ORIGINAL ARTICLE

The effects of, and interactions between, *Cardinium* and *Wolbachia* in the doubly infected spider mite *Bryobia sarothamni*

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Many arthropods are infected with vertically transmitted, intracellular bacteria manipulating their host's reproduction. Cytoplasmic incompatibility (CI) is commonly observed and is expressed as a reduction in the number of offspring in crosses between infected males and uninfected females (or females infected with a different bacterial strain). CI is often related to the presence of *Wolbachia*, but recent findings indicate that a second reproductive parasite, *Cardinium*, is also capable of inducing CI. Although both *Wolbachia* and *Cardinium* occur in arthropods and may infect the same host species, little is known about their interactions. We observed *Wolbachia* and *Cardinium* in the sexual spider mite *Bryobia sarothamni* (Acari: Tetranychidae) and investigated the effects of both bacteria on reproduction. We performed all possible crossing combinations using naturally infected strains, and show that *Cardinium* induces strong CI,

expressed as an almost complete female mortality. *B. sarothamni* is the third host species in which *Cardinium*-induced CI is observed, and this study reveals the strongest CI effect found so far. *Wolbachia*, however, did not induce CI. Even so, CI was not induced by doubly infected males, and neither singly *Wolbachia*-infected nor doubly infected females could rescue CI induced by *Cardinium*-infected males. Possibly, this is related to the differences between *Cardinium* strains infecting singly and doubly infected individuals. We found a cost of infection in single infected individuals, but not in doubly infected individuals. We show that infection frequencies in field populations ranged from completely uninfected to a polymorphic state. In none of the populations infections were fixed.

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Introduction

Vertically transmitted, intracellular bacteria that manipulate their host's reproductive mode and thereby enhance their own spread are termed reproductive parasites (O'Neill *et al.*, 1997). These manipulations include male killing, feminization, thelytokous parthenogenesis and cytoplasmic incompatibility (CI), and result in an increased proportion of infected females (the transmitting sex) in the host population. CI is the most common effect associated with reproductive parasites (O'Neill *et al.*, 1997; Stouthamer *et al.*, 1999). It is observed in crosses between infected males with females that are either uninfected (unidirectional CI) or infected with a different, incompatible symbiont strain (bidirectional CI). All other crosses are compatible. In this way, the fitness of uninfected or differentially infected females is reduced compared to the fitness of infected females. As a result, the infection will spread.

Cytoplasmic incompatibility is the result of paternal genome fragmentation in fertilized eggs. The exact

mechanisms of CI are unknown, but its cytological effects are clear. The mechanism conforms to a 'modification–rescue' system (Hoffmann and Turelli, 1997; Werren, 1997). Sperm is modified in the testes, and as a result, the paternal chromosomes show improper segregation during early mitotic divisions (Breeuwer and Werren, 1990; Reed and Werren, 1995; Tram *et al.*, 2006). This modification is 'rescued' if appropriate symbiont strains are present in the egg. Thus, fertilization is successful when both male and female are infected by symbiont strains with the same modification–rescue system. Strains that can both modify and rescue are typed as $\text{mod}^+ \text{resc}^+$. Other strain types, such as strains that do not modify but are capable of rescuing ($\text{mod}^- \text{resc}^+$) or strains that neither modify nor rescue ($\text{mod}^- \text{resc}^-$), exist as well. (Bourtzis *et al.*, 1998; Merçot and Poinot, 1998; Vala *et al.*, 2002; Zabalou *et al.*, 2004). CI leads to a reduction in the number of surviving hybrid offspring. In diploid host species, this is expressed by increased F_1 mortality. Usually, the embryos die before hatching, and therefore an increased number of unhatched eggs is observed (Hoffmann and Turelli, 1997). In haplodiploid host species, two types of CI effects—increased female mortality (the hybrid sex in haplodiploids) and increased male production—have been described (Breeuwer and Werren, 1993; Breeuwer, 1997; Vavre *et al.*, 2000; Bordenstein *et al.*, 2003; Mouton *et al.*, 2005). In the latter case, fertilized eggs develop as

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normal, haploid, males after complete elimination of the paternal chromosomes.

Cytoplasmic incompatibility caused by intracellular bacteria has been shown in a large number of arthropods (O'Neill *et al.*, 1997; Werren, 1997; Stouthamer *et al.*, 1999). In the majority of cases, CI was related to the presence of the endosymbiont *Wolbachia*, a genus in the α -Proteobacteria. *Wolbachia* is widespread among arthropods and nematodes. It is estimated that more than 20% of all arthropods are infected with *Wolbachia* (Werren *et al.*, 1995; Breeuwer and Jacobs, 1996; Jeyaparakash and Hoy, 2000; Hilgenboecker *et al.*, 2008). *Wolbachia* is involved in causing CI in many insects, isopods and mites (reviewed by Stouthamer *et al.*, 1999). More recently, CI was found to be associated with the endosymbiont *Cardinium*, which belongs to the Cytophaga–Flavobacterium–Bacteroides phylum, a phylum that is unrelated to the α -Proteobacteria. *Cardinium* has so far been found in 6–7% of all arthropods, and seems to be more common in chelicerates than in insects (Weeks *et al.*, 2003; Zchori-Fein and Perlman, 2004; Duron *et al.*, 2008). *Cardinium* is the first bacterium other than *Wolbachia* that causes CI (Hunter *et al.*, 2003). Only two cases of *Cardinium*-induced CI have been found so far—in the parasitoid wasp *Encarsia pergandiella* (Hunter *et al.*, 2003), and in the spider mite *Eotetranychus suginamensis* (Gotoh *et al.*, 2006).

Cardinium and *Wolbachia* have been found coinfecting the same host species (Weeks *et al.*, 2003; Zchori-Fein and Perlman, 2004; Gotoh *et al.*, 2006; Duron *et al.*, 2008). Although both *Cardinium* and *Wolbachia* can manipulate their host's reproductive system, little is known about their interactions. In this study, we investigate the effects of both *Wolbachia* and *Cardinium*, and their interactions, in the sexually reproducing spider mite *Bryobia sarothamni*, using isofemale lines obtained from naturally infected individuals. This phytophagous mite species feeds on common broom (*Cytisus scoparius*), and we detected both *Wolbachia* and *Cardinium* in this mite species. We test whether both bacteria independently induce CI, whether they are mutually incompatible (bidirectional CI), whether CI is induced in doubly infected individuals, and whether one bacterium can rescue CI induction of the other (see Figure 1 for an overview of tested hypotheses). We additionally investigate the distribution and infection frequency of both bacteria in five populations.

Materials and methods

Sample collection

Bryobia sarothamni mites were collected from *C. scoparius* plants from five European populations in July and August 2006 (Table 1). Populations from Vireux (FR16) and Vierves sur Viroin (BEL5 and BEL6) are less than 5 km apart, whereas the other two populations from Piriac sur Mer (FR21) and Schoorl (NL17) were more distant from each other and from the aforementioned populations. Individual mites (male and female) were either immediately analyzed in the laboratory to assess the infection frequency (see below), or individual females were reared as isofemale lines in a controlled climate room (20 °C, 60% relative humidity and 16 L:8 D photoperiod). Isofemale lines were initiated by

putting a single adult female on a single *C. scoparius* branch. Individual branches were placed in water-soaked square blocks of florist's foam covered with parafilm. Foam blocks were placed in plastic trays filled with water. Females were allowed to lay eggs for 1–2 weeks and were subsequently analyzed in the laboratory for infection status (see below). Two doubly infected (*Wolbachia* and *Cardinium*; I_{WC}), four singly *Wolbachia*-infected (I_W), one singly *Cardinium*-infected (I_C), and two uninfected lines (U) were selected and maintained for subsequent experiments (Table 1). For each of these isofemale lines, the infection status was checked during the course of the experiments by testing randomly picked individuals for infection. No changes in infection status were observed during the experiment.

Infection frequency

We screened 250 field collected individual mites from five populations for infection with *Wolbachia* and/or *Cardinium* (Table 1). DNA was extracted from single mites using the cetyltrimethylammonium bromide (CTAB) extraction method as described previously (Ros and Breeuwer, 2007). Presence of *Wolbachia* was detected using PCR amplification of *wsp* and *gltA*. Presence of *Cardinium* was detected using PCR amplification of a part of the 16S rDNA and *gyrB*. *Wsp* was amplified using the primers *wsp*-81F and *wsp*-691R (Braig *et al.*, 1998), and *gltA* using the primers *gltA*-F 5'-GAYCATGARCAR AAYGCTTCTAC-3' and *gltA*-R 5'-CCHGARTAAAAAT CAACRTTDGG-3', designed from available *Wolbachia* and *Rickettsia* genome sequences (Andersson *et al.*, 1998; McLeod *et al.*, 2004; Wu *et al.*, 2004), and tested on isolates representative of *Wolbachia* supergroups A and B. *Cardinium* 16S rDNA was amplified using the primers CLOf and CLOr1 (Weeks *et al.*, 2003). *GyrB* was amplified using primers from Groot and Breeuwer (2006).

PCR amplifications were performed in 25 μ l reactions containing 2.5 μ l 10 $\times$ Super Taq buffer (HT BioTechnology, Cambridge, UK), 1.25 μ l bovine serum albumin (10 mg ml⁻¹), 1.25 μ l MgCl₂ (25 mM), 5 μ l dNTP mix (1 mM of each nucleotide), 0.2 μ l of each primer (20 μ M each), 0.2 μ l of Super Taq (5 U μ l⁻¹; HT BioTechnology), 11.9 μ l water and 2.5 μ l of DNA extract. For 16S rDNA, no MgCl₂ and bovine serum albumin were added. PCR cycling profile for *wsp* was 35 cycles of 30 s at 95 °C, 30 s at 51 °C and 1 min at 72 °C; for *Cardinium* 16S rDNA, it was 35 cycles of 40 s at 95 °C, 40 s at 57 °C and 45 s at 72 °C; and for *gltA* and *gyrB*, it was 35 cycles of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C. Products (2 μ l) were visualized on a 1% agarose gel stained with ethidium bromide in 0.5 $\times$ TBE buffer (45 mM Tris base, 45 mM boric acid and 1 mM EDTA, pH 8.0).

PCR amplification of a part of the mitochondrial COI gene was used as a positive control for the DNA extraction. PCR was performed as described in Ros and Breeuwer (2007) with the primers COI_F3 (5'-WGTHTTAGCAGGAGCAATTACWAT-3'; modified from Toda *et al.*, 2000) and COI_R2 (5'-AAWCCTCTAA AAATRGCAATACRGC-3'; modified from Hinomoto and Takafuji, 2001). Samples for which amplification of COI failed were excluded from the dataset.

Single infections

Figure 1 Overview of research questions and associated crossing schemes needed to determine the role of *Wolbachia* and *Cardinium* in cytoplasmic incompatibility (CI) induction in singly and doubly infected host individuals.

Table 1 Origin of *Bryobia sarothamni* populations included in this study, and the name and infection status of IF lines that were used in the crossing experiments

Population	Country	Locality	Collection date	IF	Infection
BEL5	Belgium	Vierves sur Viroin	July 2006	BEL5_2 BEL5_3	I_W U
BEL6	Belgium	Vierves sur Viroin	July 2006		
FR16	France	Vireux	July 2006	FR16_1 FR16_2 ^a FR16_3 FR16_4 FR16_6 FR16_7 FR16_8	I_W I_{WC} I_W I_{WC} U I_{WC} I_W
FR21	France	Piriac sur mer	Aug 2006	FR21_2	I_C
NL17	Netherlands	Schoorl	Aug 2006		

Abbreviations: U, uninfected; IF, isofemale; I_C , *Cardinium*-infected; I_W , *Wolbachia*-infected; I_{WC} , doubly infected (*Wolbachia* and *Cardinium*).
^aThis IF line was used for 16S amplification and cloning, but was not included in the crossing experiments. Population BEL6 and NL17 were included in the assessment of infection frequencies, but not in the crossing experiments.

Screening for other reproductive parasites

To test for multiple infections by other bacteria, we amplified and cloned the 16S rDNA gene for five pooled individuals per isofemale line (one of each infection status). DNA was extracted following the CTAB method (Ros and Breeuwer, 2007). 16S rDNA was amplified using the primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1513R 5'-ACGGYTACCTTGTTACGACTT-3' (Weisburg *et al.*, 1991) in a 25 μ l reaction mix containing 2.5 μ l of 10 $\times$ Super Taq buffer, 5 μ l dNTP mix (1 mM of each nucleotide), 0.5 μ l of each primer (10 μ M each), 0.2 μ l of super Taq (5 U μ l⁻¹), 13.3 μ l water and 3 μ l of DNA extract. PCR cycling profile was 35 cycles of 1 min at 94 $^{\circ}$ C, 1 min at 54 $^{\circ}$ C and 1 min at 72 $^{\circ}$ C. PCR products were cleaned using the method of Boom *et al.* (1990). The cleaned products were ligated and bacteria were transformed using the pGEM-T Easy Vector System and JM109 competent cells (Promega, Madison WI, USA). 10–11 clones were sequenced per sample, using

the M13 forward primer. The sequence of each clone was compared to sequences present in GenBank using BLAST (www.ncbi.nlm.nih.gov/blast).

Crossing experiment

The effects of *Wolbachia* and/or *Cardinium* on host reproduction were established by combining doubly infected, singly infected and uninfected mites (see Figure 1 for crosses and their possible effects). For the crossing experiments, mites were used from nine isofemale lines that were started from naturally infected (singly or doubly) and uninfected mites collected from three populations (Table 1). Infection status of each line was assessed by screening individual females as described above. For each possible infection status (I_{WC} , I_W , I_C and U), 1–4 isofemale lines were used (Table 1). Age cohorts were produced for each of these selected lines, by groups of females laying eggs for 1–2

days on *C. scoparius* branches. Offspring of these cohorts were used in crossing experiments. Ideally, antibiotic cured isofemale lines should be used to account for host background effects. However, antibiotic treatments with tetracycline or doxycycline of doubly infected females were so far unsuccessful in creating all other types of infection among the offspring. Therefore, we used naturally infected and uninfected lines. Mites were collected as (virgin) teleiochrysalids (last resting stage before the adult reproductive phase). Pupae were placed individually on filter paper in petridishes covered with parafilm, and checked daily for emergence. Upon emergence, pairs of one female and one male were formed and placed on a single *C. scoparius* branch. Adult males and females were maximum 1 day old, except for four cases where males were 2 days old. Males were removed after 24 h. Males were only used once, except for two cases where males mated twice. Females were removed after 10 days (which generally means 8 days of egg laying; the first 2 days after emergence no eggs are produced). The number of eggs, unhatched eggs, emerging adult offspring (sons and daughters) and mortality (dead stages excluding eggs) were scored per cross.

Fitness effects

The fitness costs of infection with *Wolbachia*, *Cardinium*, or both, were assessed by comparing the fecundity of females with the four possible infection types. We compared crosses in which females were crossed with uninfected males to exclude any influence of differences in male fertility due to infection.

Statistical analyses

Groups of crosses were tested for differences in investigated traits using the software package SPSS version 15.0 (Chicago, IL, USA). Tests were performed for individual bacterial effects, and for interactions between *Wolbachia* and *Cardinium* (Table 2). Data were first tested for normality (Kolmogorov–Smirnov test) and homogeneity of group variances (Levene's test). Where possible, square-root, logarithmic or arcsine transformations were performed to attain normality and homogeneity of variances. A one-way analysis of variance was performed for each trait (number of eggs laid, unhatched eggs, sex ratio (% males), number of offspring, number of sons, number of daughters and mortality) separately to determine whether there was heterogeneity among different crosses with respect to each trait. If heterogeneity was significant, pairwise comparisons were performed using Tukey *post hoc* tests. Additional nonparametric tests (Kruskal–Wallis) were used when analysis of variance assumptions on normality and homogeneity of variances were violated and could not be met by data transformations. Crosses in which fewer than nine eggs were laid were excluded from CI analyses. We excluded cases where all male offspring in combination with normal clutch size, high egg hatchability and low juvenile mortality (that is, number of unhatched eggs and dead stages was less than four) was recorded, as it is most likely due to females not having mated. Differences in fecundity between each of the four infection types were determined using the same statistical approach as outlined above.

Results

Infection frequency

We screened 250 individual mites, collected from five field populations, for infection with *Wolbachia* and/or *Cardinium* (Figure 2). Four populations appeared to be infected—two with both *Cardinium* and *Wolbachia* and two with *Wolbachia* only. Only the population from the Netherlands (NL17) was uninfected. Population FR16 is polymorphic for infection with *Wolbachia* as well as *Cardinium*—59% of the individuals harbor both *Wolbachia* and *Cardinium*, 36% is only infected with *Wolbachia*, 2% only with *Cardinium*, and 3% is uninfected. Populations BEL5 and BEL6 are closely located to FR16, but harbor only *Wolbachia* and no *Cardinium*—53% (BEL5) and 73% (BEL6) of the individuals are *Wolbachia*-infected. The more distantly located population FR21 shows a high frequency of doubly infected individuals (89%) and no singly infected individuals; however, only nine individuals were screened for this population.

Screening for other reproductive parasites

We screened four isofemale lines (one of each infection status) for infections with other bacteria. Cloning of 16S rDNA shows that strain FR16_1 is infected with *Wolbachia* only, as no double infections with other bacteria are found. The ten clones sequenced show minimal sequence differences (<1%). Differences were randomly distributed across first, second and third codon positions, and in some cases lead to stop codons or amino-acid changes. It is, therefore, likely that these differences are sequence artifacts, and do not concern multiple infections. For *B. sarothamni* strain FR21_2, all clones were *Cardinium* sequences, except one clone, which was identified as *Ochrobactrum* spec. (α -Proteobacteria). *Ochrobactrum* has been described from nodules of *C. scoparius* (Zurdo-Piñero *et al.*, 2007), which is the host plant of *B. sarothamni*. This clone was considered as contamination and discarded. Within *B. sarothamni* strain FR16_2 both *Wolbachia* (eight clones) and *Cardinium* (two clones) were detected. This confirms the double infection found by PCR amplification. Within *B. sarothamni* strain FR16_6 neither *Wolbachia* nor *Cardinium* were detected, confirming earlier PCR results. No other reproductive parasites, such as *Rickettsia* or *Spiroplasma*, previously shown to be common in spiders (Goodacre *et al.*, 2006), were detected in any of the cloned samples. The sequences found most likely represent general contaminant bacteria—six clones were similar to *Streptococcus* spp. (human oral clones), two to the family Flavobacteriaceae (human oral clones), one to a soil bacterium (family Xanthomonadaceae) and one to an unidentified human oral bacterium.

Crossing experiments

Individual bacterial effects: The number of aborted eggs was significantly different among the four crosses in which the effect of *Cardinium* was investigated (Table 2a). In the predicted incompatible cross ($U \times I_C$; Figure 1), on average, 56.9% of all eggs did not hatch, against 3.6–10.2% in the other crosses. The sex ratio of the offspring that did hatch in the incompatible cross approached 100%; only in one of the crosses, a single female was produced. Related to the large number of

Table 2 Results of crosses between U, I_C , I_W and I_{WC} *Bryobia sarothamni* strains

Effect	Cross $F \times M$	N	Number of eggs	% Unhatched eggs	Sex ratio (% males)	Sons	Daughters	Total offspring	% Mortality (excl. eggs)
(a)									
Single effect: <i>Cardinium</i> CI?	$U \times U$	20	20.0 ± 1.3	3.6 ± 1.0 ^a	39.1 ± 4.0 ^a	5.5 ± 0.8	8.3 ± 1.1 ^a	13.8 ± 1.6 ^a	10.4 ± 2.4
	$U \times I_C$	16	19.1 ± 1.3	56.9 ± 4.4 ^b	96.9 ± 3.1 ^b	3.9 ± 0.5	0.1 ± 0.1 ^b	3.9 ± 0.5 ^b	3.0 ± 0.8
	$I_C \times U$	17	16.6 ± 0.8	10.2 ± 2.3 ^a	48.8 ± 5.9 ^{a,c}	4.7 ± 0.7	5.0 ± 0.8 ^c	9.7 ± 0.8 ^a	4.6 ± 1.0
	$I_C \times I_C$	19	17.5 ± 0.8	4.9 ± 1.1 ^a	56.9 ± 3.6 ^c	5.4 ± 0.6	4.3 ± 0.6 ^c	9.7 ± 1.0 ^a	10.3 ± 3.0
			NS	***	***	NS	***	***	NS
(b)									
Single effect: <i>Wolbachia</i> CI?	$U \times U$	20	20.0 ± 1.3	3.6 ± 1.0 ^{a,b}	39.1 ± 4.0	5.5 ± 0.8	8.3 ± 1.1	13.8 ± 1.6	10.4 ± 2.4
	$U \times I_W$	15	21.5 ± 1.5	8.6 ± 2.0 ^{a,b}	44.2 ± 4.1	6.3 ± 0.8	8.5 ± 1.1	14.7 ± 1.4	5.1 ± 1.5
	$I_W \times U$	16	17.7 ± 1.0	10.6 ± 3.5 ^b	38.6 ± 6.0	3.4 ± 0.7	6.3 ± 1.0	9.6 ± 1.2	8.4 ± 2
	$I_W \times I_W$	14	17.2 ± 1.5	1.6 ± 1.3 ^a	33.7 ± 4.8	3.9 ± 0.9	6.6 ± 1.1	10.4 ± 1.7	8.6 ± 2.6
			NS	**	NS	NS	NS	NS	NS
(c)									
Interaction: double infection CI?	$U \times U$	20	20.0 ± 1.3	3.6 ± 1.0	39.1 ± 4.0	5.5 ± 0.8	8.3 ± 1.1	13.8 ± 1.6	10.4 ± 2.4
	$U \times I_{WC}$	18	21.7 ± 1.7	15.5 ± 5.3	50.9 ± 6.5	6.6 ± 1.2	6.9 ± 1.3	13.4 ± 2.1	6.7 ± 3.7
	$I_{WC} \times U$	11	22.4 ± 1.5	4.4 ± 1.4	28.7 ± 6.5	4.4 ± 1.4	11.3 ± 1.7	15.6 ± 1.7	5.9 ± 2.5
	$I_{WC} \times I_{WC}$	9	21.8 ± 1.4	2.3 ± 1.1	33.8 ± 7.1	6.0 ± 1.4	11.7 ± 1.6	17.7 ± 1.3	5.0 ± 2.5
			NS	NS	NS	NS	NS	NS	NS
(d)									
Interaction: <i>Cardinium</i> CI rescued?	$U \times I_C$	16	19.1 ± 1.3	56.9 ± 4.4 ^a	96.9 ± 3.1 ^a	3.9 ± 0.5 ^{a,b}	0.1 ± 0.1 ^a	3.9 ± 0.5 ^a	3.0 ± 0.8
	$I_C \times I_C$	19	17.5 ± 0.8	4.9 ± 1.1 ^b	56.9 ± 3.6 ^b	5.4 ± 0.6 ^a	4.3 ± 0.6 ^b	9.7 ± 1.0 ^b	10.3 ± 3.0
	$I_W \times I_C$	12	15.6 ± 1.2	50.6 ± 6.8 ^a	100.0 ± 0.0 ^a	3.2 ± 0.8 ^{a,b}	0.0 ± 0.0 ^a	3.2 ± 0.8 ^a	7.5 ± 2.5
	$I_{WC} \times I_C$	12	17.0 ± 1.0	67.2 ± 5.7 ^a	100.0 ± 0.0 ^a	2.4 ± 0.6 ^b	0.0 ± 0.0 ^a	2.4 ± 0.6 ^a	2.3 ± 1.0
			NS	***	***	**	***	***	NS
(e)									
Interaction: remaining crosses	$U \times I_W$	15	21.5 ± 1.5	8.6 ± 1.8 ^a	44.2 ± 5.1 ^{a,b}	6.3 ± 0.9	8.5 ± 1.1	14.7 ± 1.5	5.1 ± 2.9
	$I_C \times I_W$	16	17.9 ± 1.4	10.5 ± 1.8 ^a	58.2 ± 4.9 ^a	6.8 ± 0.9	4.6 ± 1.0	11.3 ± 1.5	6.5 ± 2.8
	$I_W \times I_W$	14	17.1 ± 1.5	1.6 ± 1.9 ^b	33.7 ± 5.3 ^b	3.9 ± 1.0	6.6 ± 1.1	10.4 ± 1.6	8.6 ± 3.0
	$I_{WC} \times I_W$	16	17.6 ± 1.4	4.5 ± 1.8 ^{a,b}	34.7 ± 4.9 ^b	4.6 ± 0.9	8.0 ± 1.0	12.6 ± 1.5	11.1 ± 2.8
			NS	**	**	NS	*	NS	NS
(f)									
Interaction: remaining crosses	$U \times I_{WC}$	18	21.7 ± 1.7	15.5 ± 5.3	50.9 ± 6.5	6.6 ± 1.2	6.9 ± 1.3 ^{a,b}	13.4 ± 2.1 ^{a,b}	6.7 ± 3.7
	$I_C \times I_{WC}$	10	17.9 ± 0.9	11.2 ± 3.7	56.3 ± 5.8	6.3 ± 0.8	5.2 ± 1.1 ^a	11.5 ± 1.2 ^{a,b}	3.7 ± 1.1
	$I_W \times I_{WC}$	10	16.2 ± 1.6	6.6 ± 1.5	46.4 ± 5.6	4.3 ± 0.8	5.0 ± 1.1 ^a	9.3 ± 1.6 ^a	12.9 ± 3.0
	$I_{WC} \times I_{WC}$	9	21.8 ± 1.4	2.3 ± 1.1	33.8 ± 7.1	6.0 ± 1.4	11.7 ± 1.6 ^b	17.7 ± 1.3 ^b	5.0 ± 2.5
			NS	NS	NS	NS	**	*	NS

Abbreviations: I_C , *Cardinium*-infected; I_W , *Wolbachia*-infected; I_{WC} , doubly infected (*Wolbachia* and *Cardinium*); N, number of replicates; NS, not significant; U, uninfected. Rows a–f contain groups of crosses that were compared for each trait. Traits are listed in the top row. Values for each trait are mean ± s.e. The effect that was tested for is listed in the left column. Outcomes of statistical analyses are listed for each trait and each group of crosses. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. For each group of crosses, comparisons within a column marked with the same superscript (a, b or c) are not significantly different ($P > 0.05$) by a Tukey's *post hoc* test.

Figure 2 Infection frequencies of uninfected, singly infected (*Wolbachia* or *Cardinium*) and doubly infected (*Wolbachia* and *Cardinium*) individuals of *Bryobia sarothamni* in five European populations. n = sample size.

aborted eggs, the total number of offspring was also significantly lower in the incompatible cross. This was because of a decrease in the number of females produced, as the number of males produced, was not significantly different between the four crosses. The number of eggs produced and the mortality (of nonegg stages) were not significantly different among the four crosses.

Among the four crosses in which we tested possible *Wolbachia* CI effects, no significant differences were found between the predicted incompatible cross and the other crosses for any of the investigated traits (Table 2b). A significant difference in the number of aborted eggs was found between crosses $I_W \times U$ and $I_W \times I_W$. The low number of aborted eggs in the $I_W \times I_W$ cross was, however, likely due to a problem with the experimental setup. In the $I_W \times I_W$ cross, a high number of eggs were drowned (these eggs were not included in the computation of the number of aborted eggs).

Interactions between *Wolbachia* and *Cardinium*

Doubly infected males (I_{WC}) did not induce CI (Table 2c). No significant effects were found for any of the investigated traits for the four crosses. The average number of aborted eggs was higher in the predicted incompatible cross ($U \times I_C$), but it is not significantly different from the other three crosses.

Concluding from the results in Table 2a–c, it is believed that singly infected *Cardinium* individuals

induce strong CI, whereas singly infected *Wolbachia* or doubly infected individuals do not induce CI. We compared all possible crosses involving singly infected *Cardinium* males to investigate in which crosses the induced CI was rescued. As is apparent from Table 2d, *Cardinium*-induced CI is only rescued in crosses with *Cardinium*-infected females. Crosses involving uninfected, *Wolbachia*-infected or doubly infected females all show strong CI expressed in a reduced egg hatchability and a male-biased sex ratio. Therefore, CI induced by *Cardinium* is neither rescued by *Wolbachia* nor by doubly infected individuals.

We also investigated other possible crosses, involving *Wolbachia*-infected males (Table 2e) and doubly infected males (Table 2f). Among the four crosses involving *Wolbachia*-infected males, a significant difference in the number of aborted eggs and in the sex ratio was found; the cross between *Wolbachia*-infected females and males ($I_W \times I_W$) had a lower number of aborted eggs than the three other crosses ($U \times I_W$, $I_C \times I_W$ and $I_{WC} \times I_W$). The number is, however, also lower than that found in many other crosses, and might be due to the fact that a high number of eggs was lost in this cross due to a problem with the experimental setup (a number of eggs got wet; see above). The sex ratio in the $I_C \times I_W$ cross was higher than in the $I_W \times I_W$ and $I_{WC} \times I_{WC}$ crosses, but not higher than the $U \times I_W$ cross.

Fitness effects

We measured fitness costs of infection by comparing the fecundity of singly, doubly and uninfected females. Egg production was the highest in doubly infected females, and significantly higher than the egg production of singly (*Wolbachia* or *Cardinium*) infected females (Table 3). It was not significantly different from the egg production of uninfected females. The same pattern was observed for the number of daughters, and therefore also for the total number of offspring produced. The remaining traits were not significantly different between the four crosses.

When all crosses are included in the analysis (that is, including crosses with singly and doubly infected males), the differences in egg production are less obvious (Table 3). The number of eggs produced by doubly infected females was not significantly different from females of any of the other infection types. This is mainly due to the fact that fewer eggs were produced in crosses between doubly infected individuals and either singly *Cardinium*-, or singly *Wolbachia*-infected males (Table 2). The number of eggs produced by singly infected lines was significantly lower than the number produced by uninfected lines.

Discussion

Cardinium causes severe CI in the sexual spider mite *B. sarothamni*. CI is expressed as a strong reduction in egg hatchability and a strongly male-biased sex ratio in crosses between uninfected females and infected males. The CI expression is nearly complete: only one daughter was produced in all incompatible crosses, whereas male production was not significantly different from the compatible crosses. The observed pattern suggests that fertilized eggs, which would normally develop into females, suffer from mortality. This is concordant with the female mortality type of CI (Breeuwer, 1997; Vavre

Table 3 Fitness of U, *I_C*, *I_W* and *I_{WC}* *Bryobia sarothamni* strains

Cross F × M	N	Crossings with uninfected males							All crossings		
		Number of eggs	% Unhatched eggs	Sex ratio (% males)	Sons	Daughters	Total offspring	% Mortality (excl eggs)	Inf.	N	Nr. of eggs
U × U	20	20.0 ± 1.3 ^{a,b}	3.6 ± 1.0	39.1 ± 4.0	5.5 ± 0.8	8.3 ± 1.1 ^{a,b}	13.8 ± 1.6 ^{a,b}	10.4 ± 2.4	U	75	20.0 ± 0.7 ^b
<i>I_C</i> × U	17	16.6 ± 0.8 ^a	10.2 ± 2.3	48.8 ± 5.9	4.7 ± 0.7	5.0 ± 0.8 ^a	9.7 ± 0.8 ^a	4.6 ± 1.0	<i>I_C</i>	64	16.2 ± 0.7 ^a
<i>I_W</i> × U	16	17.7 ± 1.0 ^a	10.6 ± 3.5	38.6 ± 6.0	3.4 ± 0.7	6.3 ± 1.0 ^a	9.6 ± 1.2 ^a	8.4 ± 2.0	<i>I_W</i>	68	16.7 ± 0.4 ^a
<i>I_{WC}</i> × U	11	22.4 ± 1.5 ^b	4.4 ± 1.4	28.7 ± 6.5	4.4 ± 1.4	11.3 ± 1.7 ^b	15.6 ± 1.7 ^b	5.9 ± 2.5	<i>I_{WC}</i>	60	17.9 ± 0.7 ^{a,b}
		**	NS	NS	NS	**	*	NS			**

Abbreviations: *I_C*, *Cardinium*-infected; *I_W*, *Wolbachia*-infected; *I_{WC}*, doubly infected (*Wolbachia* and *Cardinium*); N, number of replicates; NS, not significant; U, uninfected.

Traits are listed in the top row. Values for each trait are mean ± s.e. Outcomes of statistical analyses are listed for each trait and each group of crosses. **P* < 0.05; ***P* < 0.01. For each group of crosses, comparisons within a column marked with the same superscript (a or b) are not significantly different (*P* > 0.05) by a Tukey's *post hoc* test.

et al., 2000; Bordenstein *et al.*, 2003; Mouton *et al.*, 2005). *B. sarothamni* is the third host species in which *Cardinium*-induced CI is observed, and this study reveals the strongest CI effect found for *Cardinium* so far. The other two studies on *Cardinium* CI found incomplete CI expression; in the parasitoid wasp *E. pergandiella*, 87% fewer daughters were produced in incompatible crosses, and in the spider mite *E. suginamensis*, this was 46% (Hunter *et al.*, 2003; Gotoh *et al.*, 2006). Female mortality is the only CI type found for *Cardinium* so far (Hunter *et al.*, 2003; Gotoh *et al.*, 2006). It is also the most common type of CI expression observed for *Wolbachia* in haplo-diploid species (Breeuwer, 1997; Vala *et al.*, 2000; Vavre *et al.*, 2000; Mouton *et al.*, 2005). The second CI type that has been observed in haplodiploid species is the male development type, which is expressed as an increased male production due to the conversion of fertilized diploid eggs into haploid eggs in early development (Breeuwer and Werren, 1993; Breeuwer, 1997; Vavre *et al.*, 2000; Bordenstein *et al.*, 2003; Mouton *et al.*, 2005). Until now, this type of CI has not been observed for *Cardinium*.

Although we found a strong CI effect for *Cardinium* in *B. sarothamni*, no CI was induced by *Wolbachia*. There are several potential explanations for the absence of *Wolbachia*-induced CI.

Environmental factors including age of host, remating frequency or temperature may influence the degree of CI as they affect bacterial densities in the host (Clancy and Hoffmann, 1998; Reynolds and Hoffmann, 2002; Weeks *et al.*, 2002). Bacterial densities are known to influence the level of CI expression (Breeuwer and Werren, 1993; Hoffmann and Turelli, 1997; Clancy and Hoffmann, 1998; Perrot-Minnot and Werren, 1999). Breeuwer and Werren (1993) showed that males of a strain with higher infection densities are incompatible with females from strains with lower bacterial densities. In our experiments, we controlled for these factors by using 1-day-old males and females, mating males only once, and performing experiments at a constant temperature. Therefore, it is unlikely that these factors are the reason for the lack of CI induction by *Wolbachia*.

Other factors that may influence CI expression are host genetic background or interactions between bacteria and host species (Stouthamer *et al.*, 1999; Weeks *et al.*, 2002). Contrasting results regarding host genetic background have been found; some studies revealed that host genetic

background may account for variations in CI expression (Boyle *et al.*, 1993; McGraw *et al.*, 2001; Bordenstein *et al.*, 2003), whereas others showed that the expression is independent of the host genome (Breeuwer and Werren, 1993). Other studies showed that CI may be differentially expressed in different populations of the same species, or even in individuals within a single population (Vala *et al.*, 2000; Gotoh *et al.*, 2007). In our experiments, we used singly *Wolbachia*-infected individuals from four isofemale lines from two different populations, and CI was not expressed in any of these isofemale lines, and therefore, our findings seem consistent across different host backgrounds and populations. All *Wolbachia*-infected (singly or doubly) strains used in our study harbored *Wolbachia* strains that are identical at four investigated loci (*wsp*, *ftsZ*, *groEL* and *trmD*; unpublished data). This indicates that the *Wolbachia* strains are at least highly related, if not completely identical.

A likely explanation for the lack of CI induction by *Wolbachia*-infected males is that the *Wolbachia* strain has the *mod⁻* phenotype that does not modify sperm (Bourtzis *et al.*, 1998; Merçot and Poinot, 1998; Charlat *et al.*, 2001; Vala *et al.*, 2002; Zabalou *et al.*, 2004). Males infected with *mod⁻* strains are phenotypically indistinguishable from uninfected males; they are compatible with infected as well as uninfected females. Alternatively, the *mod⁻* phenotype may be indistinguishable from the effects of the absence of *Wolbachia* in the gonadal tissues. The presence of *Wolbachia* in the gonadal tissues is an important prerequisite for successful CI induction (Veneti *et al.*, 2003). The end result, however, is the same, that is, no observable CI.

Interactions between *Wolbachia* and *Cardinium* were investigated by comparing crosses involving doubly infected individuals, and crosses between *Wolbachia*-infected and *Cardinium*-infected individuals. Doubly infected males do not induce CI, and CI induced by singly *Cardinium*-infected males is neither rescued by doubly infected nor by singly *Wolbachia*-infected females. These findings can be explained by (1) the differences in host genetic background, (2) the differences between bacterial strains, or (3) the interference between *Wolbachia* and *Cardinium*. These possibilities are explored below.

(1) As we used mites from nine naturally infected or uninfected isofemale lines obtained from three different

populations, we could not completely control the host genetic background effects. As mentioned earlier, our findings are so far consistent across different backgrounds, but we cannot fully exclude potential impact of using mites from different populations. (2) Another explanation for the lack of CI induction in doubly infected individuals might be related to the genetic differences between the *Cardinium* strains in singly and doubly infected individuals. Singly and doubly infected individuals were collected from different populations (FR16 and FR21, respectively). We were unable to obtain singly and doubly infected isofemale lines from a single mite population. Ideally, one would like to generate singly infected lines from doubly infected isofemale lines to rule out confounding effects of host and bacterial genotype. Unfortunately, antibiotic treatment of doubly infected isofemales lines has so far not resulted in offspring infected with only *Cardinium* or *Wolbachia*. Genotyping of *Cardinium* strains from the two populations showed that they are slightly different: 0.7% difference at 16S rDNA (3bp out of 407) and 0.8% at *gyrB* (5bp out of 631) (unpublished data). It is possible that the modifier-rescue system of the *Cardinium* strain found in doubly infected individuals is different from that of the strain found in singly infected individuals, similar to the variation found for modifier-rescue systems of *Wolbachia*. (3) An interesting alternative explanation is that *Wolbachia* and *Cardinium* interfere in such a way, that *Cardinium* can neither rescue nor modify in the presence of *Wolbachia*. Currently, we cannot distinguish between the alternative explanations, although the second explanation seems feasible because of the differences that we found between the strains.

There are three other studies that investigated CI in individuals infected with both *Wolbachia* and *Cardinium*; Gotoh *et al.* (2006) detected no CI effect in the spider mite *Tetranychus pueraricola*, whereas Perlman *et al.* (2006) found a strong CI effect (increased female mortality) in the whitefly parasitoid *Encarsia inaron*. White *et al.* (2009) show that in the latter case, the CI is induced by *Wolbachia*, with no CI induction or rescue by *Cardinium*. As in the latter study, we also found an inability of the *Wolbachia* strain to rescue *Cardinium* modification; crosses between *Wolbachia*-infected females and *Cardinium*-infected males were incompatible. Apparently, these strains differ in their modifier-rescue system.

Costs related to the infection are important for determining the spread and maintenance of infections within populations (Werren, 1997; Hoffmann *et al.*, 1998). We found a cost of infection with either *Wolbachia* or *Cardinium*; fewer eggs and daughters are produced by singly infected females compared with uninfected or doubly infected females. The latter observation that a fitness cost is imposed on singly infected individuals, but not on doubly infected individuals is surprising. This fitness advantage of doubly infected females (compared with singly infected individuals) is only apparent when these females are mated with either uninfected or doubly infected males, but not when mated to singly infected males. Apparently, the infection status also influences male fertility. Fitness effects of *Wolbachia* are diverse; there are several examples known where infection with *Wolbachia* led to decrease in fecundity of infected females (Vala *et al.*, 2000; Perrot-Minnot *et al.*, 2002) had no significant effect (Hoffmann *et al.*, 1996; Poinot and

Merçot, 1997; Bordenstein and Werren, 2000; Montenegro *et al.*, 2006), or led to an increase in fecundity (Vavre *et al.*, 1999; Dobson *et al.*, 2004). In a few other arthropods and in all nematodes, *Wolbachia* is obligatory, and thus required for host fertility (Bandi *et al.*, 2001; Pannebakker *et al.*, 2007). *Cardinium* is known to increase fecundity in *Metaseiulus occidentalis* (Weeks and Stouthamer, 2004).

Fitness costs are important determinants of infection frequencies in the field. This study is the first to compare the fitness between singly and doubly infected females, with a surprising outcome. Our finding may have implications for the persistence and spread of *Wolbachia* and/or *Cardinium* in populations. Doubly infected individuals have a higher fitness (measured as fecundity) than singly infected individuals. This observation, combined with the fact that single *Cardinium* infections induce CI—and therefore stimulate the spread of singly infected *Cardinium* individuals—may result in complex infection dynamics. We investigated infection frequency in five populations, and found two populations that were infected with both *Wolbachia* and *Cardinium*. In population FR16 (140 individuals), the majority (59%) of individuals harbor both *Wolbachia* and *Cardinium*, and singly infected *Cardinium* individuals are extremely rare (2%). In population FR21 (nine individuals), most individuals are doubly infected. These polymorphisms might be transient states, for example, as *Cardinium* and/or *Wolbachia* recently infected the population and fixation of infection has not been reached yet. However, polymorphism may persist under certain conditions. Factors determining the infection frequencies are the level of CI induced, the costs of infection, efficiency of vertical transmission and rate of possible horizontal transmission (Werren, 1997; Hoffmann *et al.*, 1998). Horizontal transmission allows invasion without any manipulation, and was recently suggested as a possible explanation for the invasion of *Cardinium* in spiders (Duron *et al.*, 2008). Even so, a high temperature or naturally occurring antibiotics may cure individuals of infection (van Opijnen and Breeuwer, 1999). It is also unknown how densities are regulated in doubly infected individuals, and how both *Wolbachia* and *Cardinium* are faithfully transmitted to the next generation (Stouthamer *et al.*, 1999; Perlman *et al.*, 2006). More detailed transmission studies should answer such questions. Furthermore, other factors, such as the role of symbionts in resistance to parasitoids, may influence the frequency of symbionts in populations (Oliver *et al.*, 2005). In most studies on CI, infection frequencies in the field have not been estimated. More detailed studies including field estimates will improve our understanding of infection dynamics and the fundamental factors determining symbiont frequencies (Weeks *et al.*, 2002).

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