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Wolbachia Strengthens *Cardinium*-Induced Cytoplasmic Incompatibility in the Spider Mite *Tetranychus piercei* McGregor

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Abstract *Wolbachia* and *Cardinium* are maternally inherited intracellular bacteria that can manipulate the reproduction of their arthropod hosts, such as by inducing cytoplasmic incompatibility (CI). Although the reproductive alteration induced by *Wolbachia* or *Cardinium* have been well investigated, the effects of these two endosymbionts co-infecting the same host are poorly understood. We found that *Tetranychus piercei* McGregor is naturally infected with *Wolbachia* and *Cardinium*. We performed all possible crossing combinations using naturally infected and cured strains, and the results show that *Wolbachia* induced a weak level of CI, while *Cardinium*-infected and doubly infected males caused severe CI. *Wolbachia* and *Cardinium* could not rescue CI each other; however, *Wolbachia* boosted the expression of *Cardinium*-induced CI. Quantitative PCR results demonstrated that CI was associated with the infection density of *Wolbachia* and *Cardinium*.

Introduction

Many arthropods are infected with diverse bacterial endosymbionts. These intracellular bacteria manipulate their host's reproduction in such a way as to enhance their own spread through a population [38]. These manipulations include male killing, feminization, thelytokous parthenogenesis, and cytoplasmic incompatibility (CI, see below), and result in an increased proportion of infected females (the transmitting sex) in the host population. One of the most common of these endosymbionts is *Wolbachia*, which belong to the alpha subdivision of the phylum Proteobacteria and which are widespread among arthropods and nematodes. It has been estimated that more than 20 % of all arthropod species are infected with *Wolbachia* [37, 38]. CI is the most common effect of *Wolbachia* and has been described in arachnids and most groups of insects [7, 26, 37].

CI is a form of conditional infertility in which the cross between infected male and uninfected female are incompatible, whereas the reciprocal cross and self-crosses are compatible. In addition, bidirectional incompatibility can occur when two host lines are infected with different *Wolbachia* strains [2, 27]. The mechanisms of CI can be explained by the "modification–rescue" system [15, 36]. This system contains the *Wolbachia*-induced modification of sperm and the *Wolbachia*-induced rescue of that modification upon fertilization [36]. Following fertilization with a *Wolbachia*-modified sperm, the result is normal development in embryos from eggs harboring at least the same *Wolbachia* types as the father. Otherwise, abnormal mitosis occurs lacking the father's *Wolbachia* type(s), which typically results in embryo's death. Many studies have shown that incompatibility was manifested as a disruption of pronuclear chromatin condensation followed by missegregation of chromosomes during mitosis [9, 39]. The great majority of studies have shown that there is diversity among *Wolbachia* strains in both the modification and rescue function. A *Drosophila* study showed that several *Wolbachia* strains that cannot generate modifications in host sperm can still rescue the modifications caused by

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other strains as long as the two strains are sufficiently closely related [4]. Transfer of nine distantly related *Wolbachia* strains into the same host background showed that a given *Wolbachia* variant can possess multiple rescue determinants corresponding to different CI systems [43]. Some superinfections, i.e., infections with more than one strain of *Wolbachia*, can have additive effects. For example, a superinfected male is unidirectionally incompatible with both single-infected and uninfected females. The strength of CI caused by superinfected males is similar to the strength of CI from the strongest single infection when male is mated to singly infected or uninfected females [10, 20].

CI was once thought to be a unique phenotype of *Wolbachia*. *Cardinium* was the second bacterial lineage discovered to induce CI in arthropods [16]. *Cardinium*, which belong to the Cytophaga–Flavobacterium–Bacteroides phylum, have been found in 6–7 % of all arthropods [11, 35]. *Cardinium*-induced CI have been found in the parasitoid wasp *Encarsia pergandiella*, the spider mite *Eotetranychus suginamensis*, the spider mite *Bryobia sarothamni*, and the carmine spider mite *Tetranychus cinnabarinus* [12, 16, 24, 42]. The mechanism of *Cardinium*-induced CI is unclear.

Many factors, including host genotype, the bacterial strain, bacterial density, and sperm cyst infection, have been proposed to affect the expression of CI [22, 31, 32, 34]. Bacterial density has been found to strongly affect the strength of CI in insects [3, 8]. The strength of CI differs in the planthoppers *Laodelphax striatellus* and *Sogatella furcifera*, and appears to be due to different amounts of *Wolbachia* in males [21]. It has also shown a positive correlation between the density of *Wolbachia* within pole cells and CI levels in *Drosophila* [30]. The bug *Orius strigicollis* is superinfected with two strains of *Wolbachia*, *w*Ous1 and *w*Ous2. *w*Ous1 interferes with the ability of *w*Ous2 to cause CI by suppressing *w*Ous2 densities [33]. In addition, *Cardinium* density also affects CI strength in the carmine spider mite *T. cinnabarinus*, and a threshold level of *Cardinium* density may be required for the induction of CI [42]. Although the factors that influence strength of *Wolbachia*-induced CI have been widely investigated, the CI induced by doubly infected *Wolbachia* and *Cardinium* has been rarely reported.

Wolbachia and *Cardinium* have been found to co-infect the same host species [11, 12, 35, 44], especially in the spider mite. The reproductive phenotype and interactions of a co-infection of *Wolbachia* and *Cardinium* have been studied in the spider mite *B. sarothamni* and the parasitic wasp *Encarsia inaron* [24, 40]. In *B. sarothamni*, *Cardinium*-infected males induced severe CI; however, CI could not be induced by doubly infected and *Wolbachia*-infected males. The different phenotypes caused by *Cardinium* are related to the different *Cardinium* strains infected in singly and doubly infected individuals [24]. However, in *E. inaron*, *Wolbachia* caused CI, but *Cardinium* did not [40].

In this study, to elucidate how double infection of *Wolbachia* and *Cardinium* affects CI, we investigated a species of spider mite that is known to have both infections, *Tetranychus piercei* McGregor, using isofemale lines obtained from naturally infected and cured individuals. Specifically, we attempted to determine whether (1) one or both of the bacteria caused CI, (2) one of the bacteria could affect the expression and rescue of CI of the other, and (3) bacterial density affected the expression and rescue of CI.

Materials and Method

Sample Collection and Culture

The spider mite *Tetranychus piercei* McGregor was collected from soybean [*Glycine max* (L.) Merr.] leaves in Yulong, Yunnan Province, southwest of China. The singly *Cardinium*-infected line was generated by treating doubly infected mites with high temperatures (34 ± 1 °C, L:D = 16:8, RH 60 %) for six successive generations [28]. Most F_6 individuals were singly infected *Cardinium*, but a small proportion had two symbionts. Progeny of the singly *Cardinium*-infected F_6 females were retained, and PCR screening procedure was repeated for the following generations to ensure stable transmission of the *Cardinium*. To obtain the singly *Wolbachia*-infected line, penicillin G (0.1 %, w/v) was used to treat doubly infected mites for four successive generations [18, 42]. Progeny of the singly *Wolbachia*-infected F_4 females were retained, and PCR screening procedure was repeated for the following generations to ensure stable transmission of the *Wolbachia*. The uninfected line was generated by treating doubly infected mites with tetracycline solution (0.1 %, w/v) for six successive generations [42]. For the sixth and following generations, 45 individuals were taken from the population and checked by PCR to confirm that the line was uninfected. These lines were maintained in a mass-rearing environment without antibiotics for about six generations before use, to avoid the potential side effects of antibiotic treatment. Mites were reared on a leaf of the common bean (*Phaseolus vulgaris* L.) placed on a water-saturated sponge mat in Petri dishes (9 cm in diameter) at 25 ± 1 °C, 60 % r.h. and under 16 L:8D photoperiod.

Screening of Symbiont Infection

We used PCR to check *Wolbachia* and *Cardinium* infection status during the initiation of the laboratory cultures. DNA was extracted from single mites using the

cetyltrimethylammonium bromide (CTAB) extraction method as described previously [23]. All PCR reactions were run in 25 µl buffer using the TAKARA Taq kit (No. R001B; Takara Co., Ltd., Shiga, Japan): 16.3 µl H_2O, 2.5 µl 10× buffer, 1.5 µl of 2.5 mM dNTP, 1.5 µl of 25 mM $MgCl_2$, 0.2 µl of 5 U/µl Taq, 2 µl sample, and 0.5 µl each of 20 µm forward and reverse primer. *Wolbachia* was detected by amplification of *wsp* and *ftsZ* genes by PCR, using the primers *wsp*-81F/*wsp*-691R [6] and *ftsZ*-F/*ftsZ*-R [1]. Reactions were cycled 35 times at 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 1 min. *The* presence of *Cardinium* was detected using PCR amplification of a part of the 16S rDNA and *gyrB*. *Cardinium* 16S rDNA was amplified using the primers CLOf and CLOr [35]. The *gyrB* gene was amplified using primers from Groot and Breeuwer [14]. Each PCR was run for one cycle at 94 °C for 2 min, 35 cycles at 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. Reagent negative and positive controls were included in the reactions. The PCR products were electrophoresed in a 1.0 % agarose gel in TAE/EtBr for 30 min at 120 V, 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, singly infected, and uninfected mites (see Table 1 for crosses and their possible effects). Single females in the teleiochrysalis stage (the last developmental stage before adult emergence) were placed with a 1 day-old adult virgin male from the same culture on the same leaf disk. Males were discarded 2 days after the females reached adulthood. The mated females were allowed to oviposit for 5 days. Both males and females were checked by PCR to confirm their infection status. The eggs on the leaf disks were checked daily to determine unhatchability, mortality in immature stages, and the sex ratio (% males).

Groups of crosses were tested for differences in investigated traits using the software package SPSS version 16.0 (Chicago, IL, USA). Tests were performed for individual bacterial effects and for interactions between *Wolbachia* and *Cardinium* (Table 1). Data were first tested for normality (Kolmogorov–Smirnov test) and homogeneity of group variances (Levene's test). Which possible, square-root, logarithmic or arcsine transformations were performed to attain normality and homogeneity of variance. A one-way analysis of variance was performed for each trait (number of eggs laid, unhatched eggs, and 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.

Quantitative PCR of Bacteria

Wolbachia and *Cardinium* infection levels were determined by Q-PCR. Ten of doubly infected *Wolbachia* and *Cardinium*, singly *Wolbachia*-infected and singly *Cardinium*-infected mites (male and female) of 1 day-old were collected separately. Adult mites were prepared and individually subjected to DNA extraction using TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.4.0. The sample DNA extracted from mites was diluted to 1 ng/µl with deionized and distilled water for consistent Q-PCR assay. The *wsp* gene of *Wolbachia* and the 16S rDNA gene of *Cardinium* were quantified using the ABI PRISM 7300 Real-Time PCR System. The *Wolbachia* primers were designed specifically to amplify the 124 bp region of the *Wolbachia wsp* gene (WF5′-AGCAATCCTT TAGTAACAGAG-3′ and WR5′-ATTAGCACCATAAGA ACCA-3′). The *Cardinium* primers were designed specifically to amplify the 88 bp region of the16S rDNA gene (CF5′-ATGGCATGTACAAAGGGAAGC-3′ and CR5′-T TGCAGACCTCAATCCGAAC-3′). SYBR green was used to monitor the amplification reaction. The 20 µl reaction mixture consisted of 10 µl 2× SYBR Premix Ex Taq, 0.4 µl of 10 µM of each primer, 0.4 µl 50× ROX Reference Dye, 2 µl DNA template and 6.8 µl dH_2O. The Q-PCR cycling conditions included 1 cycle (30 s 95 °C) followed by 40 cycles (5 s 95 °C, 31 s 60 °C), and finally, 1 cycle (15 s 95 °C, 1 min 60 °C, 15 s 95 °C). Three replicates were run and averaged for each DNA sample. Negative controls were included in all amplification reactions. The PCR products of primers specific for the *wsp* gene of *Wolbachia* and the 16S rDNA gene of *Cardinium* were amplified by conventional PCR, then the PCR products was purified using the AxyPrep^{TM} DNA Gel Extraction kit (AXYGEN) and cloned into a pGEM-T Easy vector (Promega). A series of DNA standards prepared from plasmid DNA was used and standard curves were plotted using a tenfold dilution series from 10^4 to 10^8 copies numbers. Ct values in each dilution were measured using a Q-PCR to generate the standard curves for *wsp* and 16S rDNA. The slopes of the standard curves for *wsp* and 16S rDNA were −3.44 and −3.45, respectively. From the slopes, a high amplification efficiency of 0.95 was determined for both *wsp* and 16S rDNA in the investigated range. 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.

Table 1 Results of different infected colonies of the Yunnan (YN) population

Effect	Cross (F×M)	*N*	Number of eggs	% Unhatched eggs	Sex ratio (% males)	% Mortality
(a) Single effect: *Wolbachia* CI?	U×U	32	31.8 ± 0.9	3.5 ± 1.4[a]	14.8 ± 1.9[a]	4.2 ± 1.5
	U×Iw	34	34.4 ± 2.7	32.0 ± 1.5[b]	22.0 ± 1.3[b]	9.1 ± 1.5
	Iw×U	35	34.1 ± 1.6	3.1 ± 1.4[a]	15.9 ± 1.1[a]	7.1 ± 1.4
	Iw×Iw	33	33.4 ± 1.8	6.0 ± 1.6[a]	15.4 ± 1.2[a]	7.1 ± 1.6
			NS	***	*	NS
(b) Single effect: *Cardinium* CI?	U×U	32	31.8 ± 1.5	3.5 ± 1.4[a]	14.8 ± 1.0[a]	4.2 ± 1.5
	U×Ic	25	34.2 ± 1.4	61.9 ± 1.4[b]	35.0 ± 1.7[b]	13.2 ± 1.7
	Ic×U	26	30.9 ± 2.7	5.3 ± 1.3[a]	13.3 ± 1.9[a]	6.2 ± 1.5
	Ic×Ic	27	37.2 ± 1.4	3.8 ± 1.6[a]	11.1 ± 1.1[a]	4.8 ± 1.5
			NS	***	***	NS
(c) Interaction: double infection CI?	U×U	32	31.8 ± 1.9	3.5 ± 1.4[a]	14.8 ± 1.4[a]	4.2 ± 1.5[a]
	U×Iwc	32	33.1 ± 1.4	88.5 ± 1.4[b]	72.2 ± 1.3[b]	17.9 ± 1.4[b]
	Iwc×U	30	31.8 ± 1.5	6.9 ± 1.7[a]	14.7 ± 1.9[a]	4.6 ± 1.6[a]
	Iwc×Iwc	28	31.5 ± 1.3	5.2 ± 1.3[a]	13.4 ± 1.1[a]	6.2 ± 1.4[a]
			NS	***	***	***
(d) *Wolbachia* modifies	U×Ic	25	34.2 ± 1.8	61.9 ± 1.4[a]	35.0 ± 1.7[a]	13.2 ± 1.7
the strength of	Iw×Iwc	30	31.8 ± 1.6	80.1 ± 1.5[b]	93.9 ± 1.5[b]	15.3 ± 1.5
Cardinium-induced CI?	Iw×Ic	28	30.5 ± 1.4	60.9 ± 1.5[a]	53.2 ± 1.2[c]	16.6 ± 0.9
			NS	***	***	NS
(e) *Wolbachia* affects	Ic×Ic	27	37.2 ± 2.0	3.8 ± 0.9	11.1 ± 1.4	4.8 ± 1.4
Cardinium-induced CI rescue?	Iwc×Ic	25	36.5 ± 1.7	3.7 ± 1.4	14.5 ± 1.7	5.2 ± 1.5
	Iwc×Iwc	28	31.5 ± 1.8	5.2 ± 1.6	13.4 ± 1.3	6.2 ± 1.2
			NS	NS	NS	NS
(f) *Cardinium* modifies	U×Iw	34	34.4 ± 1.6	32.0 ± 1.4	22.0 ± 1.7	9.1 ± 1.4
the strength of	Ic×Iwc	26	35.6 ± 1.3	30.4 ± 1.3	25.8 ± 1.3	9.2 ± 1.6
Wolbachia-induced CI?	Ic×Iw	24	35.6 ± 1.4	31.5 ± 1.4	26.4 ± 1.8	7.9 ± 1.5
			NS	NS	NS	NS
(g) *Cardinium* affects	Iw×Iw	33	33.4 ± 1.9	6.0 ± 1.5	15.9 ± 1.9	4.1 ± 1.3
Wolbachia-induced CI rescue?	Iwc×Iw	26	32.8 ± 1.5	5.8 ± 1.4	16.5 ± 1.9	4.4 ± 1.3
	Iwc×Iwc	28	31.5 ± 1.6	5.2 ± 1.2	13.4 ± 1.4	6.2 ± 1.5
			NS	NS	NS	NS

Iw Wolbachia-infected, *Ic Cardinium*-infected, *Iwc* doubly infected (*Wolbachia* and *Cardinium*), *U* uninfected, *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

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 Tukey's post hoc test

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Results

Cytoplasmic Incompatibility of Doubly Infected and Singly Infected Lines

Effects of Wolbachia and Cardinium Alone

By comparing the four crosses in which we tested possible *Wolbachia* CI effects, we found that *Wolbachia* caused weak level of CI (Table 1a). In the incompatible cross (U×Iw), on average, 32.0 % of all eggs did not hatch, compared with 3.45–6.03 % in the other three crosses. The sex ratio of the offspring that did hatch in the incompatible cross approached 22.0 %; the number of eggs produced and the mortality were not significantly different among the four crosses.

In the case of *Cardinium*, the number of unhatched eggs was significantly different among the four crosses (Table 1b). In the incompatible cross U×Ic, approximate 61.9 % of all eggs did not hatch, compared with 3.5–5.3 % in the other crosses. The male ratio was significantly higher

in the incompatible cross than in the other crosses. This was because of a decrease in the number of female progenies, as the number of male progenies, was not significantly different among the four crosses. Therefore, *Cardinium* is capable of causing severe CI.

Effects of Double Infection

Strong CI can be induced by doubly infected males (Iwc) (Table 1c). The unhatched eggs and the sex ratio (% males) were high in the incompatible cross (U×Iwc) which is significantly different from the other three crosses. The strength of CI induced by doubly infected males (Iwc) also was found to be higher than that induced by the singly infected males (Iw and Ic).

Interactions Between Wolbachia and Cardinium

In order to find out whether *Wolbachia* can influence the CI strength of *Cardinium,* different crosses were investigated. As shown in Table 1d, an average of 80 % of all eggs did not hatch in the cross Iw×Iwc, and the unhatched rate was significantly higher than the crosses U×Ic (61.9 %) and Iw×Ic (60.9 %). The presence of *Wolbachia* in both male and female of a cross seemed to change *Cardinium*-induced CI expression. The results indicated that *Wolbachia* can promote the strength of *Cardinium*-induced CI. In addition, the crosses Ic×Ic, Iwc×Ic, and Iwc×Iwc (Table 1e) showed that *Wolbachia* does not affect *Cardinium*-induced CI rescue. Moreover, there was no significant difference between the crosses U×Ic and Iw×Ic (Table 1d), which indicates that CI induced by *Cardinium* cannot be rescued by *Wolbachia.*

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

Wolbachia and Cardinium Densities in Singly and Doubly Infected Lines

At day 1, the numbers of *Wolbachia* in the singly and doubly infected males were 0.55 and 0.47 $\times$ 10^6 (Fig. 1a). The density of *Wolbachia* in the doubly infected males was significantly lower than that in singly *Wolbachia*-infected males ($P < 0.001$). However, the numbers of *Cardinium* in the singly and doubly infected males were 0.49 and 0.57 $\times$ 10^6 (Fig. 1a), respectively. The number of

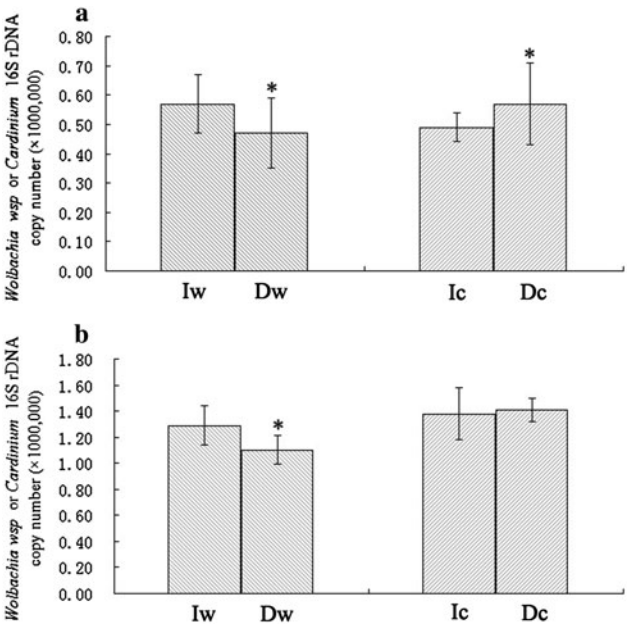

Fig. 1 Infection densities of *Wolbachia* and *Cardinium* in singly infected and doubly infected males (**a**) and females (**b**). *Iw* densities of *Wolbachia* in singly *Wolbachia*-infected mites, *Ic* densities of *Cardinium* in singly *Cardinium*-infected mites, *Dw* densities of *Wolbachia* in doubly infected mites, and *Dc* densities of *Cardinium* in doubly infected mites. *Asterisks* indicate statistically significant differences (Mann–Whitney *U* test, $P < 0.001$) *Error bars* ± 1 standard error

Cardinium in the doubly infected males was significantly higher than that in singly *Cardinium*-infected males ($P < 0.001$). In the doubly infected males, the multiplication of *Wolbachia* was suppressed while the multiplication of *Cardinium* is promoted.

In 1 day-old mites, the densities of *Wolbachia* and *Cardinium* were clearly higher in females than that of males. The numbers of *Wolbachia* in the singly and doubly infected females were 1.29 and 1.10 $\times$ 10^6 (Fig. 1b), respectively. The densities of *Wolbachia* were lower in doubly infected females than in singly infected females. However, the numbers of *Cardinium* in the singly and doubly infected females were 1.38 and 1.41 $\times$ 10^6 (Fig. 1b), respectively. The densities of *Cardinium* were slightly higher in doubly infected females than in singly infected females, but it was not statistically significant ($P > 0.05$).

Discussion

Cytoplasmic Incompatibility

Both of *Wolbachia* and *Cardinium* can cause CI in our study. CI was expressed as a reduction in egg hatchability

and a male-biased sex ratio in crosses between uninfected females and infected males in our testing crosses. This is concordant with the female mortality type of CI [7, 29]. *Wolbachia* caused a weak level of CI. The *wsp* gene sequence (unpublished) of this *Wolbachia* indicates that the strain belongs to the Ori subgroup. The *Wolbachia* belonging to the Ori subgroup were shown to cause a wide range of CI in Chinese populations of *T. urticae* [41]. Moreover, *Cardinium*-infected males caused severe CI, and the CI induced by doubly infected males was more severe than that caused by males singly infected with *Cardinium* or *Wolbachia*. The males singly infected by *Cardinium* also caused severe CI in the spider mite *B. sarothamni*; however, singly *Wolbachia*-infected and doubly infected males did not induce CI [24]. On the contrary, in doubly infected *E. inaron*, *Wolbachia*, and not *Cardinium*, caused CI of the female mortality type [40].

Symbionts Affect Each Other in the Expression and Rescue of CI

CI induced by singly *Cardinium*-infected males cannot be rescued by singly *Wolbachia*-infected females, and CI induced by singly *Wolbachia*-infected males cannot be rescued by singly *Cardinium*-infected females. These results are consistent with the result of studies in *B. sarothamni* and *E. inaron* [24, 40]. However, the rate of unhatched eggs was significantly higher in the Iw×Iwc cross than in the U×Ic cross. This suggests that *Wolbachia* increased the severity of *Cardinium*-induced CI. Although multiple infections of *Wolbachia* and other symbionts are common and the relative contributions of *Wolbachia* and *Cardinium* to CI modification and rescue have been studied in a doubly infected *E. inaron* [40], to the best of our knowledge, the present results are the first to show that *Wolbachia* can increase the severity of *Cardinium*-induced CI.

Factors Affect the Levels of CI

Host genotype has been proposed to affect expression of CI [17]. In our experiments, singly infected lines were generated from doubly infected isofemale lines to rule out effects of host genotype. Bacterial strains have also been shown to be important in the expression of CI [4], and it is possible that *Wolbachia* has evolved a new phenotype that is beneficial for *Cardinium*-induced CI in *T. piercei* McGregor. Transferring *Wolbachia* into other *Cardinium*-infected *T. piercei* McGregor would be necessary to verify this new phenotype.

A likely explanation is that *Wolbachia* promoted the strength of *Cardinium*-induced CI by promoting *Cardinium* densities when doubly infected males mated to uninfected females. *Cardinium* density has been suggested to be a critical factor for CI intensity in *T. cinnabarinus*, and a threshold level of *Cardinium* density may be required for the induction of CI [42]. We found that the multiplication of *Wolbachia* is suppressed while the multiplication of *Cardinium* is promoted in doubly infected males (Fig. 1a). It may also explain why the CI induced by doubly infected males was more severe than the CI induced by males singly infected with *Cardinium* or *Wolbachia*. Maintenance of infection by multiple CI-inducing symbionts is therefore often evolutionarily favored [29]. The synergy between *Wolbachia* and *Cardinium* appears to agree with their general co-occurrence in the host tissues. Cohabitation in the same host provides ample opportunities for interactions among symbionts that can either facilitate or limit symbiotic existence. For example, in the sweet potato whitefly *Bemisia tabaci*, secondary symbionts were found to share bacteriocytes with primary symbiont, which allowed them to be vertically transmitted by "hitching a ride" with primary symbiont [13]. In this way, symbionts co-infecting the same host can better manipulate the reproduction of their host.

In addition, the *Wolbachia* density in doubly infected males was lower than that in males singly infected with *Wolbachia*, although the rate of unhatched eggs in the Ic×Iwc cross was similar to that in the I×Iw cross. The severity of *Wolbachia*-induced CI in doubly infected males was not affected by the *Wolbachia* density. Similarly, in three *Drosophil*a species (*D. simulans* Hawaii, *D. sechellia*, and *D. auraria*), CI was positively correlated with bacterial density, but in all the other *Drosophila* species including *D. simulans* Riverside, CI was not correlated with bacterial density [5]. There are two possible explanations for this result. On the one hand, a threshold level of bacterial density is required for inducing CI. Below the threshold density of *Wolbachia*, the penetrance of CI began to fall off. Densities above the threshold appeared to have no additional effect on the strength of CI. The threshold density could vary between different *Wolbachia* strains [25]. In doubly infected males, the *Wolbachia* density may have not been below the threshold density, so the level of CI did not fall. On the other hand, because of the selective pressure acting on both partners, various mechanisms should have evolved to control infection density within an appropriate range, which leads to the idea that both symbiont and host genotype may contribute to the regulation of infection density [19]. For example, in the adzuki bean beetles *Callosobruchus chinensis*, the host genotypes could suppress *Wolbachia* density by becoming co-infected with different *Wolbachia* strains [17].

The density of *Cardinium* in doubly infected females was not significantly different from that in females singly infected with *Cardinium* (Fig. 1b), which is different from the case in males. The biological effects of symbionts may

largely depend on their infection densities. A reduced infection density may result in imperfect vertical transmission and a consequent loss of infection. High infection density may also have pathological effects on the host, and hence have negative effects on their fitness [17]. In the parasitoid wasp *Nasonia vitripennis*, bacterial density was correlated with compatibility differences between males and females. Males from strains with high bacterial densities were incompatible with females from strains with lower densities [8]. However, this was not the case in our study. More work is needed to investigate the relations between degree of CI and density of *Wolbachia* and *Cardinium* in doubly infected females.

In brief, in the same host body, various interactions are expected to occur between coexisting symbionts. We investigated the interactions of both *Wolbachia* and *Cardinium* in the spider mite *T. piercei*, and found that the level of CI induced by *Wolbachia* was not related to *Wolbachia* density, but that *Wolbachia* promoted the strength of *Cardinium*-induced CI. These findings should help to design further studies of the interactions between *Wolbachia* and *Cardinium* in doubly infected hosts and help to develop a method for controlling arthropod pests using these two endosymbionts.

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