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Reproductive compensation favours male-killing *Wolbachia* in a live-bearing host

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Wolbachia are maternally inherited, cellular endosymbionts that can enhance their fitness by biasing host sex ratio in favour of females. Male killing (MK) is an extreme form of sex-ratio manipulation that is selectively advantageous if the self-sacrifice of *Wolbachia* in males increases transmission through females. In live-bearing hosts, females typically produce more embryos than can be carried to term, and reproductive compensation through maternal resource reallocation from dead males to female embryos could increase the number of daughters born to infected females. Here, we report a new strain of MK *Wolbachia* (*w*Csc2) in the pseudoscorpion, *Cordylochernes scorpioides*, and present the first empirical evidence that reproductive compensation favours the killing of males in a viviparous host. Females infected with the *w*Csc2 strain produced 26 per cent more and significantly larger daughters than tetracycline-cured females. In contrast to the previously described *w*Csc1 MK *Wolbachia* strain in *C. scorpioides*, *w*Csc2 infection was not accompanied by an increase in the rate of spontaneous brood abortion. Characterization of the *w*Csc1 and *w*Csc2 strains by multi-locus sequence typing and by *Wolbachia* surface protein (*wsp*) gene sequencing indicates that the marked divergence between these two MK strains in their impact on host reproductive success, and hence in their potential to spread, has occurred in association with homologous recombination in the *wsp* gene.

Keywords: *Wolbachia*; male killing; reproductive compensation; *Cordylochernes scorpioides*; recombination; viviparity

1. INTRODUCTION

Wolbachia are obligate intracellular bacteria that infect an estimated 66 per cent of insect species (Hilgenboecker *et al.* 2008), and also occur in nematodes, amphipods, isopods and arachnids (Charlat *et al.* 2003; Rowley *et al.* 2004; Zeh *et al.* 2005; Baldo *et al.* 2007; Werren *et al.* 2008). As with other genetic elements present in the cytoplasm of animal cells, these cellular endosymbionts are transmitted to offspring only through eggs, and can therefore enhance their fitness by biasing host sex ratio in favour of females (Cosmides & Tooby 1981). *Wolbachia* employ diverse mechanisms for manipulating host sex ratio, including feminizing genetic males (Bouchon *et al.* 1998; Hiroki *et al.* 2002; Negri *et al.* 2006) and inducing parthenogenesis (Stouthamer *et al.* 1993). Alternatively, female bias can be achieved by killing male embryos early in development. Male killing (MK) by *Wolbachia* has been demonstrated in beetles (Hurst *et al.* 1999; Fialho & Stevens 2000), butterflies (Hurst *et al.* 1999; Dyson *et al.* 2002), fruit flies (Hurst *et al.* 2000; Dyer & Jaenike 2004) and a single pseudoscorpion species (Zeh *et al.* 2005; Zeh & Zeh 2006*a*).

While *Wolbachia* clearly benefit from feminization or parthenogenesis induction by converting dead-end males into microbe-transmitting females, the fitness gain derived from MK is less apparent. Kin selection theory predicts that MK can be favoured, if *Wolbachia* lethality in male embryos increases the transmission of clonally related *Wolbachia* lineages in female siblings (Werren 1987; Hurst 1991). Such fitness compensation may occur in egg-laying hosts through cannibalism, lowered risk of inbreeding or reduced sibling competition for resources (Hurst 1991; Hurst & Majerus 1993). In live-bearing hosts, females typically produce more embryos than can be carried to term. Reproductive compensation, a viviparity-specific form of fitness compensation in which maternal resources are reallocated from dead embryos to viable siblings (Charlesworth 1994), provides a direct, physiological mechanism that could increase the number and/or quality of daughters born to infected females, thereby promoting the spread of MK endosymbionts.

The discovery of an MK strain of *Wolbachia* in a highly female-biased line of the harlequin beetle-riding pseudoscorpion *Cordylochernes scorpioides* (henceforth, the *w*Csc1-infected line; Zeh *et al.* 2005; Zeh & Zeh 2006*a*) afforded a unique opportunity to assess the capacity of reproductive compensation to favour MK in a live-bearing host. Unlike most terrestrial arthropods, pseudoscorpions are viviparous, with embryos developing in an external, translucent brood sac overlying the female's genital aperture (Weygoldt 1969; Zeh & Zeh 2006*b*). Although *w*Csc1-infected females that carried broods to term produced 10 per cent more daughters than uninfected females, this reproductive compensation was heavily outweighed by a high rate of spontaneous brood abortion in infected females, thereby limiting the spread of the *w*Csc1 strain (Zeh & Zeh 2006*a*).

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Here, we report a second strain of MK *Wolbachia* (henceforth, *w*Csc2) in *C. scorpioides* that can potentially spread through populations by means of reproductive compensation. Using a combination of inheritance studies, antibiotic treatment, early-stage embryo counts and assessment of female reproductive success, we show that the killing of male embryos in *w*Csc2-infected females significantly increases the number and size of female offspring, relative to those produced by tetracycline-cured females, but does not increase the rate of spontaneous brood abortion. Although *w*Csc1 and *w*Csc2 differ extensively in their *wsp* gene sequences, characterization by multi-locus sequence typing (MLST; Baldo *et al.* 2006*b*) revealed a close evolutionary relationship between the two strains. Phylogenetic and recombination analyses indicate that the marked divergence between these two MK strains in their impact on host reproductive success, and hence in their potential to proliferate, has occurred in association with homologous recombination in the *wsp* gene of the *w*Csc2 strain.

2. MATERIAL AND METHODS

(a) *Pseudoscorpions*

Experimental females were derived from an iso-female line characterized by extremely female-biased sex ratios through four generations in the laboratory. This *w*Csc2-infected line was established from a field-collected dam that produced 18 females and zero males in the laboratory, following the collection of 174 *C. scorpioides* individuals from the Parque Nacional Soberanía (PNS) in the Republic of Panamá in January 2006. In each generation, the line was maintained at a population size of 250–300 adults by outcrossing *w*Csc2-infected females from approximately 15 full-sib families to males from a large laboratory population (800–1000 adults per generation), also established from the PNS collections in 2006. These males were randomly selected from approximately 30 full-sib families whose sex ratios were not significantly female biased ($p > 0.05$). Four generations of outcrossing to unrelated males resulted in a set of *w*Csc2-infected, full-sib families that differed substantially in their nuclear genetic backgrounds.

(b) *Phenotypic and fitness consequences of wCsc2 infection*

(i) *Tetracycline treatment*

To determine the effect of *Wolbachia* curing on female fecundity (number of early-stage embryos produced), female reproductive success (number of nymphs born) and offspring sex ratio in the *w*Csc2-infected line, we performed a split-brood experiment in which the first-instar nymphs (protonymphs) produced by each of 13 *w*Csc2-infected-line females (the parental or P generation) were randomly assigned at birth to either an untreated (control) or a tetracycline-treated rearing regime (for details of rearing methods and tetracycline treatment, see Zeh *et al.* 2005; Zeh & Zeh 2006*a*). Dietary supplementation with tetracycline was terminated when the treated offspring (the F1 generation) reached sexual maturity. Four untreated and four treated F1 female offspring from each P-generation female were then mated to males from the laboratory population. To control for the effects of nuclear genetic background across treatments, within full-sib families, the tetracycline-treated and untreated females were mated to the same set of four males, i.e. males were mated in random order to one untreated female and one treated female.

After mating, *C. scorpioides* females exhibit one of the three possible outcomes: (i) failure to produce a brood of embryos; (ii) production of a brood of embryos but subsequent spontaneous abortion of the entire brood, or (iii) brood production and carrying of embryos to term, with all protonymphs birthing simultaneously. In order to determine whether sex-ratio distortion in the *w*Csc2-infected line occurs through feminization or MK, we exploited the 'external womb' form of viviparity in *C. scorpioides*, and obtained within-female counts of both early-stage embryos produced by and protonymphs born to the F1 dams. Broods were permitted to develop to the stage at which individual embryos were first clearly distinguishable (approx. 5 days after mating) before being photographed, using an Olympus SZ60 stereomicroscope equipped with a Ultra 20 digital camera. For this early-stage brood photomicroscopy, each female was gently restrained on her dorsum under a glass slide, and a digital image of the entire brood recorded for subsequent embryo counting, using NIH ImageJ (v. 1.37). Females were then returned to their individual vials for continued monitoring of their reproductive status.

Within 48 h of birth, nymphs were collected from the female's brood nest and counted. As males and females are indistinguishable as nymphs, we reared 25 protonymphs to the adult stage (the F2 generation) from each of 12 treated and 12 untreated females in order to assess offspring sex ratio in the two treatments. These adult offspring were photographed, again using an Olympus SZ60 stereomicroscope equipped with an Ultra 20 digital camera. NIH ImageJ (v. 1.37) was then used to measure cephalothorax length, the trait most closely correlated with female reproductive success (Newcomer *et al.* 1999).

To assess the efficacy of tetracycline treatment in curing *w*Csc2 infection, *Wolbachia*-specific PCR, using the MLST *ftsZ* primer pair (see below), was performed on DNA samples extracted from 12 of the F1 tetracycline-treated dams, as well as both positive and negative controls. We confirmed that the quality of the DNA samples was suitable for amplification by carrying out a second PCR, using a primer pair designed from an approximately 600 bp region of *C. scorpioides* mitochondrial COI gene (GTAGGWCTTTGCTATAGAA TACTTATTCG and AAGTTCTAAAATTTCGATCAGT WAGAAG).

(ii) *Test for paternal inheritance of female-biased sex ratio*

To determine whether female-biased sex ratio in the *w*Csc2-infected line is inherited exclusively through females, surviving males from the untreated feeding regime were mated to females from the laboratory population. From each successful brood, 25 protonymphs were reared to adults to estimate offspring sex ratio.

(iii) *Statistical analyses*

Tetracycline effects on brood sex ratio, number of early-stage embryos, number of protonymphs, number of female protonymphs produced per F1 female (see below) and the size of adult F2 females were analysed using a general linear mixed model (GLMM) approach, as implemented in PROC GLIMMIX in SAS, v. 9.2 (SAS 2008). Antibiotic treatment was modelled as a fixed effect, with the P-generation dam included as a random effect to avoid pseudoreplication. To control for possible effects of tetracycline treatment on

female fecundity, the number of early-stage embryos produced by each female was included as a covariate in analyses of the number of protonymphs born and the number of female protonymphs born. The few treated and untreated females that did not produce a brood sac within 45 days of mating were excluded from analyses of early-stage embryo and nymph production because such females never become gravid (Newcomer *et al.*1999). To assess the potential for reproductive compensation to enhance *Wolbachia* transmission through increased embryonic survival of females, we restricted comparison of the number of daughters born (proportion of female adult offspring × number of nymphs born) to untreated and tetracycline-treated dams to only those broods for which we had directly determined offspring sex ratio by rearing 25 nymphs to the adult stage.

(c) *Multi-locus sequence typing and* wsp *gene sequencing of* wCsc1 *and* wCsc2 Wolbachia *strains*

(i) *DNA extraction, polymerase chain reaction and sequencing*

DNA was extracted from whole individuals, using the Invitrogen Chargeswitch gDNA Micro Tissue Kit, following the manufacturer's instructions. PCR was carried out, using the standard primers for the five *Wolbachia* MLST loci, *gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA* (Baldo *et al.* 2006*b*; http://pubmlst.org/wolbachia/). For PCR amplification of the *wsp* gene, a conserved primer pair (CTACRTTCGCTT RCAATACAACG and TTCTGCACCAAYAGTGCTGT AAAS) was designed from the alignment of published *wsp* sequences from 34 *Wolbachia* strains (Zeh *et al.* 2005).

PCR reactions were carried out in a 25 µl volume containing approximately 10 ng of genomic DNA, 2.5 µl of Expand Long Template buffer 2 (Roche), 350 µM dNTPs, 750 nM primers, and 1 U of a 5 : 1 mixture of TITANIUM *Taq* (ClonTech) and *Pfu*Ultra (Stratagene) DNA polymerases. PCR amplification conditions involved a 1 min hot start at 95°C, followed by 34 iterations of the following cycle: melting at 93°C for 25 s, annealing at 52, 54 or 58°C for 40 s (52°C for *gatB*, *coxA*, *hcpA*, *ftsZ*; 54°C for *wsp*; 58°C for *fpbA*) and extension at 68°C for 60 s.

PCR templates were prepared for sequencing by electrophoresing 12 µl of the reaction mixture through a 0.8 per cent agarose gel stained with ethidium bromide. Amplification products were excised from gels and purified with Promega Wizard minicolumns. BigDye reactions (6 µl) containing approximately 100 ng of purified PCR product and 3.2 pmol of PCR primer were sequenced with both forward and reverse PCR primers, using an Applied Biosystems Prizm 3730 DNA Analyser. Sequences were edited from the chromatograms using the FinchTV v. 1.4.0 viewer (Geospiza Inc.), and contig sequences were assembled from forward and reverse sequences in Geneious Pro 4.5 (Biomatters Ltd). For both the *w*Csc1 and *w*Csc2 strains, stable inheritance of *Wolbachia* gene sequences was confirmed by sequencing the DNA of at least one individual from each of three generations of the infected *C. scorpioides* lines.

(ii) *Phylogenetic analyses and tests for recombination*

DNA sequences of individual MLST genes, the concatenated MLST genes and the *wsp* gene for the *w*Csc1 and *w*Csc2 *Wolbachia* strains were compared with sequence data for *Wolbachia* strains infecting 28 other host taxa (see electronic supplementary material, table S1). MLST sequences were obtained from the *Wolbachia* MLST website (http://pubmlst.org/wolbachia/; Jolley *et al.* 2004), and we selected only strains for which both MLST and *wsp* gene sequences were available. Representatives from the A, B, D and F *Wolbachia* supergroups, including five MK strains from lepidopteran and dipteran hosts, were included in the phylogenetic analyses. Sequences were aligned in Geneious with ClustalW (Larkin *et al.* 2007), and phylogenetic analyses were conducted using maximum parsimony and maximum-likelihood (ML) methods in PAUP v. 4.0b10 (Swofford 2002). For ML, the best-fit evolutionary model for each dataset was determined through AIC likelihood ratio tests, using Modeltest 3.7 (Posada & Crandall 1998). Comparison of MLST and *wsp* ML tree topologies was performed using 1000 bootstrap replications of the Shimodaira–Hasegawa (SH) test (Shimodaira & Hasegawa 1999), as implemented in the LSCORES command in PAUP. The SH test involved fitting the MLST- and *wsp*-derived tree topologies to the MLST dataset to determine whether they were significantly different in their likelihood (−ln L) scores. To facilitate visual comparison of trees, *Wolbachia* phylograms were rooted with the *Brugia malayi Wolbachia* sequence (Fenn *et al.* 2006). It should be noted, however, that the most appropriate taxon for rooting arthropod *Wolbachia* sequences remains in question (Bordenstein *et al.* 2009).

Tests for homologous recombination between the 30 *Wolbachia* strains, that is, recombination involving replacement in a recipient sequence by homologous DNA from a donor genome (Baldo *et al.* 2006*a*), were performed with the Recombination Detection Program, v. 3 (Martin *et al.* 2005*b*), using the following methods to identify horizontal gene transfer (HGT) events: 3Seq (Boni *et al.* 2007), Bootscan (Martin *et al.* 2005*a*), Chimaera (Posada & Crandall 2001), GENECONV (Padidam *et al.* 1999), MaxChi (Maynard Smith 1992), Phylpro (Weiller 1998), RDP (Martin & Rybicki 2000) and SiScan (Gibbs *et al.* 2000).

(d) *Polymerase chain reaction assays for presence of non-*Wolbachia *male-killing bacteria*

To test for possible multiple infections in the *w*Csc1- and *w*Csc2-infected lines, we performed PCR assays for non-*Wolbachia* bacteria known to be associated with MK in other arthropod species (Duron *et al.* 2008). Using DNA samples that tested positive for *wsp* and all five MLST loci, PCR reactions were performed with the following primer pairs: R1 and R2 for *Rickettsia sp.*; ArsF and ArsR2 for *Arsenophonus nasoniae*; FlavF and FlavR for *Flavobacterium sp.*; SpixoF and SpixoR for *Spiroplasma ixodetis*, and SpoulF and SpoulR for *Spiroplasma poulsonii*. The sequences and annealing temperatures for these primer pairs are described in table 2 of Duron *et al.* (2008).

3. RESULTS

(a) *Phenotypic and fitness consequences of* wCsc2 *infection*

(i) *Tetracycline treatment*

Wolbachia-specific PCR confirmed the efficacy of tetracycline treatment, with all 12 of the tested, treated F1 dams failing to produce an *ftsZ* amplification product. Untreated (*w*Csc2-infected) and tetracycline-treated (cured) females did not differ in the proportion that failed to become gravid, spontaneously aborted their entire broods or gave birth to protonymphs (Fisher

exact test, $p = 0.825$). Dam mortality, exclusion of females that did not become gravid and, in a few cases, failure to obtain an accurate embryo or protonymph count reduced the number of females included in analyses of fecundity and reproductive success to 37 untreated and 41 tetracycline-treated females. After controlling for the effect of female body size by including cephalothorax length as a covariate in the GLMM ($p < 0.001$), tetracycline treatment resulted in a marginally significant reduction in the number of early-stage embryos produced by treated dams (T) compared with untreated controls (C) ($\bar{x}_T \pm$ s.e. $= 80.15 \pm 2.45$, $\bar{x}_C = 87.64 \pm 2.45$, $F_{1,12} = 4.77$, $p = 0.05$). Despite this slight reduction in fecundity, tetracycline treatment significantly increased female reproductive success. With the number of early-stage embryos included as a covariate ($F_{1,51} = 21.35$, $p < 0.001$), treated dams gave birth to a least-squares mean of 53.90 ± 2.51 nymphs, compared with 33.42 ± 2.64 for untreated females ($F_{1,12} = 32.11$, $p < 0.001$). As the rate of spontaneous brood abortion did not differ between treated (8.70%) and *w*Csc2-infected females (9.52%) (Fisher exact test, $p = 1.000$), the lower reproductive success of the untreated dams was attributable to a higher rate of embryonic mortality in broods that were successfully carried to term ($\bar{x}_C$ of embryos dying before birth $= 56.8 \pm 2.6\%$ $\bar{x}_T = 32.0 \pm 2.5\%$, $F_{1,22} = 46.52$, $p < 0.001$).

Tetracycline treatment also restored offspring sex ratio to 1 : 1. In the subset of nymphs reared to the adult stage from 12 untreated and 12 treated females, untreated females produced a highly significant excess of female offspring (paired $t_{11} = 21.098$, $p \ll 0.001$), but there was no significant difference in the number of male versus female offspring produced by treated dams (paired $t_{11} = 0.964$, $p = 0.356$). The mean proportion of daughters (p_{female}) produced by untreated females was 0.951 ± 0.022, compared with a mean for tetracycline-treated females of 0.477 ± 0.023. Among females whose broods were assessed for sex ratio, *w*Csc2-infected dams gave birth to a mean of 34.89 ± 1.81 female protonymphs, 26 per cent more daughters than the mean of 27.68 ± 1.90 for cured females ($F_{1,9} = 6.65$, $p = 0.0297$). In addition, adult female offspring of untreated dams were significantly larger, as measured by cephalothorax length, than those born to tetracycline-treated dams ($\bar{x}_C = 1.34 \pm 0.01$ mm, $\bar{x}_T = 1.30 \pm 0.01$ mm, $F_{1,22} = 7.64$, $p = 0.011$). By contrast, adult male size did not differ significantly between treatments ($\bar{x}_C = 1.27 \pm 0.03$ mm, $\bar{x}_T = 1.30 \pm 0.01$ mm, $F_{1,21} = 1.13$, $p = 0.301$). Survivorship from the protonymph to the adult stage was high ($\bar{x}_T = 0.880 \pm 0.019$, $\bar{x}_C = 0.857 \pm 0.019$) and did not differ between the two treatments ($F_{1,11} = 0.67$, $p = 0.429$), indicating that the extreme female bias exhibited by the *w*Csc2-infected line cannot be attributed to MK after birth.

(ii) *Test for paternal inheritance of female-biased sex ratio*

Because of the low number of surviving *w*Csc2-infected males ($n = 15$) and their apparent low fertility, only five females mated to these males gave birth to nymphs. From the subset of offspring randomly selected for rearing to adulthood, the five dams produced 56 female and 47 male offspring. A paired *t*-test revealed no significant difference in the number of male versus female offspring (paired $t_4 = 0.864$, $p = 0.4363$), indicating that female-biased sex ratio in the *w*Csc2-infected line is not transmitted through males.

(b) *Assays for multiple infections in* wCsc1 *and* wCsc2 *lines*

Within each *C. scorpioides Wolbachia* strain, *wsp* and MLST sequences obtained from pseudoscorpion individuals across three matrilineal generations were identical and sequence traces were free of multiple peaks, suggesting infection by a single *Wolbachia* strain. In addition, PCR tests for the presence of non-*Wolbachia* MK bacteria (*Rickettsia sp.*, *A. nasoniae*, *Flavobacterium* sp.; *S. ixodetis* and *S. poulsonii*) were all negative.

(c) *Multi-locus sequence typing and* wsp *gene sequencing of* wCsc1 *and* wCsc2 Wolbachia *strains*

(i) *Phylogenetic affinities of the* C. scorpioides Wolbachia *strains*

Bidirectional sequencing yielded nucleotide data ranging from 422 to 478 bp for the five MLST loci. While the *w*Csc1 and *w*Csc2 strains are very similar or identical at these loci, with pair-wise identities ranging from 97 to 100 per cent, NCBI BLAST searches revealed that the two *C. scorpioides* strains show considerable divergence from all other *Wolbachia* strain sequences in GenBank. Across the five loci, the sequences with the highest BLAST similarity scores ranged from 89 to 93 per cent identical to the *C. scorpioides Wolbachia* sequences (data not shown). Gene fragments for MLST loci were aligned and trimmed, consistent with sequences recorded in the *Wolbachia* MLST database, to a length of 369–444 bp, depending on the locus. The loci were combined in order of their location within the *w*Mel *Wolbachia* reference genome (*gatB–coxA–hcpA–ftsZ–fbpA*; Baldo *et al.* 2006*b*), resulting in a concatenated MLST sequence alignment of 2079 bp. Likelihood and parsimony methods yielded broadly concordant phylogenies, with the *w*Csc1 and *w*Csc2 strains clustering as monophyletic sister taxa for all MLST loci. The phylogeny based on concatenated MLST sequences placed the pseudoscorpion *Wolbachia* clade near the base of the tree, as the sister group to A + B supergroup strains (figure 1). This topology was largely consistent with phylogenies based on individual MLST loci, with the exception of *coxA*, which clustered both the pseudoscorpion and F-group clades with supergroup B strains.

For the *wsp* gene, sequences from the 30 *Wolbachia* strains were aligned across a 495 bp region. In contrast to MLST loci, *wsp* sequences from the *w*Csc1 and *w*Csc2 strains are highly divergent (uncorrected '*p*' = 0.200), with the two strains widely separated in the *wsp* phylogeny (figure 2). As the basal arthropod-infecting strain in this tree, *w*Csc1 is most similar in its *wsp* sequence to *Wolbachia* from *B. malayi* and *Ephestia kuehniella*, with uncorrected *p* distances of 0.158 and 0.148, respectively. By contrast, the more-derived *w*Csc2 *wsp* sequence exhibits the greatest similarity to the *Wolbachia* strain infecting *Teleogryllus taiwanemma* (uncorrected $p = 0.180$). Comparison of the MLST and *wsp* trees revealed a highly significant difference in their topologies

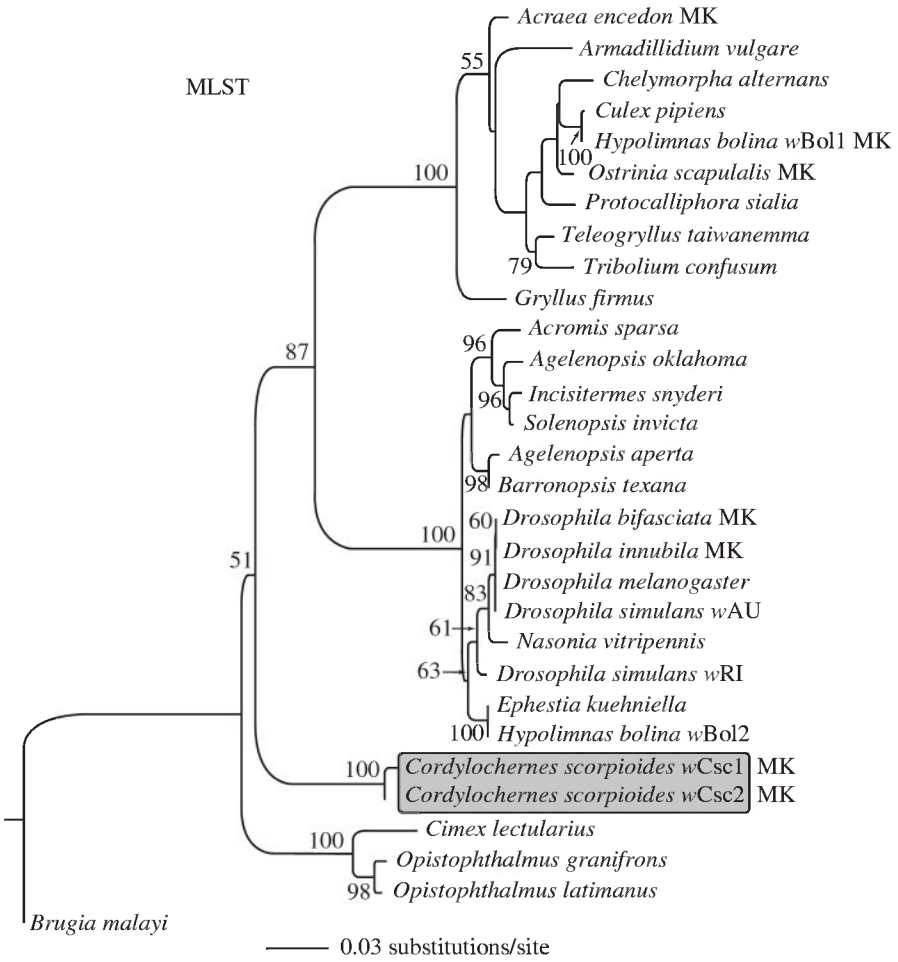

Figure 1. Maximum-likelihood phylogram of the 30 *Wolbachia* strains based on a 2079 bp alignment of the concatenated MLST loci. *Wolbachia* strains are identified by their host taxon. The single most likely tree was determined using a GTR + G + I model of substitution (−ln L score = 8965.36, tree length = 1233, consistency index = 0.562). *Wolbachia* strains implicated in MK are indicated by 'MK.' Values at nodes denote per cent support for clades based on 500 bootstrap replicates.

(MLST tree: −ln L score = 8965, *wsp* tree = 10 696, ln L difference = 1731, SH test, $p < 0.001$).

(ii) *Tests for recombination*

For the recombination tests, the *wsp* sequence was inserted between the *ftsZ* and *fbpA* gene fragments in the concatenated MLST sequence for each strain. For the entire dataset, the analyses identified 19 statistically significant recombination events, 13 of which involved the *wsp* gene. Evidence for the *w*Csc2 *wsp* gene as a recombinant sequence was particularly strong, with the 3Seq, Chimaera, MaxChi and RDP algorithms all yielding *p*-values of <0.001. The single, major parental sequence in the *w*Csc2 *wsp* recombinant was identified as *w*Csc1, with minor parental sequences from a highly diverged group of seven very similar *wsp* sequences (*A. encedon*, *A. oklahoma*, *B. texana*, *P. sialia*, *S. invicta*, *T. taiwanemma* and *T. confusum*).

4. DISCUSSION

To our knowledge, this is the first study to demonstrate empirically that reproductive compensation in a live-bearing host provides a selective advantage to MK by a cellular endosymbiont. *Cordylochernes scorpioides* females infected with the newly identified *w*Csc2 *Wolbachia* strain gave birth to an average of 20 daughters for every son. This highly female-biased sex ratio was restored to 1 : 1 in cured females and was attributable to the killing of male embryos during development. Infected females suffered no reduction in the number of early-stage embryos produced but gave birth to 38 per cent fewer protonymphs than their tetracycline-treated counterparts. Infected females did, however, produce 26 per cent more and significantly larger daughters than cured females through the reallocation of maternal resources from dead male embryos to female siblings. MK thus appears to be a highly adaptive strategy in the *w*Csc2 strain, with *Wolbachia* self-sacrifice in *C. scorpioides* males increasing the transmission of clonally related *Wolbachia* through host females.

Our assessment of the benefits of MK could potentially be confounded by *Wolbachia*-independent effects of tetracycline treatment on male versus female mortality during development. Two lines of evidence, however, strongly suggest that this is not the case. First, the sex ratios of offspring produced by naturally uninfected and antibiotic-cured females are very similar. Based on a survey of the broods of 153 field-collected *C. scorpioides* females ($n > 3000$ offspring), Zeh *et al.* (2005) reported an overall proportion male of 0.49. When the few significantly female-biased broods ($ns = 8$) were excluded from analysis, the mean proportion male was 0.52, a value nearly identical to that produced by the tetracycline-treated females in this study (proportion

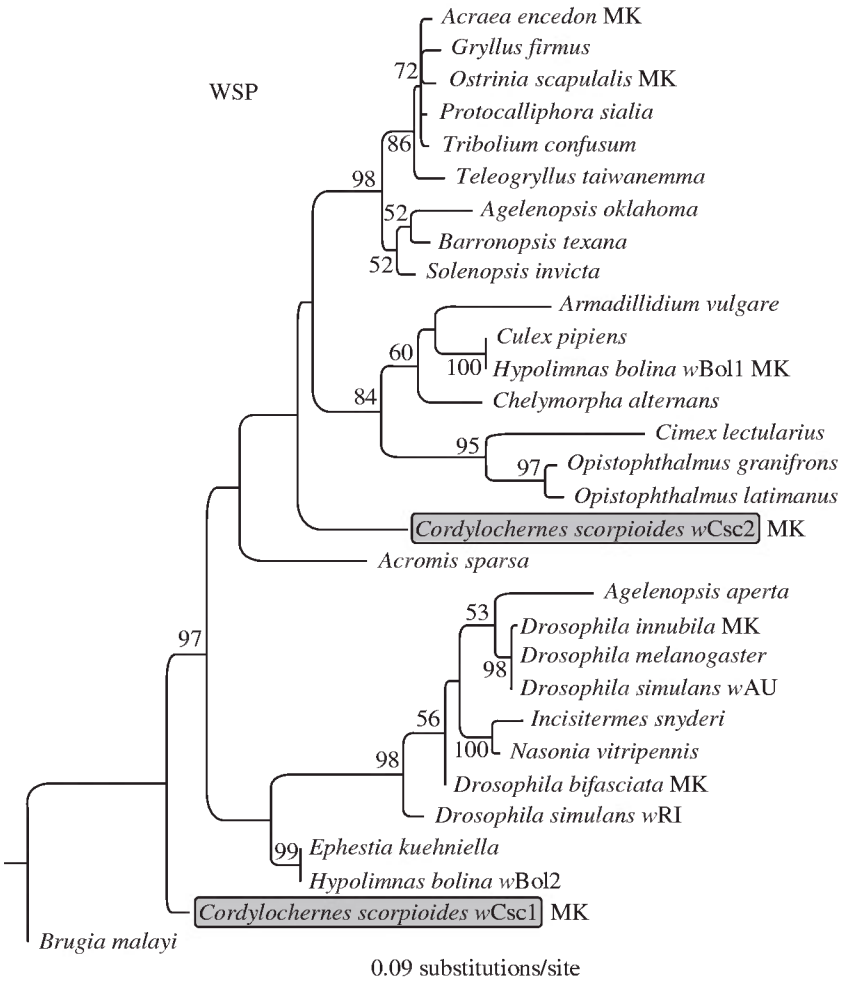

Figure 2. Maximum-likelihood phylogram of the 30 *Wolbachia* strains based on a 495 bp alignment of the *wsp* locus. *Wolbachia* strains are identified by their host taxon. The single most likely tree was determined using a GTR + G + I model of substitution (−ln L score = 4890.89, tree length = 990, consistency index = 0.452). *Wolbachia* strains implicated in MK are indicated by MK. Values at nodes denote per cent support for clades based on 500 bootstrap replicates.

male = 0.53), and to the offspring sex ratio of *w*Csc1-infected females cured with tetracycline in a previous study (proportion male = 0.51; Zeh *et al.* 2005). In addition, survival from the protonymph to the adult stage in the study reported here was high and nearly identical in the control (mean survivorship = 0.86) and tetracycline treatments (mean survivorship = 0.88). As the two treatments differed markedly in sex ratio but not in survivorship, there is essentially no scope for gender-based differences in post-embryonic mortality associated with tetracycline treatment. The finding that population sex ratio deviates only slightly from 1 : 1 is consistent with the results of a published PCR survey (Zeh *et al.* 2005), indicating low *Wolbachia* infection frequency (approx. 10%) in *C. scorpioides* from central Panama. Although this previous survey did not distinguish between strain types, preliminary results from a survey currently in progress suggest an overall *Wolbachia* infection rate of 8.6 per cent, with the *w*Csc2 strain four times as abundant as the *w*Csc1 strain (D. W. Zeh 2009, unpublished data).

Phylogenetic analysis of the five MLST loci indicates that *w*Csc2 and the previously described *w*Csc1 strain from *C. scorpioides* (Zeh *et al.* 2005; Zeh & Zeh 2006*a*) diverged only recently from a common ancestor. Over the 2063 nucleotides that constitute their MLST concatenated sequences, the two strains exhibit only 14 substitutions. Despite this close evolutionary relationship, *w*Csc1 and *w*Csc2 differ substantially in their capacity to benefit from reproductive compensation in *C. scorpioides* female hosts. While both strains are effective killers of male embryos that bias sex ratio in favour of females, in the *w*Csc1 strain, any gain from reproductive compensation in broods carried to term is strongly outweighed by a high rate of spontaneous brood abortion and the resultant overall reduction in the number of daughters born to infected females compared with uninfected controls (Zeh & Zeh 2006*a*). By contrast, in this study, rate of spontaneous brood abortion among *w*Csc2-infected females was low and unaffected by tetracycline treatment.

Interestingly, this difference between the *w*Csc1 and *w*Csc2 strains in their impact on female reproductive success in *C. scorpioides* is associated with extensive divergence in their *wsp* gene sequences, with 112 nucleotide substitutions, several indels and 48 amino acid differences over 521 aligned nucleotides. Phylogenies based on MLST and *wsp* gene sequences were topologically highly incongruent, suggesting HGT, a hypothesis that was strongly supported by our recombination detection analyses. Evidence indicates that *w*Csc2 *wsp* gene is a

mosaic sequence derived from recombination between the *w*Csc1 *wsp* sequence and the *wsp* sequence from one or more closely related *wsp* genes present in .*Wolbachia* strains that infect insects and spiders. The ensemble of seven *wsp* gene sequences identified as minor parental sequences in the recombinant *w*Csc2 sequence may signify that the true minor parental sequence was not present in the 30 analysed strains but is very similar to the sequences of these seven strains. Phylogenetic and recombination analyses thus indicate that the marked divergence between the *w*Csc1 and *w*Csc2 MK strains in their impact on host reproductive success, and hence in their potential to proliferate, has occurred in association with homologous recombination in the *wsp* gene.

Comparative studies of arthropod-infecting *Wolbachia* strains have revealed the highly chimaeric nature of *Wolbachia* genomes, with extensive evidence for recombination in *wsp* (Baldo *et al.* 2005), as well as within and between housekeeping genes such as *gltA*, *dnaA*, *ftsZ* and *groEL* (Baldo *et al.* 2006*a*). Comparative sequencing studies also suggest strong, diversifying selection acting on the *wsp* locus (Baldo *et al.* 2005), reflecting a history of antagonistic coevolution between arthropod hosts and their *Wolbachia* endosymbionts (Jiggins *et al.* 2002). In *C. scorpioides*, it remains to be determined whether *wsp* recombination is the cause of the reduced rate of spontaneous brood abortion in *w*Csc2-infected females or is simply a diagnostic marker of evolutionary divergence between the *w*Csc1 and *w*Csc2 *Wolbachia* strains.

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REFERENCES

Baldo, L., Lo, N. & Werren, J. H. 2005 Mosaic nature of the *Wolbachia* surface protein. *J. Bacteriol.* **187**, 5406–5418. (doi:10.1128/JB.187.15.5406-5418.2005)

Baldo, L., Bordenstein, S., Wernegreen, J. J. & Werren, J. H. 2006*a* Widespread recombination throughout *Wolbachia* genomes. *Mol. Biol. Evol.* **23**, 437–449. (doi:10.1093/molbev/msj049)

Baldo, L. *et al.* 2006*b* Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Appl. Environ. Microbiol.* **72**, 7098–7110. (doi:10.1128/AEM.00731-06)

Baldo, L., Prendini, L., Corthals, A. & Werren, J. H. 2007 *Wolbachia* are present in southern African scorpions and cluster with supergroup F. *Curr. Microbiol.* **55**, 367–373. (doi:10.1007/s00284-007-9009-4)

Boni, M. F., Posada, D. & Feldman, M. W. 2007 An exact nonparametric method for inferring mosaic structure in sequence triplets. *Genetics* **176**, 1035–1047. (doi:10.1534/genetics.106.068874)

Bordenstein, S. R., Paraskevopoulos, C., Hotopp, J. C. D., Sapountzis, P., Lo, N., Bandi, C., Tettelin, H., Werren, J. H. & Bourtzis, K. 2009 Parasitism and mutualism in *Wolbachia*: what the phylogenomic trees can and cannot say. *Mol. Biol. Evol.* **26**, 231–241. (doi:10.1093/molbev/msn243)

Bouchon, D., Rigaud, T. & Juchault, P. 1998 Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proc. R. Soc. Lond. B* **265**, 1081–1090. (doi:10.1098/rspb.1998.0402)

Charlat, S., Hurst, G. D. D. & Mercot, H. 2003 Evolutionary consequences of *Wolbachia* infections. *Trends Genet.* **19**, 217–223. (doi:10.1016/S0168-9525(03)00024-6)

Charlesworth, B. 1994 The evolution of lethals in the *t*-haplotype system of the mouse. *Proc. R. Soc. Lond. B* **258**, 101–107. (doi:10.1098/rspb.1994.0149)

Cosmides, L. M. & Tooby, J. 1981 Cytoplasmic inheritance and intragenomic conflict. *J. Theor. Biol.* **89**, 83–129. (doi:10.1016/0022-5193(81)90181-8)

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J. & Hurst, G. D. 2008 The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biol.* **6**, 27. (doi:10.1186/1741-7007-6-27)

Dyer, K. A. & Jaenike, J. 2004 Evolutionarily stable infection by a male-killing endosymbiont in *Drosophila innubila*: molecular evidence from the host and parasite genomes. *Genetics* **168**, 1443–1455. (doi:10.1534/genetics.104.027854)

Dyson, E. A., Kamath, M. K. & Hurst, G. D. D. 2002 *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer. *Heredity* **88**, 166–171. (doi:10.1038/sj.hdy.6800021)

Fenn, K., Conlon, C., Jones, M., Quail, M. A., Holroyd, N. E., Parkhill, J. & Blaxter, M. 2006 Phylogenetic relationships of the *Wolbachia* of nematodes and arthropods. *PLoS Pathog.* **2**, 887–899. (doi:10.1371/journal.ppat.0020094)

Fialho, R. F. & Stevens, L. 2000 Male-killing *Wolbachia* in a flour beetle. *Proc. R. Soc. Lond. B* **267**, 1469–1473. (doi:10.1098/rspb.2000.1166)

Gibbs, M. J., Armstrong, J. S. & Gibbs, A. J. 2000 Sister-scanning: a Monte Carlo procedure for assessing signals in recombinant sequences. *Bioinformatics* **16**, 573–582. (doi:10.1093/bioinformatics/16.7.573)

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. & Werren, J. H. 2008 How many species are infected with *Wolbachia*?—a statistical analysis of current data. *FEMS Microbiol. Lett.* **281**, 215–220. (doi:10.1111/j.1574-6968.2008.01110.x)

Hiroki, M., Kato, Y., Kamito, T. & Miura, K. 2002 Feminization of genetic males by a symbiotic bacterium in a butterfly, *Eurema hecabe* (Lepidoptera: Pieridae). *Naturwissenschaften* **89**, 167–170. (doi:10.1007/s00114-002-0303-5)

Hurst, L. D. 1991 The incidences and evolution of cytoplasmic male killers. *Proc. R. Soc. Lond. B* **244**, 91–99. (doi:10.1098/rspb.1991.0056)

Hurst, G. D. D. & Majerus, M. E. N. 1993 Why do maternally inherited microorganisms kill males. *Heredity* **71**, 81–95. (doi:10.1038/hdy.1993.110)

Hurst, G. D. D. *et al.* 1999 Male-killing *Wolbachia* in two species of insect. *Proc. R. Soc. Lond. B* **266**, 735–740. (doi:10.1098/rspb.1999.0698)

Hurst, G. D. D., Johnson, A. P., von der Schulenburg, J. H. G. & Fuyama, Y. 2000 Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. *Genetics* **156**, 699–709.

Jiggins, F. M., Hurst, G. D. D. & Yang, Z. H. 2002 Host-symbiont conflicts: positive selection on an outer membrane protein of parasitic but not mutualistic Rickettsiaceae. *Mol. Biol. Evol.* **19**, 1341–1349.

Jolley, K. A., Chan, M.-S. & Maiden, M. C. J. 2004 mlstdbNet—distributed multi-locus sequence typing

(MLST) databases. *BMC Bioinform.* **5**, 86. (doi:10.1186/1471-2105-5-86)

Larkin, M. A. *et al.* 2007 CLUSTALW and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948. (doi:10.1093/bioinformatics/btm404)

Martin, D. & Rybicki, E. 2000 RDP: detection of recombination amongst aligned sequences. *Bioinformatics* **16**, 562–563. (doi:10.1093/bioinformatics/16.6.562)

Martin, D. P., Posada, D., Crandall, K. A. & Williamson, C. 2005*a* A modified bootscan algorithm for automated identification of recombinant sequences and recombination breakpoints. *Aids Res. Hum. Retroviruses* **21**, 98–102. (doi:10.1089/aid.2005.21.98)

Martin, D. P., Williamson, C. & Posada, D. 2005*b* RDP2: recombination detection and analysis from sequence alignments. *Bioinformatics* **21**, 260–262. (doi:10.1093/bioinformatics/bth490)

Maynard Smith, J. 1992 Analyzing the mosaic structure of genes. *J. Mol. Evol.* **34**, 126–129.

Negri, I., Pellecchia, M., Mazzoglio, P. J., Patetta, A. & Alma, A. 2006 Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an XX/XO sex-determination system. *Proc. R. Soc. B* **273**, 2409–2416. (doi:10.1098/rspb.2006.3592)

Newcomer, S. D., Zeh, J. A. & Zeh, D. W. 1999 Genetic benefits enhance the reproductive success of polyandrous females. *Proc. Natl Acad. Sci. USA* **96**, 10 236–10 241. (doi:10.1073/pnas.96.18.10236)

Padidam, M., Sawyer, S. & Fauquet, C. M. 1999 Possible emergence of new geminiviruses by frequent recombination. *Virology* **265**, 218–225. (doi:10.1006/viro.1999.0056)

Posada, D. & Crandall, K. A. 1998 MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818. (doi:10.1093/bioinformatics/14.9.817)

Posada, D. & Crandall, K. A. 2001 Evaluation of methods for detecting recombination from DNA sequences: computer simulations. *Proc. Natl Acad. Sci. USA* **98**, 13 757–13 762. (doi:10.1073/pnas.241370698)

Rowley, S. M., Raven, R. J. & McGraw, E. A. 2004 *Wolbachia pipientis* in Australian spiders. *Curr. Microbiol.* **49**, 208–214. (doi:10.1007/s00284-004-4346-z)

SAS 2008 *The SAS system for Windows. Release 9.2*. Cary, NC: SAS Institute.

Shimodaira, H. & Hasegawa, M. 1999 Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* **16**, 1114–1116.

Stouthamer, R., Breeuwer, J. A. J., Luck, R. F. & Werren, J. H. 1993 Molecular-identification of microorganisms associated with parthenogenesis. *Nature* **361**, 66–68. (doi:10.1038/361066a0)

Swofford, D. L. 2002 *PAUP**: Phylogenetic analysis using parsimony (*and other methods), Version 4.0B10. Sunderland, MA: Sinauer Associates.

Weiller, G. F. 1998 Phylogenetic profiles: a graphical method for detecting genetic recombinations in homologous sequences. *Mol. Biol. Evol.* **15**, 326–335.

Werren, J. H. 1987 The coevolution of autosomal and cytoplasmic sex-ratio factors. *J. Theor. Biol.* **124**, 317–334. (doi:10.1016/S0022-5193(87)80119-4)

Werren, J. H., Baldo, L. & Clark, M. E. 2008 *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741–751. (doi:10.1038/nrmicro1969)

Weygoldt, P. 1969 *The biology of pseudoscorpions*. Cambridge, MA: Harvard University Press.

Zeh, D. W., Zeh, J. A. & Bonilla, M. M. 2005 *Wolbachia*, sex ratio bias and apparent male killing in the harlequin beetle riding pseudoscorpion. *Heredity* **95**, 41–49. (doi:10.1038/sj.hdy.6800666)

Zeh, J. A. & Zeh, D. W. 2006*a* Male-killing *Wolbachia* in a live-bearing arthropod: brood abortion as a constraint on the spread of a selfish microbe. *J. Invert. Pathol.* **92**, 33–38. (doi:10.1016/j.jip.2006.02.004)

Zeh, J. A. & Zeh, D. W. 2006*b* Outbred embryos rescue inbred half-siblings in mixed-paternity broods of live-bearing females. *Nature* **439**, 201–203. (doi:10.1038/nature04260)