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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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 &
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 that 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; Gotth 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 sarotheamni (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 (Gotth 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 co-infecting 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
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 cm²) 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 H2O, 2.5 µl 10×buffer, 1.5 µl of 2.5 mM dNTP, 1.5µl of 25 mM MgCl₂, 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
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 teliochrysalis 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); wConQ-F, wConQ-R (Zhao et al. 2013a).

The 20 ul final volume reaction mixture consisted of 10 ul 2xSYBR®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 10^4 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.
### 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)

<table>
<thead>
<tr>
<th>Effect</th>
<th>Cross</th>
<th>F × M</th>
<th>N</th>
<th>Number of eggs</th>
<th>Hatchability (%)</th>
<th>Survival rate in immature stage (%)</th>
<th>Female offspring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) <em>Cardinium</em> CI?</td>
<td>U×U</td>
<td>32</td>
<td>24.94 ±1.27a</td>
<td>96.53 ±0.57b</td>
<td>93.61 ±1.37b</td>
<td>83.86 ±0.79b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U×Ic</td>
<td>20</td>
<td>26.25 ±1.84a</td>
<td>29.58 ±2.73a</td>
<td>84.74 ±2.85a</td>
<td>48.75 ±7.31a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ic×U</td>
<td>31</td>
<td>33.90 ±0.66b</td>
<td>97.88 ±0.51b</td>
<td>96.00 ±0.65b</td>
<td>87.20 ±0.71b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ic×Ic</td>
<td>28</td>
<td>24.86 ±0.96a</td>
<td>95.65 ±0.74b</td>
<td>91.64 ±1.61ab</td>
<td>84.15 ±1.02b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F3-107b</td>
<td>14.276***</td>
<td>282.805***</td>
<td>3.679 *</td>
<td>30.529***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) <em>Wolbachia</em> CI?</td>
<td>U×U</td>
<td>32</td>
<td>24.94 ±1.27</td>
<td>96.53 ±0.57b</td>
<td>93.61 ±1.37a</td>
<td>83.86 ±0.79b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U×Iw</td>
<td>31</td>
<td>24.65 ±0.79</td>
<td>83.53 ±1.59a</td>
<td>94.66 ±0.95ab</td>
<td>86.45 ±0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iw×U</td>
<td>40</td>
<td>25.08 ±0.71</td>
<td>96.93 ±0.64b</td>
<td>95.77 ±0.72ab</td>
<td>86.91 ±0.79</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iw×Iw</td>
<td>26</td>
<td>26.31 ±1.03</td>
<td>98.55 ±0.45b</td>
<td>97.97 ±0.55b</td>
<td>85.77 ±1.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F3-125b</td>
<td>0.585 NS</td>
<td>46.737 ***</td>
<td>2.831 *</td>
<td>2.672 NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c) Double infection CI?</td>
<td>U×Iwc</td>
<td>26</td>
<td>32.69 ±1.38b</td>
<td>29.94 ±3.88a</td>
<td>90.84 ±2.65a</td>
<td>43.31 ±6.53a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iwc×U</td>
<td>55</td>
<td>25.44 ±0.66a</td>
<td>95.46 ±0.70b</td>
<td>96.13 ±0.56ab</td>
<td>86.90 ±0.74b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iwc×Iwc</td>
<td>26</td>
<td>33.65 ±0.72b</td>
<td>99.15 ±0.35c</td>
<td>99.11 ±0.31b</td>
<td>88.23 ±0.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F3-135b</td>
<td>17.986***</td>
<td>249.507***</td>
<td>5.008 *</td>
<td>51.15 ***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(d) <em>Wolbachia</em> modifies the strength of <em>Cardinium</em>-induced CI?</td>
<td>U×Ic</td>
<td>20</td>
<td>26.25 ±1.84b</td>
<td>29.58 ±2.73</td>
<td>84.74 ±2.85</td>
<td>48.75 ±7.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iw×Iwc</td>
<td>27</td>
<td>22.41 ±0.82ab</td>
<td>40.92 ±2.94</td>
<td>87.88 ±2.40</td>
<td>63.72 ±4.80</td>
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</tr>
<tr>
<td></td>
<td>Iw×Ic</td>
<td>26</td>
<td>20.35 ±0.62a</td>
<td>36.18 ±3.41</td>
<td>91.41 ±2.67</td>
<td>55.46 ±4.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F2-70b</td>
<td>5.694**</td>
<td>2.931 NS</td>
<td>2.443 NS</td>
<td>1.732 NS</td>
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<tr>
<td>(e) <em>Wolbachia</em> affects <em>Cardinium</em>-induced CI rescue?</td>
<td>Ic×Ic</td>
<td>28</td>
<td>24.86 ±0.96a</td>
<td>95.65 ±0.74a</td>
<td>91.64 ±1.61a</td>
<td>84.15 ±1.02a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iwc×Ic</td>
<td>31</td>
<td>32.84 ±1.02b</td>
<td>95.30 ±0.70a</td>
<td>95.46 ±0.61a</td>
<td>87.74 ±0.62b</td>
<td></td>
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<tr>
<td></td>
<td>Iwc×Iwc</td>
<td>26</td>
<td>33.65 ±0.72b</td>
<td>99.15 ±0.35b</td>
<td>99.11 ±0.31b</td>
<td>88.23 ±0.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F2-82b</td>
<td>28.430***</td>
<td>13.734***</td>
<td>12.924***</td>
<td>6.826**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(f) <em>Cardinium</em> modifies the strength of <em>Wolbachia</em>-induced CI?</td>
<td>U×Iw</td>
<td>31</td>
<td>24.65 ±0.79b</td>
<td>83.53 ±1.59</td>
<td>94.66 ±0.95</td>
<td>86.45 ±0.95b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Icw×Ic</td>
<td>45</td>
<td>26.80 ±0.58c</td>
<td>82.34 ±1.85</td>
<td>94.42 ±1.04</td>
<td>86.95 ±0.76b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Icw×Iw</td>
<td>29</td>
<td>21.59 ±0.62a</td>
<td>86.75 ±2.32</td>
<td>95.99 ±0.78</td>
<td>81.35 ±1.41a</td>
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<tr>
<td></td>
<td>F2-102b</td>
<td>17.610 ***</td>
<td>2.722 NS</td>
<td>0.568 NS</td>
<td>8.261 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g) <em>Cardinium</em> affects <em>Wolbachia</em>-induced CI rescue?</td>
<td>Icw×Iw</td>
<td>26</td>
<td>26.31 ±1.03b</td>
<td>98.55 ±0.45</td>
<td>97.97 ±0.55</td>
<td>85.77 ±1.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Icw×Iwc</td>
<td>25</td>
<td>18.08 ±0.81a</td>
<td>98.01 ±0.70</td>
<td>96.02 ±0.97</td>
<td>86.66 ±0.95</td>
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</tr>
<tr>
<td></td>
<td>Icw×Iwc</td>
<td>26</td>
<td>33.65 ±0.72c</td>
<td>99.15 ±0.35</td>
<td>99.11 ±0.31</td>
<td>88.23 ±0.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F2-74b</td>
<td>73.534***</td>
<td>0.595 NS</td>
<td>3.931 NS</td>
<td>1.643 NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 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).
### 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 (Table 1b). 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* 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, 1wc/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

### Table 2. Primers used for PCR assays included in this study

<table>
<thead>
<tr>
<th>Target species</th>
<th>Target gene</th>
<th>Assay type</th>
<th>Primer sequence</th>
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<tr>
<td>Cardinium</td>
<td>16S rDNA</td>
<td>Diagnostic PCR</td>
<td>CLOF (5'-CGGGTGTTAAAAATTGAGCGTG-3')</td>
<td>Weeks et al. 2003</td>
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<td>CLOR (5'-ACCTMTTCTTAACTCAAGCT-3')</td>
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<td>wsp</td>
<td>Diagnostic PCR</td>
<td>wsp-81F (5'-TGGTCCAATAATAGGGAAGAAC-3')</td>
<td>Zhou et al. 1998</td>
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<td>wsp-691R (5'-AAAAATTTAAACGCTACTCA-3')</td>
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<tr>
<td>Cardinium</td>
<td>16S rDNA</td>
<td>SYBR</td>
<td>CLOF (5'-CCTGGGCTAAATGTATTTT-3')</td>
<td>Xie et al. 2010</td>
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<td>CLOR (5'-AAAAATTTAAACGCTACTCA-3')</td>
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<tr>
<td>Wolbachia</td>
<td>wsp</td>
<td>SYBR</td>
<td>wConQ-F (5'-CTCGTTACTTCGGTTCTTAGGC-3')</td>
<td>Zhao et al. 2013a</td>
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<td>wConQ-R (5'-TTAAACGCTACTCAAGCTG-3')</td>
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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)(X²= 17.65, df = 1, p<0.001 for U vs Ic; X²= 15.3, df = 1, p<0.001 for U vs Iw ; X²= 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).
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 truncatus* (Zhao et al. 2013b). The contributions of *Cardinium* and *Wolbachia* varied in doubly infected hosts. *Wolbachia* but not
Cardinium induced CI in E. inaron (White et al. 2009). Wolbachia induced a weak 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 weak 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 weak 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 consistent 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 (Perlman 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 doubly 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.
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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