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Article

Cytoplasmic incompatibility and fitness benefits in the two-spotted spider mite *Tetranychus urticae* (red form) doubly infected with *Wolbachia* and *Cardinium*

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Abstract

Maternally inherited *Wolbachia* and *Cardinium* are widely distributed among arthropods, and their presence usually causes modifications of the reproduction and fitness of the host. Although co-infections of *Cardinium* and *Wolbachia* in the same host is common, yet relatively little is known about the multiple infections on host or the individual effects of each symbiont. In this study, we investigated the effects of, and interaction between, *Wolbachia* and *Cardinium* in the doubly infected two-spotted spider mite *Tetranychus urticae* (red form) in China. The individual cytoplasmic incompatibility (CI) level, bacteria density, fecundity, and host longevity were examined. Our results indicate that *Wolbachia* induced a week level of CI, while *Cardinium*-infected and doubly infected males causes severe CI. *Wolbachia* and *Cardinium* could not modify the CI strength and rescue CI each other. *Wolbachia* inhibited the proliferation of *Cardinium* in double-infected mites. The infection with *Cardinium* alone enhanced the fecundity of infected females. Interestingly, we found survival benefit in *Wolbachia*-infected, *Cardinium*-infected and the doubly infected females. We discuss the results observed with respect to the spread of bacterial infection in natural populations.

Key words: *Wolbachia*, *Cardinium*, *Tetranychus urticae* (red form), cytoplasmic incompatibility, fitness

Introduction

Vertically transmitted, intracellular bacteria are widespread in arthropods, inducing a variety of phenotypes in their host, from obligate nutritional mutualism to facultative reproductive parasitism (Moran 2006; Werren *et al*. 2008). *Wolbachia* are one of the most abundant intracellular bacteria in some classes of arthropods and nematodes, estimated to infect 52% of arthropod species (Weinert *et al*. 2015). The success of *Wolbachia* can be attributed in large part to its ability to manipulate the reproduction of its host to promote the spread of infection into the host population. *Wolbachia* have been implicated in all types of reproductive manipulations discovered to date, including cytoplasmic incompatibility (CI) (Bourtzis *et al*. 1996), male killing (Hurst *et al*. 1999), induction of thelytokous parthenogenesis (Stouthamer *et al.* 1993), and feminization of genetic males (Rousset *et al.* 1992). CI is the most common effect of *Wolbachia* infection. 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). In addition to the effect on reproduction, *Wolbachia* can also influence the fitness of the host, such as increase the survival and fecundity of infected females and enhance tolerance to viral infections (Dobson *et al*. 2004; Hedges *et al*. 2008; Zug &

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Hammerstein 2015). However, their manifestation appears to significantly vary among different host species, *Wolbachia* strains, and host genotypes (Fry *et al*. 2004; Xie *et al*. 2011).

Cardinium is a second maternally inherited reproductive parasite has been found to manipulate host reproduction by inducing cytoplasmic incompatibility (Hunter *et al*. 2003), feminization (Weeks *et al*. 2001) and thelytokous parthenogenesis (Zchori-Fein *et al*. 2001, 2004). *Cardinium* has been found only in 13% of arthropod species tested so far, and appears to be less widespread than *Wolbachia* (Weinert *et al*. 2015). So far, only a few studies have investigated fitness effect of *Cardinium* on its hosts. *Cardinium* increased fecundity in the predatory mite *Metaseiulus occidentalis* (Weeks & Stouthamer 2004), increased survival when singly infecting the parasitoid wasp *Encarsia inaron* (White *et al*. 2011) and shortened the developmental time of nymphs of *Sogatella furcifera* (Zhang *et al*. 2012a).

Infection by multiple symbionts is fairly common (Duron *et al*. 2008). Several studies have reported co-infections of *Wolbachia* and *Cardinium* in the same host species (Weeks *et al*. 2003; Zchori-Fein & Perlman 2004; Gotoh *et al*. 2007; Zhang *et al*. 2016). Although both *Wolbachia* and *Cardinium* can manipulate host reproduction, yet relatively little is known about the multiple infections on host or the individual effects of each symbiont. The results of previous investigations into the reproductive effect of co-infection of *Wolbachia* and *Cardinium* have been mixed. *Cardinium* but not *Wolbachia* induced CI in *Bryobia sarothamni* (Ros & Breeuwer 2009), while *Wolbachia* but not *Cardinium* induced CI in *Encarsia inaron* (White *et al*. 2009). Neither *Cardinium* nor *Wolbachia* induced CI in *Tetranychus pueraricola* (Gotoh *et al*. 2007). *Wolbachia* induced a week level of CI, while *Cardinium*-infected and doubly infected males caused severe CI in *Tetranychus piercei* (Zhu *et al*. 2012). *Cardinium* induced strong CI, double infection caused partial CI, and *Wolbachia* did not induce CI in *S. furcifera* (Zhang *et al*. 2012b). In addition, studies on the fitness consequences of multiple infections for host, nor the individual cost or benefit derived from each symbiont are very limited.

Wolbachia and *Cardinium* have been found coinfecting the spider mite *T. cinnabarinus* in China (Xie *et al*. 2010), but their effects and interactions are still unknown. In this study, we examined the strength of CI and bacteria density in *T. urticae* (red form, formerly called *T. cinnabarinus* in China) to determine the relative contributions of *Wolbachia* and *Cardinium*, and to examine potential interaction occurring between the symbionts, using isofemale lines obtained from naturally infected and cured individuals. We also investigated the fitness costs, benefits, or both of different infection status in *T. urticae* (red form). We addressed the following questions: 1) Do *Wolbachia* and *Cardinium* affect the reproduction of *T. urticae* (red form)? 2) Could one of the bacteria affect the expression and rescue of CI of the other? 3) Does the bacteria density affect the expression and rescue of CI? 4) Do fitness costs and/or benefits influence the infection frequency of endosymbionts?

Materials and methods

Preparation of spider mite lines

The two-spotted spider mite *Tetranychus urticae* (red form) was collected from soybean (*Glycine max*) plants in Zhenjiang, Jiangsu in July 2007. A screening of 40 adult females for *Cardinium* and *Wolbachia* by PCR (see below) showed that 6 females were singly infected with *Cardinium*, while the others were doubly infected with *Cardinium* and *Wolbachia*. The nucleotide sequence of the *wsp* gene from *Wolbachia* has been submitted to the GenBank database (GenBank number: KX463505). *Wolbachia* infected in the Zhenjiang population of *T. urticae* (red form) classified into subgroup *Con* belonging to supergroup B, according to the *wsp* gene sequences. The nucleotide sequences of the 16S rDNA genes amplified from the doubly infected line and singly

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Cardinium-infected line (GenBank number: GU731426) showed the two lines had the identical sequences, indicating that they harbored the same strain of *Cardinium*. Mites were reared on a leaf of the common bean (*Phaseolus vulgaris L.*) placed on a water-saturated sponge mat in Petri dishes (dia. 9) at 25±1°C, 60% r.h. and under L16: D8 conditions.

To generate the doubly infected (Iwc) and singly *Cardinium*-infected (Ic) lines, females from the teleiochrysalis stage were allowed to lay eggs without being crossed with males. The eggs were reared until adulthood (males). After the males reached sexual maturity, they were backcrossed with the mother. After the cross, the female adults were transferred to new leaf discs and were allowed to lay eggs for 3-5 days. Females were each checked for *Wolbachia* and *Cardinium* infection by PCR amplification. The eggs were separately reared on new leaf discs depending on the infection status of the mother. The above process was continued for three to four generations until a 100% doubly infected line and a 100% singly *Cardinium*-infected line were obtained.

To generate the singly *Wolbachia*-infected (Iw) and uninfected (U) lines, we treated doubly infected mites with antibiotics. Small leaf discs (ca 3 cm^2) from the common bean were placed on a cotton bed soaked in either tetracycline solution (0.1%, w/v) to eliminate both *Cardinium* and *Wolbachia* or in penicillin G solution (0.1%, w/v) to eliminate *Cardinium* only (Morimoto *et al.* 2006), and kept for 24 h before they were used for rearing the newly hatched larvae. Distilled water was added daily to keep the cotton bed wet. The cotton and the leaf discs were replaced every 4 days. Four generations later, progeny of the singly *Wolbachia*-infected females were retained, and PCR screening procedure was repeated for the following generations to ensure stable transmission of the *Wolbachia*. Six generations later, mites were checked by PCR to confirm that the line was free of *Cardinium* and *Wolbachia*. For the sixth and following generations, a sample of 40 mites was taken from the population and checked by PCR to confirm that the lines were uninfected. These lines were maintained in a mass-rearing environment without antibiotic for about four generations (2 months) before use, to avoid potential side-effect of the antibiotic treatment.

DNA extraction and PCR amplification

DNA was extracted by homogenizing a single female adult in a 25 µl mixture of STE buffer (100 mM NaCl, 10mM Tris-HCl, 1 mM EDTA, pH 8.0) and proteinase K (10 mg/ml, 2 microliters) in a 1.5ml Eppendorf tube. The mixture was incubated at 37°C for 30 min and then 95°C for 5 min. The samples were centrifuged briefly, and used immediately for the PCR reactions or stored at −20°C for later use.

To check for *Wolbachia* and *Cardinium* infection, all PCR reactions were run in 25 µl of buffer using the TAKARA Taq kit (No. R001B; Takara Co., Ltd.): 16.3 µl H_2O, 2.5 µl 10×buffer, 1.5 µl of 2.5 mM dNTP, 1.5µl of 25 mM $MgCl_2$, 0.2µl Taq (1 U), 2 µl sample and 1 µl of primers (20 pmol each). The primers used for detection of *Cardinium* were CLOf and CLOr1 (Weeks *et al.* 2003), which amplified *ca*. 450 bp of 16S rDNA (Table 2). Each PCR was run for one cycle of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s and a final extension of 5 min at 72 °C. *Wolbachia* was detected by using *wsp* gene primers wsp-81F and wsp-691R (Zhou *et al.* 1998), which amplify a 599bp product (Table 2). Reactions were cycled 35 times at 94°C for 30 s, 52°C for 45 s and 72°C for 1 min. Reagent negative and positive controls were included in the reactions. For samples failing to amplify using *Wolbachia* and *Cardinium* specific primers, primers COI-forward and COI-reverse (Navajas *et al.* 1996) were used to amplify mitochondria DNA as a positive control for template DNA quality. The PCR products were electrophoresed in a 1.0% agarose gel in TBE/EtBr for 40 min at 60 mA, and then photographed on a UV transilluminator.

Crossing experiment

The effects of *Wolbachia* and/or *Cardinium* on host reproduction were established by combining

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doubly infected (*Wolbachia* and *Cardinium*), singly infected (*Wolbachia* or *Cardinium*) and uninfected mites, resulting in 16 treatments (Table 1). Single females in the teleiochrysalis stage (the last developmental stage before adult emergence) were placed with adult virgin males from either the same or a different culture on the same leaf disk. We used young virgin males produced as a cohort by groups of females isolated as teliochrysalids laying eggs for 1–2 days. Males were discarded 2 days after the females reached adulthood and mated females were allowed to oviposit for 5 days. The eggs on the leaf discs were checked daily to determine hatchability, survival rate in immature stages and sex ratio (% daughters).

Data were analyzed with one-way analysis of variance (ANOVA) and means were compared using the Tukey-HSD test (SPSS 17.0). To normalize the data, log transformation was used for the number of eggs laid per female and an arcsine square root transformation was used for egg hatchability, survival rate and female ratio.

Fitness effects on host fecundity and survival

The effects of different infection types (*Wolbachia*-infected, *Cardinium*-infected, doubly infected, and uninfected) on female fecundity were tested by comparing the number of eggs laid in 5d by infected and uninfected females, in crosses involving uninfected males. Females were crossed with uninfected males to exclude any influence of differences in male fertility because of infection.

Differences in host longevity were observed in comparisons of the four different infection types. We measured age-specific survival of the U, Iw, Ic, and Iwc lines by placing 9 virgin females and 9 virgin males of the same infection status on the same leaf. Three leaves were used for each infection status. The leaves were monitored every day, and dead females were removed and counted until all females had died. Survivor curves for individual hosts were compared using the Kaplan-Meier log-rank test (Dobson *et al*. 2004).

Wolbachia *and* Cardinium *density measurement*

Wolbachia and *Cardinium* infection levels were determined by Q-PCR using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Six of doubly infected, singly *Wolbachia*-infected and singly *Cardinium*-infected mites (male and female) of 1 day-old were collected separately. DNA of single mites was extracted using the above method. SYBR green was used to monitor the amplification reaction. The following primers (Table 2) were designed specifically to amplify the 133 bp region of the *Cardinium* 16S rDNA gene and the 112bp region of the *Wolbachia wsp* gene: CLOF, CLOR (Xie *et al*. 2010); *w*ConQ-F, *w*ConQ-R (Zhao *et al*. 2013a).

The 20 ul final volume reaction mixture consisted of 10 ul 2×SYBR®*PremixEx Taq*™ (*Applied Biosystems*), 10 uM of each primer, 50×ROX Reference Dye and 2ul of DNA template. The RTQ-PCR cycling conditions included 1 cycle (10s 95°C) followed by 40 cycles (5s 95°C, 31s 60°C), and finally 1 cycle (15s 95°C, 1min 60°C, 15s 95°C)**.** Three replicates were run and averaged for each DNA sample. Negative controls were included in all amplification reactions. Standard curves were plotted using a 10-fold dilution series consisting of 10^{-8} to 10^{-4} dilutions of the DNA standards prepared from plasmid DNA. The quality and concentration of all purified standard DNA are measured by OD absorbance at 260 nm. The number of molecules in all samples is determined from the threshold cycles in the PCR based on a standard curve. Statistical analysis was performed using the Mann-Whitney *U*-test, as the data were not normally distributed.

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TABLE 1. Results of Compatibility of crosses between U (uninfected), Ic (*Cardinium*-infected), Iw (*Wolbachia*-infected) and Iwc (doubly infected) colonies in the Jiangsu population of *Tetranychus urticae* (red form)

Effect	Cross F ×M	N	Number of eggs	Hatchability (%)	Survival rate in immature stage (%)	Female offspring (%)
(a) *Cardinium* CI?	U×U	32	24.94±1.27a	96.53±0.57b	93.61±1.37b	83.86±0.79b
	U×Ic	20	26.25±1.84a	29.58±2.73a	84.74±2.85a	48.75±7.31a
	Ic×U	31	33.90±0.66b	97.88±0.51b	96.00±0.65b	87.20±0.71b
	Ic×Ic	28	24.86±0.96a	95.65±0.74b	91.64±1.61ab	84.15±1.02b
	$F_{3,\ 107}$ [b]		14.276***	282.805***	3.679 *	30.529***
(b) *Wolbachia* CI?	U×U	32	24.94±1.27	96.53±0.57b	93.61±1.37a	83.86±0.79
	U×Iw	31	24.65±0.79	83.53±1.59a	94.66±0.95ab	86.45±0.95
	Iw×U	40	25.08±0.71	96.93±0.64b	95.77±0.72ab	86.91±0.79
	Iw×Iw	26	26.31±1.03	98.55±0.45b	97.97±0.55b	85.77±1.11
	$F_{3,\ 125}$ [b]		0.585 NS	46.737 ***	2.831 *	2.672 NS
(c) Double infection CI?	U×U	32	24.94±1.27a	96.53±0.57b	93.61±1.37a	83.86±0.79b
	U×Iwc	26	32.69±1.38b	29.94±3.88a	90.84±2.65a	43.31±6.53a
	Iwc×U	55	25.44±0.66a	95.46±0.70b	96.13±0.56ab	86.90±0.74b
	Iwc×Iwc	26	33.65±0.72b	99.15±0.35c	99.11±0.31b	88.23±0.87b
	$F_{3,135}$ [b]		17.986***	249.507***	5.008**	51.159***
(d) *Wolbachia* modifies the strength of *Cardinium*-induced CI?	U×Ic	20	26.25±1.84b	29.58±2.73	84.74±2.85	48.75±7.31
	Iw×Iwc	27	22.41±0.82ab	40.92±2.94	87.88±2.40	63.72±4.80
	Iw×Ic	26	20.35±0.62a	36.18±3.41	91.41±2.67	55.46±5.49
	$F_{2,\ 70}$ [b]		5.694**	2.931 NS	2.443 NS	1.732 NS
(e) *Wolbachia* affects *Cardinium*-induced CI rescue?	Ic×Ic	28	24.86±0.96a	95.65±0.74a	91.64±1.61a	84.15±1.02a
	Iwc×Ic	31	32.84±1.02b	95.30±0.70a	95.46±0.61a	87.74±0.62b
	Iwc×Iwc	26	33.65±0.72b	99.15±0.35b	99.11±0.31b	88.23±0.87b
	$F_{2,\ 82}$ [b]		28.430***	13.734***	12.924***	6.826**
(f) *Cardinium* modifies the strength of *Wolbachia* -induced CI?	U×Iw	31	24.65±0.79b	83.53±1.59	94.66±0.95	86.45±0.95b
	Ic×Iwc	45	26.80±0.58c	82.34±1.85	94.42±1.04	86.955±0.76b
	Ic×Iw	29	21.59±0.62a	86.75±2.32	95.99±0.78	81.35±1.41a
	$F_{2,\ 102}$ [b]		17.610 ***	2.722 NS	0.568 NS	8.261 **
(g) *Cardinium* affects *Wolbachia* -induced CI rescue?	Iw×Iw	26	26.31±1.03b	98.55±0.45	97.97±0.55	85.77±1.11
	Iwc×Iw	25	18.08±0.81a	98.01±0.70	96.02±0.97	86.66±0.95
	Iwc×Iwc	26	33.65±0.72c	99.15±0.35	99.11±0.31	88.23±0.87
	$F_{2,74}$ [b]		73.534***	0.595 NS	3.931 NS	1.643 NS

N number of replicates, and NS not significant

Rows a–g contain groups of crosses that were compared for each trait. Traits are listed in the top row. Values for each trait are mean ± SE. 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. $P < 0.05$(*), $P < 0.01$ (**) and $P < 0.001$ (***) (ANOVA). Values in a column followed by different letters are significantly different at $P < 0.05$ (Tukey HSD test).

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TABLE 2. Primers used for PCR assays included in this study

Target species	Target gene	Assay type	Primer sequence	Reference
Cardinium	16S rDNA	Diagnostic PCR	CLOf (5'-GCGGTGTAAAATGAGCGTG-3') CLOr1(5'-ACCTMTTCTTAACTCAAGCCT-3')	Weeks *et al.* 2003
Wolbachia	*wsp*	Diagnostic PCR	wsp-81F(5'-TGGTCCAATAAGTGATGAAGAAAC-3') wsp-691R (5'-AAAAATTAAACGCTACTCCA-3')	Zhou *et al.* 1998
Cardinium	16S rDNA	SYBR	CLOF (5'-CCTGGGCTAGAATGTATTTTG-3') CLOR (5'-AAAGGGTTTCGCTCGTTATAG-3')	Xie *et al.* 2010
Wolbachia	*wsp*	SYBR	*w*ConQ-F (5'-CTCGTTACTTCGGTTCTTATGGC-3') *w*ConQ- R(5'-TTAAACGCTACTCCAGCTTCTGC-3')	Zhao *et al.* 2013a

Results

Effects of Cardinium *and/or* Wolbachia *on host reproduction*

We found strong evidence that *Cardinium* induced CI in *T. urticae* (red form) (Table 1a). In the predicted incompatible cross (U/Ic), the progeny hatchability (29.58±2.73%) was significantly lower than those of the other crosses (95.65±0.74% to 97.88±0.51%). Because of the large numbers of aborted eggs, the female ratio was also significantly lower in the predicted incompatibility crosses than in the other crosses. This was due to a decrease in the number of females produced, as the number of males produced was not significantly different among the four crosses.

In the cross between uninfected females and *Wolbachia*-infected males (U/Iw), on average, 83.53±1.59% of all eggs hatched, against 96.53–98.55% in the other three crosses(Table1b).No differences in survival rate at immature stages and sex ratio were observed between the predicted incompatible cross and the other crosses (Table 1b). These results suggested that *Wolbachia* in *T. urticae* (red form) did not have the perceptible ability to manipulate the host sex ratio and induced week CI.

Double infection also induced strong CI in *T. urticae* (red form) (Table 1c). The cross U/Iwc showed significantly reduced hatchability 29.94±3.88% and sex ratio 43.31±6.53% among the four combinations.

We conclude that singly *Cardinium*-infected males and doubly infected males induced strong CI, whereas singly *Wolbachia*-infected males induced week CI. No significant difference was observed between the CI strength induced by doubly infected and singly *Cardinium*-infected males.

Interactions between Cardinium *and* Wolbachia

To found out whether the presence of *Wolbachia* can modify the CI strength of *Cardinium*, different crosses were investigated. Results are presented in Table 1d. We did not find evidence that *Wolbachia* modified *Cardinium*-induced CI. Meanwhile, we found that *Wolbachia*-infected females could not rescue *Cardinium*-induced CI. In addition, the Ic/Ic, Ic/Iwc, Iwc/Iwc crosses showed that *Wolbachia* did not affect *Cardinium*-induced CI rescue (Table 1e).

Similarly, the crosses (U/Iw, Ic/Iwc, Ic/Iw) and (Iw/Iw, Iwc/Iw, Iwc/Iwc) were investigated to find out whether *Cardinium* could influence the CI strength of *Wolbachia* (Table 1f, g). These crosses showed that *Cardinium* could not change the strength of *Wolbachia*-induced CI and *Wolbachia*-induced CI rescue. In addition, CI induced by *Wolbachia*-infected males could not be rescued by *Cardinium*.

Effects on host fecundity

To exclude the influence of any infection-induced differences in male fertility, we compared the

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number of eggs laid in the first 5 d by infected and uninfected females crossed with uninfected males (Fig. 1). The results showed the fecundity of singly *Cardinium*-infected females (33.90±0.66) to be significantly higher than the fecundity of uninfected, singly *Wolbachia*-infected and doubly infected females (24.94±1.27 to 25.44±0.66), indicating that *Cardinium* can promote the fecundity of infected females (p<0.001, Tukey-HSD test).

Effects on host longevity

The effects of *Wolbachia* and/or *Cardinium* on host longevity were tested by comparing the life spans of the four different infection types. The results are presented in Fig. 2. The singly *Cardinium*-infected (20.44±0.53 days), singly *Wolbachia*-infected (20.04±1.17 days) and doubly infected females (18.15±1.19 days) lived much longer than uninfected females (14.67±0.96 days) in *T. urticae* (red form)(X^2= 17.65, df = 1, p<0.001 for U vs Ic; X^2= 15.3, df = 1, p<0.001 for U vs Iw ; X^2= 8.20, df = 1, p=0.004 for U vs Iwc, respectively, Kaplan-Meier log-rank test).The life spans of singly *Cardinium*-infected, singly *Wolbachia*-infected and doubly infected females showed no significant difference (p>0.05, Kaplan-Meier log-rank test). Survival curves showed that no *Cardinium*-infected females died in the first 15 days, while 34% *Wolbachia*-infected females, 37% doubly infected females and 60% uninfected females died 15 days after emergence. The results indicated *Wolbachia* and *Cardinium* could prolong female host longevity.

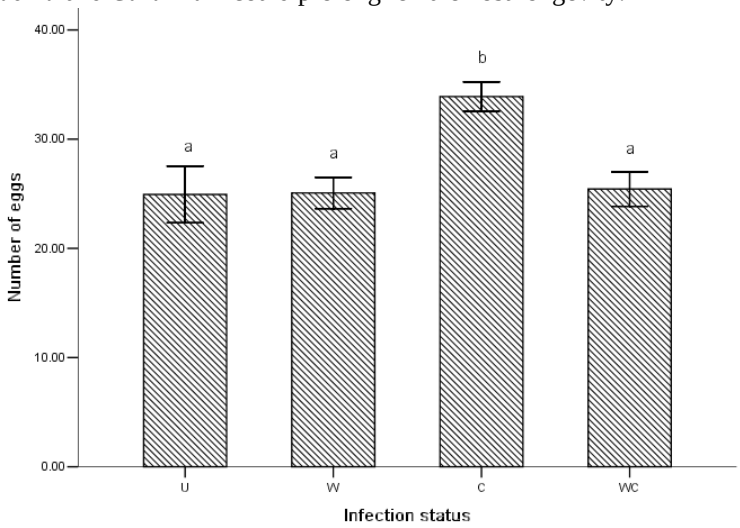

FIGURE 1. Mean fecundity over 5d of uninfected (U), singly *Wolbachia*-infected (W), singly *Cardinium*-infected (C) and doubly infected (WC) females crossed with uninfected males. a and b represent statistic groups (Tukey HSD test, $p < 0.05$).

Bacteria density measurement

We examined infection density of *Cardinium* and *Wolbachia* in singly infected and doubly infected females and males. The numbers of *Cardinium* in the singly and doubly infected males were 0.81 and 0.55× 10^7 per ml, respectively, indicating the density of *Cardinium* in the doubly infected males was significantly lower than that in singly *Cardinium*-infected males (p<0.001, Mann-Whitney *U*-test). The density of *Wolbachia* showed no differences between the doubly infected and singly *Wolbachia*-infected males (Fig. 3b).

The densities of *Wolbachia* and *Cardinium* were clearly higher in females than that of males. No difference in *Cardinium* density (Fig. 3a) and *Wolbachia* density (Fig. 3b) were observed between singly infected and doubly infected females. The densities of *Wolbachia* were significantly higher than *Cardinium* in both singly infected and doubly infected mites (Fig. 3a, b).

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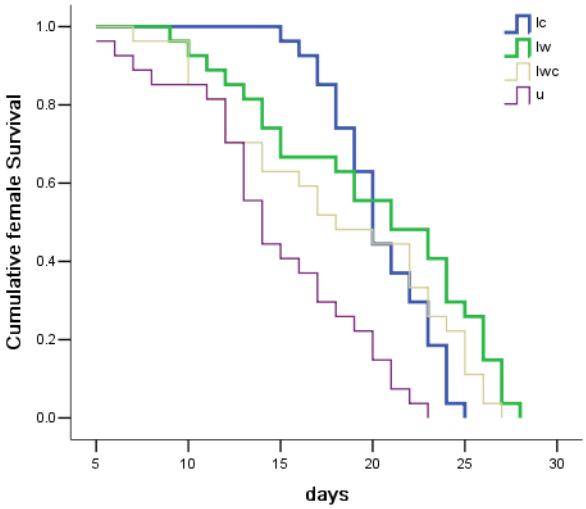

FIGURE 2. Comparison of *Wolbachia* and *Cardinium* effects on female longevity in *T. urticae* (red form). U, uninfected strain; Iw, singly *Wolbachia*-infected strain; Ic, singly *Cardinium*-infected strain; Iwc, doubly infected strain. Survivor curves for individual hosts were compared using the Kaplan-Meier log-rank test.

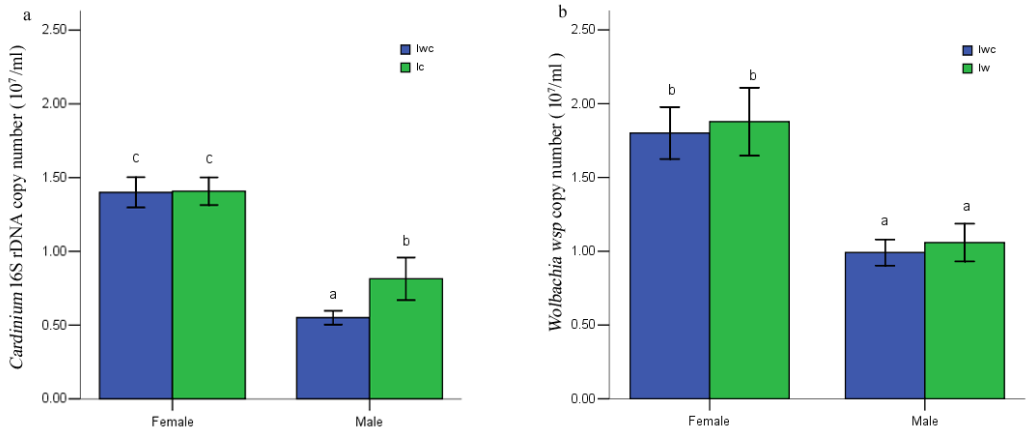

FIGURE 3. Infection density of *Cardinium* (a) and *Wolbachia* (b) in singly infected and doubly infected females and males. Iw, singly *Wolbachia*-infected strain; Ic, singly *Cardinium*-infected strain; Iwc, doubly infected strain. Copy numbers per ml were determined by quantitative PCR using the 16S rDNA gene of *Cardinium* and *wsp* gene of *Wolbachia*. Each point is the average of three measurements of six samples. Bars indicate standard errors. a, b, and c represent statistic groups (Mann-Whitney *U*-test, $p<0.05$).

Discussion

We found strong evidence that double infection with *Cardinium* and *Wolbachia* induced strong CI in *T. urticae* (red form) and CI was expressed as a reduction in egg hatchability and a male-biased sex ratio in crosses between uninfected females and infected males. The co-infection of *Cardinium* and *Wolbachia* had been shown to induce CI in *E. inaron* (White *et al*. 2009), *T. piercei* (Zhu *et al.* 2012), *S. furcifera* (Zhang *et al.* 2012b) and *Tetranychus truncates* (Zhao *et al.* 2013b).The contributions of *Cardinium* and *Wolbachia* varied in doubly infected hosts. *Wolbachia* but not

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Cardinium induced CI in *E. inaron* (White *et al*. 2009). *Wolbachia* induced a week level of CI, but strengthened *Cardinium*-induced CI in *T. piercei* (Zhu *et al.* 2012). In *S. furcifera*, *Cardinium* induced strong CI, double infection caused partial CI, and *Wolbachia* did not induce CI (Zhang *et al.* 2012b). In this study, *Cardinium* induced strong level of CI in *T. urticae* (red form). Although the density of *Wolbachia* was significantly higher than *Cardinium* in the Zhenjiang population of *T. urticae* (red form), *Wolbachia* induced week CI reducing hatchability but without sex-ratio distortion. This is the first record of the reproductive effect of *Wolbachia* belonged to the *Con* subgroup of group B in spider mite. Male age and bacteria density did not influence the *Wolbachia*-induced CI expression (unpublished data). *Wolbachia*-induced week CI may due to the other factors such as bacteria strain, host genetic background or the interaction between bacteria and host species (Reynolds & Hoffmann 2002; Sakamoto *et al*. 2005; Xie *et al*. 2011).

No significant differences were observed between the CI strength induced by doubly infected and singly *Cardinium*-infected males. There are two potential explanations: 1) *Cardinium* played crucial role in CI induction in doubly infected line. *Wolbachia*-induced CI may be covered by *Cardinium* when they modified the same sperm. 2) Both *Cardinium* and *Wolbachia* induced CI in doubly infected line, but *Cardinium*-induced CI strength was weaker than singly infected line. Negative interference between *Wolbachia* and *Cardinium* was observed, resulting in lower *Cardinium* density in the doubly infected males. *Cardinium*-induced CI expression was affected by bacteria density in *T. cinnabarinus* (Xie *et al*. 2010). The decreased *Cardinium* density in doubly infected males may lead to a decline of *Cardinium*-induced CI strength. This is consisted with the higher hatchability and female sex ratio of cross Iw/Iwc compared with cross I/Ic, though the difference was not significant.

In addition to the effect on reproduction, *Wolbachia* and *Cardinium* could also influence the fitness of the host. *Cardinium* infection had a positive effect on fecundity in *T. urticae* (red form). Fecundity-enhancing *Cardinium* strain was also found in the predatory mite *M. occidentalis* (Weeks & Stouthamer 2004). By contrast, *Cardinium* provided fitness cost to fecundity of infected females in *B. sarothamni* (Ros & Breeuwer 2009). The possible mechanisms underlying these effects are still unclear. *Wolbachia* infection in *Drosophila mauritiana* leads to increase mitotic activity of germline stem cells and to decrease apoptosis in the germarium (Fast *et al*. 2011). *Cardinium* probably affects host fecundity in a similar way. Like *Wolbachia*, fitness effects of *Cardinium* on fecundity are important for the spread and maintenance of infection within populations (Perlmam *et al*. 2008). Although *Wolbachia* have been found to enhance fecundity in several species (Dobson *et al*. 2004; Fry *et al*. 2004; Xie *et al*. 2011; Serga *et al*. 2014), no fecundity change was observed in *Wolbachia*-infected females in this study. Co-infection of *Cardinium* and *Wolbachia* in females also did not affect fecundity. The fitness benefit on *Cardinium*-infected individual, but not on doubly infected individuals is surprising. A likely explanation is that co-infection of *Wolbachia* and *Cardinium* in a single host increased metabolic cost of bearing two symbionts, resulting in no fitness effect on fecundity of double infected female. This fitness advantage of *Cardinium*-infected females was only apparent when these females were mated with uninfected males. Apparently, the infection status also influenced male fertility.

Both *Wolbachia* and *Cardinium* prolonged the longevity of the host in *T. urticae* (red form). Our result is the first report that all the three infection status affected longevity in female spider mites. *Wolbachia* showed both positive and negative effects on longevity in different studies (Dobson *et al*. 2002; Fry *et al*. 2004; Xie *et al*. 2011; Zhao *et al*. 2013a). *Cardinium*'s positive effect on longevity was only found in the parasitoid wasp *E. inaron* (White *et al*. 2011). So far, no fitness benefit on longevity was found in double infected females. In *Drosophila*, *Wolbachia* influence the expression level of the genes involved in lifespan regulation (Maistrenko *et al*. 2016). The mechanistic basis for *Cardinium*'s positive effect on longevity should be investigated in the future.

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Fitness effects and CI strength are important determinants of infection frequencies in the field (Hoffmann *et al*. 1990; Turelli & Hoffmann 1995). The benefit effect on fecundity and longevity, combined with the CI induced by *Cardinium* and *Wolbachia* in *T. urticae* (red form) can stimulate the rapid spread of infection. This explained why there were no uninfected individuals in the nature population of *T. urticae* (red form). *Wolbachia* induced weaker CI than *Cardinium* and double infection. Models predict that *Wolbachia* should be lost from natural populations unless beneficial to the host or perfectly transmitted from mothers to offspring (Turelli & Hoffmann 1995). Although *Wolbachia* provide survival benefit to host, no significant difference was observed compared with *Cardinium*-infected and doubly infected females. Our data indicate that no *Cardinium*-infected females died in the first 15 days (the main oviposition period of spider mite), while 34% *Wolbachia*-infected females died 15 days after emergence. This would result in a relatively higher number of offspring produced by *Cardinium*-infected females, and therefore it gradually reduced the number of *Wolbachia*-infected individuals. That is why we did not found singly *Wolbachia*-infected individuals in the field. The infection frequency of doubly infected (85%) was much higher than singly *Cardinium*-infected (15%) in the field. The week unidirectional CI in the cross Ic/Iwc may provide double infected female hosts with a reproductive advantage relative to singly *Cardinium*-infected females. Another likely explanation is that double infection could confer some untested fitness benefit to the host, which was at an advantage over singly *Cardinium*-infected females, such as elevate resistance to parasitoids and insecticides (Oliver *et al.* 2005; Duron *et al*. 2006). More detailed studies will improve our understanding of infection dynamics and the fundamental factors determining symbiont frequencies.

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