia* bidirectional CI is a subject of current debate (Werren, 1997b; Coyne and Orr, 2004). There is good evidence that *Wolbachia* infections do play a role in barriers to current gene exchange between species (*Nasonia*, Breeuwer and Werren, 1993; Bordenstein *et al*., 2001; *Drosophila*, Shoemaker *et al*., 1999; Jaenike *et al*., 2006). Although genetic incompatibilities are also involved in these examples, *Wolbachia*-driven incompatibility seems to have evolved early enough to have played a role in the initial speciation process. However, in these cases, as in virtually all studies of current barriers to gene exchange, it is very difficult to identify the order in which barriers have arisen (Bordenstein, 2003). As a result, the extent to which endosymbionts are important in insect speciation remains unclear. The field crickets *G. firmus* and *G. pennsylvanicus* exhibit a very clear unidirectional incompatibility and have been cited as a possible example of *Wolbachia*-induced CI. The data presented here strongly suggest that this is not the case.

Acknowledgements

We thank Rodman G Getchell for helping with the RT-PCR, Steve Bogdanowicz for technical advice in all aspects of this study and Jose A Andres for statistical advice. This project was supported by NSF grant DEB-0639904 to RGH. The work of MEC was supported by NSF grant EF-0328363 to J Werren.

References

Barr AR (1980). Cytoplasmic incompatibility in natural populations of a mosquito, *Culex pipiens*. *Nature* **283**: 71–72.

Bordenstein SR (2003). Symbiosis and the origin of species. In: Miller T, Bourtzis K (eds). *Insect Symbiosis*. CRC Press: New York.

Bordenstein SR, O'Hara FP, Werren JH (2001). *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Science* **409**: 675–677.
Bourtzis K, Nirgianaki A, Markakis G, Savakis C (1996). *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. *Genetics* **144**: 1063–1073.
Boyle L, O'Neill SL, Robertson HM, Karr TL (1993). Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. *Science* **260**: 1796–1799.
Breeuwer JAJ, Stouthamer R, Barns SM, Pelletier DA, Weisburg WG, Werren JH (1992). Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid wasp genus *Nasonia* based on 16S ribosomal DNA sequences. *Insect Mol Biol* **1**: 25–36.
Breeuwer JAJ, Werren JH (1990). Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* **346**: 558–560.
Breeuwer JAJ, Werren JH (1993). Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. *Genetics* **135**: 565–574.
Breeuwer JAJ, Werren JH (1995). Hybrid breakdown between two haploid species: the role of nuclear and cytoplasmic genes. *Evolution* **49**: 705–717.
Bressac C, Rousset F (1993). The reproductive incompatibility system in *Drosophila simulans*: DAPI-staining analysis of the *Wolbachia* symbionts in sperm cysts. *J Invert Pathol* **61**: 226–230.
Burpee DM, Sakaluk SK (1993). Repeated matings offset the costs of reproduction in female field crickets. *Evol Ecol* **7**: 240–250.
Callaini G, Dallai R, Riparbelli MG (1997). *Wolbachia*-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of *Drosophila simulans*. *J Cell Sci* **110**: 271–280.
Callaini GM, Riparbelli G, Giordano R, Dallai R (1996). Mitotic defects associated with cytoplasmic incompatibility in *Drosophila simulans*. *J Invert Pathol* **67**: 55–64.
Clark ME, Bailey-Jourdain C, Ferree PM, England S, Sullivan W, Windsor D *et al.* (2008). *Wolbachia* modification of sperm does not always require residence within developing sperm. *Heredity* (in press).
Clark ME, Veneti Z, Bourtzis K, Karr TL (2002). The distribution and proliferation of the intracellular bacteria *Wolbachia* during spermatogenesis in *Drosophila*. *Mech Dev* **111**: 3–15.
Clark ME, Veneti Z, Bourtzis K, Karr TL (2003). *Wolbachia* distribution and cytoplasmic incompatibility during sperm development: the cyst as the basic cellular unit of CI expression. *Mech Dev* **120**: 185–198.
Coyne JA (1992). Genetics and speciation. *Nature* **355**: 511–515.
Coyne JA, Orr HA (2004). *Speciation*. Sinauer Associates: Sunderland, MA.
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.
Duron O, Weill M (2006). *Wolbachia* infection influences the development of *Culex pipiens* embryos in incompatible crosses. *Heredity* **96**: 493–500.
Giordano R, Jackson JJ, Robertson HM (1997). The role of *Wolbachia* bacteria in reproductive incompatibilities and hybrid zones of *Diabrotica* beetles and *Gryllus* crickets. *Proc Natl Acad Sci USA* **94**: 11439–11444.
Giordano R, O'Neil SL, Robertson HM (1995). *Wolbachia* infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana*. *Genetics* **140**: 1307–1317.
Harrison RG (1983). Barriers to gene exchange between closely related cricket species. I. Laboratory hybridization studies. *Evolution* **37**: 245–251.
Harrison RG (1985). Barriers to gene exchange between closely related cricket species. II. Life cycle variation and temporal isolation. *Evolution* **39**: 244–259.
Harrison RG, Bogdanowicz SM (1997). Patterns of variation and linkage disequilibrium in a field cricket hybrid zone. *Evolution* **51**: 493–505.
Harrison RG, Rand DM (1989). Mosaic hybrid zones and the nature of species boundaries. In: Otte D, Endler JA (eds). *Speciation and its Consequences*. Sinauer: Sunderland, MA. pp 111–133.
Harrison RG, Rand DM, Wheeler WC (1987). Mitochondrial DNA variation in field crickets across a narrow hybrid zone. *Mol Biol Evol* **4**: 144–158.
Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008). How many species are infected with Wolbachia?—a statistical analysis of current data. *FEMS Microbiol Lett* **281**: 215–220.
Hoerauf A, Nissen-Pahle K, Schmetz C, Henkle-Duhrsen K, Blaxter ML, Büttner DW *et al.* (1999). Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. *J Clin Invest* **103**: 11–17.
Hoerauf A, Volkmann L, Hamelmann C, Adjei O, Autenrieth IB, Fleischer B *et al.* (2000). Endosymbiotic bacteria in worms as targets for a novel chemotherapy in filariasis. *Lancet* **355**: 1242–1243.
Hoffmann AA, Clancy D, Duncan J (1996). Naturally-occurring *Wolbachia* infection in *Drosophila simulans* that does not cause cytoplasmic incompatibility. *Heredity* **76**: 1–8.
Hurst GD, Jiggins FM, Robinson SJW (2001). What causes inefficient transmission of male-killing *Wolbachia* in *Drosophila*? *Heredity* **87**: 220–226.
Hurst GD, Johnson AP, Schulenburg JH, Fuyama Y (2000). Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. *Genetics* **156**: 699–709.
Hurst GDD, Jiggins FM, 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.
Ikeda H (1970). The cytoplasmically-inherited 'sex-ratio' condition in natural and experimental populations of *Drosophila bifasciata*. *Genetics* **65**: 311–333.
Ivy TM, Sakaluk SK (2005). Polyandry promotes enhanced offspring survival in decorated crickets. *Evolution* **59**: 152–159.
Jaenike J, Dyer KA, Cornish C, Minhas MS (2006). Asymmetrical reinforcement and *Wolbachia* infection in *Drosophila*. *PLoS Biol* **4**: e325.
Jeyaprakash A, Hoy MA (2000). Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* **9**: 393–405.
Lassy CW, Karr TL (1996). Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans*. *Mech Dev* **57**: 47–58.
Mandel MJ, Ross C, Harrison RG (2001). Do *Wolbachia* infections play a role in unidirectional incompatibilities in a field cricket hybrid zone? *Mol Ecol* **10**: 703–709.
Maroja LS (2008). Barriers to gene exchange in a field cricket hybrid zone. PhD dissertation, Cornell University, Ithaca, NY.
Marshall JL (2004). The *Allonemobius–Wolbachia* host–endosymbiont system: evidence for rapid speciation and against reproductive isolation driven by cytoplasmic incompatibility. *Evolution* **58**: 2409–2425.
McGraw EA, Merritt DJ, Droller NJ, O'Neill SL (2002). *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc Natl Acad Sci USA* **99**: 2918–2923.
Navajas M, Tsagkarakov A, Lagnel J, Perrot-Minnot M-J (2000). Genetic differentiation in *Tetranychus urticae* (Acari: Tetranychidae): polymorphism, host races or sibling species? *Exp Appl Acarol* **24**: 365–376.
O'Neill SL, Giordano R, Colbert AME, Karr TL, Robertson HM (1992). 16S rRNA phylogenetic analysis of the bacterial

endosymbionts associated with cytoplasmic incompatibility in insects. *Proc Natl Acad Sci USA* **89**: 2699–2702.

O'Neill SO, Karr TL (1990). Bidirectional cytoplasmic incompatibility between conspecific populations of *Drosophila simulans*. *Nature* **348**: 178–180.

Poinsot D, Bourtzis K, Markakis G, Savakis C, Merlot H (1998). *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: host effect and cytoplasmic incompatibility relationships. *Genetics* **150**: 227–237.

R Development Core Team (2006). *R: a language and environment for statistical computing*. Vienna, Austria (http://www.R-project.org).

Riparbelli MG, Giordano R, Callaini G (2007). Effects of *Wolbachia* on sperm maturation and architecture in *Drosophila simulans* Riverside. *Mech Dev* **124**: 699–714.

Ross CL, Harrison RG (2002). A fine-scale spatial analysis og the mosaic hybrid zone between *Gryllus firmus* and *Gryllus pennsylvanicus*. *Evolution* **56**: 2296–2312.

Ross CL, Harrison RG (2006). Viability selection on overwintering eggs in a field cricket mosaic hybrid zone. *Oikos* **115**: 53–68.

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.

Sakaluk SK, Schaus JM, Eggert AK, Snedden WA, Brady PL (2002). Polyandry and fitness of offspring reared under varying nutritional stress in decorated crickets. *Evolution* **56**: 1999–2007.

Shoemaker DD, Katju V, Jaenike J (1999). *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria*. *Evolution* **53**: 1157–1164.

Simmons LW (1988). The calling song of the field cricket *Gryllus bimaculatus* (De Geer): constraints on transmission and its role in intermale competition and female choice. *Anim Behav* **36**: 380–394.

Stouthamer R, Breeuwer JAJ, Hurst DD (1999). *Wolbachia Pipientis*: microbial manipulator of arthropod reproduction. *Ann Rev Microbiol* **53**: 71–102.

Stouthamer R, Breeuwer JAJ, Luck RF, Werren JH (1993). Molecular identification of microorganisms associated with parthenogenesis. *Nature* **361**: 66–68.

Telschow A, Hammerstein P, Werren JH (2005a). The effect of *Wolbachia* versus genetic incompatibilities on reinforcement and speciation. *Evolution* **59**: 1607–1619.

Telschow A, Yamamura N, Werren JH (2005b). Bidirectional cytoplasmic incompatibility and the stable coexistence of two *Wolbachia* strains in parapatric host populations. *J Theor Biol* **235**: 265–274.

Tram U, Sullivan W (2002). Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science* **296**: 1124–1126.

Vala F, Breeuwer JA, Sabelis MW (2000). *Wolbachia* induced 'hybrid breakdown' in the two-spotted spider mite *Tetranychus urticae* Koch. *Proc R Soc Lond B Biol Sci* **267**: 1931–1937.

Veneti Z, Clark ME, Zabalou S, Karr TL, Savakis C, Bourtzis K (2003). Cytoplasmic incompatibility and sperm cyst infection in different *Drosophila–Wolbachia* associations. *Genetics* **164**: 545–552.

Wagner WE, Kelley RJ, Tucker KR, Harper CJ (2001). Females receive a life-span benefit from male ejaculates in a field cricket. *Evolution* **55**: 994–1001.

Weeks AR, Reynolds KT, Hoffmann AA (2002). *Wolbachia* dynamics and host effects: what has (and has not) been demonstrated? *Trends Ecol Evol* **17**: 257–262.

Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA (2007). From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS Biol* **5**: e114.

Werren JH (1997a). Biology of *Wolbachia*. *Annu Rev Entomol* **42**: 587–609.

Werren JH (1997b). *Wolbachia* and speciation. In: Howard D, Berlocher S (eds). *Endless Species and Speciation*. Oxford University Press: New York.

Werren JH, Windsor D, Guo RL (1995). Distribution of *Wolbachia* among neotropical arthropods. *Proc Royal Soc Lond B* **262**: 197–204.

Werren JH, Windsor DM (2000). *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proc R Soc Lond B Biol Sci* **267**: 1277–1285.

West SA, Cook JM, Werren JH, Godfray HC (1998). *Wolbachia* in two insect host–parasitoid communities. *Mol Ecol* **7**: 1457–1465.

Willett C, Ford MJ, Harrison RG (1997). Inferences about the origin of a field cricket hybrid zone from a mitochondrial DNA phylogeny. *Heredity* **79**: 484–494.

Yen JH, Barr AR (1971). New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens*. *Nature* **232**: 657–658.

Zchori-Fein E, Gottlieb Y, Coll M (2000). *Wolbachia* density and host fitness components in *Muscidifurax uniraptor* (Hymenoptera: pteromalidae). *J Invertebr Pathol* **75**: 267–272.