Symbiont-conferred reproduction and fitness benefits can favour their host occurrence

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Abstract
Double infections of Wolbachia and Spiroplasma are frequent in natural populations of Tetranychus truncatus, a polyphagous mite species that has been a dominant species in China since 2009. However, little is known about the causes and ecological importance of such coexistences. In this study, we established T. truncatus strains with different infection types and then inferred the impact of the two endosymbionts on host reproduction and fitness. Double infection induced cytoplasmic incompatibility, which was demonstrated by reduction in egg hatchability of incompatible crosses. However, doubly infected females produced more eggs relative to other strains. Wolbachia and Spiroplasma did not affect host survival, whereas doubly infected females and males developed faster than other strains. Such reproduction and fitness benefits provided by double infections may be associated with the lower densities of each symbiont, and the quantitative results also confirmed competition between Wolbachia and Spiroplasma in doubly infected females. These symbiont-conferred beneficial effects maintain stable prevalence of the symbionts and also help drive T. truncatus outbreaks in combination with other environmental factors.

KEYWORDS
double infection, fitness, reproduction, symbiont, Tetranychus truncatus

1 | INTRODUCTION

Heritable bacterial endosymbionts are widespread in arthropods. They utilize diverse strategies to spread within host populations, such as provision of essential nutrients (Gündüz & Douglas, 2009; Hosokawa, Koga, Kikuchi, Meng, & Fukatsu, 2010), defense against parasites (Hedges, Brownlie, O’Neill, & Johnson, 2008; Vorburger, Gehrer, & Rodriguez, 2010), and reproductive manipulation (Ma, Vavre, & Beukeboom, 2014).

Endosymbionts have traditionally been grouped into primary and secondary symbionts. Primary symbionts are mutualists that tend to have a nutritional value, which is the case in the typical example of aphids and Buchnera aphidicola (Baumann, 2005). In contrast, secondary symbionts are often facultative symbionts from the host’s perspective and have a much broader array of effects. Most symbionts are exclusively or predominantly vertically transmitted; however, the often-found incongruence between host and symbiont phylogenies indicates that these infections are generally short-lived on macro-evolutionary timescales. In theory, once a symbiont invades a novel host, it must immediately be capable of replication and colonization of the female germ line. Hurst and Darby (2009) also speculated that high rates of horizontal transmission, reproductive manipulation, and direct fitness benefits to the host will favour maintenance of secondary symbionts within the host population. The high prevalence of several secondary symbionts without obvious parasitic phenotypes can sometimes be linked to such direct fitness effects; for example, increased performance and sex ratio bias of infected whiteflies sufficiently explain the spread of Rickettsia symbionts (Himler et al., 2011).
Among the secondary symbionts, Wolbachia is by far the most widely documented and infects a large proportion of arthropod species (Werren, Baldo, & Clark, 2008). Wolbachia is notorious for its reproductive parasitism, which ensures its spread despite lowering host fitness. However, even for reproductive parasites, it can be beneficial to enhance host fitness. Indeed, recent years have seen a growing body of evidence on Wolbachia-associated fitness benefits (reviewed in Zug & Hammerstein, 2015). Spiroplasma is a wall-less bacterium associated with diverse arthropods and plants in which it has commensal, pathogenic, or mutualistic effects (Cisak et al., 2015). Some Spiroplasma species and strains are known as male killers in fruit flies, ladybird beetles, planthoppers, and butterflies, wherein infected females produce all-female or female-biased offspring (Jiggins, Hurst, Jiggins, VD Schulpenburg, & Majerus, 2000; Sanada-Morimura, Matsumura, & Noda, 2013; Tinsley & Majerus, 2006; Williamson et al., 1999). Independent studies have shown that the two symbionts simultaneously infect some arthropods (Duron et al., 2008; Enigl & Schausberger, 2007; Goodacre, Martin, Thomas, & Hewitt, 2006; Jaenike, Stahlhut, Boelio, & Unckless, 2010; Shokal et al., 2016). In Drosophila neotestacea, recent spread and mutualism account for the positive association between Wolbachia and Spiroplasma (Jaenike et al., 2010). However, the ecological and evolutionary importance of such coexistences remains underexplored.

Tetranychus truncatus is a polyphagous species that infests more than 60 host plant species (Bolland, Gutierrez, & Fletchmann, 1998). It is mainly distributed in East and Southeast Asia, and there is report of its presence in the USA (Migeon & Dorkeld, 2006–2017). T. truncatus have already become the dominant pest in China since 2009 based on analysis of spider mite sampling data from 2002 to 2015. T. truncatus are host to several symbionts; for example, previous studies revealed that T. truncatus is infected with diverse Wolbachia strains (Zhang et al., 2013), and double infections of Wolbachia and Cardinium are frequent in some natural populations (Zhao, Zhang, & Hong, 2013). Recently, high prevalence of Spiroplasma was identified in T. truncatus, and there was a substantial positive association between Wolbachia and Spiroplasma in some populations (Zhang, Chen, Yang, Qiao, & Hong, 2016).

Given the high rate of co-transmission of these two symbionts and recent T. truncatus outbreaks, we investigated the mutualistic phenotypes of these two symbionts within T. truncatus. We established different T. truncatus strains with the same host genetic background and investigated the effect of Wolbachia alone or in combination with Spiroplasma on T. truncatus reproduction and fitness. Furthermore, the densities of the two symbionts were monitored and compared during host development to infer their competition relationship.

2 | MATERIALS AND METHODS

2.1 | Wolbachia and Spiroplasma prevalence in T. truncatus

Adult T. truncatus were collected from six populations in northern China (Figure 1). Individual mites were either immediately analyzed in the laboratory to assess infection frequency (see below) or individual females were reared as isofemale lines on leaves of common bean (Phaseolus vulgaris L.) placed on a water-saturated sponge mat in Petri dishes (dia. 9) at 25 ± 1°C, 60% humidity, and under L16–D8 conditions.

Subsequently, 16–24 field-collected individual mites were screened for infection with Wolbachia and/or Spiroplasma. DNA was extracted from single mites using a genomic DNA extraction kit (TaKaRa, Dalian, China). All DNA samples were first PCR-screened for the mitochondrial gene COI as a control for quality (Navajas, Gutierrez, Lagnell, & Boursot, 1996). Wolbachia and Spiroplasma presence was detected using PCR amplifications of wsp and 16S rRNA, respectively. wsp was amplified using the primers wspF1 and wspR1 (Baldo et al., 2006), and 16S rRNA was amplified using the primers SpitsJ04 and SpitsN55 (Jaenike, Polak, Fiskin, Helou, & Minhas, 2007). In addition, to confirm the presence of other two reproductive endosymbionts, Cardinium and Rickettsia, in T. truncates. PCR amplifications were performed using primers Ch-F and Ch-R (Zchori-Fein & Perlman, 2004), RICS741F and RCIT1197R (Davis, Ying, Brunner, Pantoja, & Ferwerda, 1998) that were widely used to detect Cardinium and Rickettsia, respectively. Each reaction was carried out on a Veriti machine (ABI Biosystems, USA) in a 25 μl volume containing 12.5 μl 2× Taq Master Mix (Vazyme Biotech, China), 0.5 μl primer (20 μmol/L each), and 1 μl of DNA extract. Positive and negative controls were included in PCRs.
host biology. In this study, we firstly established four *T. truncatus* strains: mites infect with both *Wolbachia* and *Spiroplasma* (designated as W+S+), *Wolbachia* only (W+S−), *Spiroplasma* only (W−S+), and no symbionts (W−S−). To eliminate the effects of host genetic background, the above-mentioned four *T. truncatus* strains were derived from lines collected in Shenyang, Liaoning (Pop 3 in Figure 1). The original field females included three strains, W+S+, W+S−, and W−S+. The W−S− strains were obtained by raising infected mites on the common bean placed on a cotton bed soaked in tetracycline solution (0.1%, w/v) for three generations. These lines were maintained in a mass-rearing environment without antibiotics for approximately six generations to allow recovery from tetracycline treatment. For each of these lines, infection status was checked during the course of the experiments.

2.3 | Crossing experiments

To test whether *Wolbachia* and *Spiroplasma* cooperate to cause cytoplasmic incompatibility (CI) in *T. truncatus*, crossing experiments were performed (listed as male × female, Figure 2). 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. Each cross used 16 leaf disks. Males were discarded 2 days after the females reached adulthood. The mated females were allowed to oviposit for 5 days. The eggs on the leaf disks were checked daily to determine hatchability and the sex ratio (% females). Prior to analyses, data were first tested for normality (Kolmogorov–Smirnov test, SPSS 17.0) and homogeneity of group variances (Levene’s test, SPSS 17.0). Which possible, square root, logarithmic or arcsine transformations were performed to attain normality and homogeneity of variance. In this study, log transformation was used for the number of eggs laid per female, and arcsine square root transformation was used for egg hatchability and sex ratio. Data were then analyzed with one-way analysis of variance (ANOVA), and the means were compared using the Tukey HSD test (SPSS 17.0).

The male-killing phenotype of *Spiroplasma* in arthropods results in the production of female-biased offspring sex ratios. Here, the sex ratio data of the four *T. truncatus* strains were analyzed to determine if male-killing occurred.

2.4 | Survival assay

To test for potential effects of symbionts on host survival, we measured the survival of different *T. truncatus* strains by placing eight virgin females and eight virgin males from the same strains on the same leaf. Three leaves were used for each line, and females were transferred to

![Figure 2](image-url)  
**Figure 2** Results of crosses between different *Tetranychus truncatus* strains. Number of eggs (a), egg hatch percent (b), and female proportion of offspring (c) are shown. Results are mean ± SEM, and a and b represent statistically different groups (Tukey-HSD test, *p* < .05)
fresh leaves every 3 days. The number of dead females was recorded daily. Survivor curves for individual hosts were compared using the Kaplan–Meier method and log-rank test (Dobson, Rattanadechakul, & Marsland, 2004).

2.5 Development assay

The effect of symbionts on mite development was assessed. Thirty virgin and 30 mated females (which produce male and female offspring, respectively) were placed on a leaf disk and allowed to lay eggs for 8 hr. The eggs were individually moved to new small leaf disks. The small disks were monitored every 8 hr, and the stage of mite was recorded until adulthood. The development time of every stage was calculated. Log transformation was used for the total development time of each mite line, and differences were analyzed using the Mann–Whitney test (SPSS 17.0).

2.6 Wolbachia and Spiroplasma densities

The Wolbachia and Spiroplasma densities in individual mites were estimated by real-time quantitative PCR (QPCR). QPCR was carried out with the ABI PRISM 7300 Sequence Detection System (Applied Biosystems). We used primers designed to amplify a 141-bp fragment of wsp from Wolbachia (wQF1, 5′-GAGCAGCGAATGTAAGCATA-3′, and wQR1, 5′-AATAACGAGCACCACCCAATA-3′) and a 141-bp fragment of 16S rRNA from Spiroplasma (sQF1, 5′-TGTAGTTCTCAGGGATTGTTTTCTC-3′, and sQR1, 5′-CGCTTCCACCATCGCTT-3′). The PCR products of primers specific for wsp for Wolbachia and 16S rRNA for Spiroplasma were amplified by conventional PCR; then, the PCR products were purified using the AxyPrep TM DNA Gel Extraction kit (AXYGEN) and cloned into a pEASY-T1 vector (TransGen Biotech, Beijing, China). A series of DNA standards prepared from plasmid DNA was used, and standard curves were plotted using a 10-fold dilution series from $10^4$ to $10^9$ copy numbers. Ct values in each dilution were measured by QPCR to generate the standard curves for wsp and 16S rRNA. The 20 μl reaction mixture consisted of 10 μl 2× SYBR Premix Ex Taq (Applied Biosystems), 0.4 μl 10 mmol/L of each primer, 0.4 μl 50× ROX Reference Dye, 2 μl DNA template, and 6.8 μl H₂O in single wells of a 96-well plate (PE Applied Biosystems). The QPCR cycling conditions included one cycle (10 s at 95°C) followed by 40 cycles (5 s at 95°C and 31 s at 60°C), and finally one cycle (15 s at 95°C, 1 min at 60°C and 15 s at 95°C).

Eight each of singly and doubly infected females of 2-, 4-, 6-, 8-, and 12-days-old were collected. DNA of individual mites was extracted as described above. Three replicates were run and averaged for each DNA sample. Negative controls were included in all amplification reactions. From the slopes, a high amplification efficiency of 0.95 was determined for both wsp and 16S rRNA 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.

3 RESULTS

3.1 Double infections of Wolbachia and Spiroplasma are frequent in T. truncatus

Among 132 wild-caught adult mites that were collected from six sites, 16.7% and 3.8% were infected with only Wolbachia or Spiroplasma, respectively, and 79.5% were infected with both symbionts. Double infections with high prevalence were present in all screened populations (Figure 1). The infections of Cardinium and Rickettsia were not detected in these examined samples.

Sequencing of Wolbachia wsp identified two haplotypes that differed by one base (wTru1 in Pop 2, 4, 5, and 6; wTru5 in Pop 1 and 3). However, identical Spiroplasma 16S rRNA sequences were observed among infected individuals.

3.2 Wolbachia and Spiroplasma have diverse effects on host reproduction

To clarify the effects of Wolbachia and Spiroplasma on host reproduction, egg number, egg hatchability, and offspring sex ratio of the 16 crosses were compared (Figure 2 A, B, C). Among the four crosses in which we tested possible Wolbachia CI effects, the egg hatchability of the predicted compatible crosses (W+S− × W+S−) was significantly lower than that in other crosses, whereas the female proportion was significantly higher in this cross. Doubly infected males appeared to induce CI, which was inferred based on significant reductions of egg hatchability and female proportion of offspring in the predicted incompatible crosses. However, no significant effects were found for other crosses in which singly Spiroplasma-infected males and Spiroplasma-uninfected males were investigated. In addition, nonsignificant differences in female proportion of the four strains indicated that Spiroplasma does not cause male killing in T. truncatus.

The effects of Wolbachia and Spiroplasma on host fecundity were also measured. The doubly infected females mostly showed the highest egg production, except for the crosses that involved the singly Wolbachia-infected males, in which singly Spiroplasma-infected females produced more eggs. In general, egg production of singly infected females and uninfected females did not significantly differ across all crosses.

3.3 Wolbachia and Spiroplasma do not affect host survival

The longevities of four female lines were compared to discover the effects of infection. As shown in Figure 3, there was no significant effect of Wolbachia and Spiroplasma on female host survival (log-rank test, p = .87).

3.4 Double infections can shorten host developmental rate

The development times of females and males from different strains are presented in Figure 4A, B, respectively. Doubly infected females reached adulthood (10.8 ± 0.004) significantly earlier than
singly infected females by 1–2 days (W+S−: 12.38 ± 0.13, W−S+: 12.11 ± 0.13) and uninfected females (11.81 ± 0.003) (p < .0001).

Additionally, time to adulthood was significantly shorter in uninfected than singly infected females (p < .05), and the latter two had no significant difference (p = .97). Similarly, doubly infected (7.27 ± 0.19) and uninfected males (6.99 ± 0.25) developed faster than singly infected males (W+S−: 7.83 ± 0.15, W−S+: 8.13 ± 0.11) (p < .05), whereas no significant difference was detected between doubly infected and uninfected males (p = .41), singly Wolbachia- and singly Spiroplasma-infected males (p = .31).

3.5 | Wolbachia and Spiroplasma densities are higher in singly than doubly infected females

The Wolbachia density steadily increased (except for 2-day-old to 4-day-old adults of the singly infected strain) as adult females developed in both strains. At three developmental stages, 2, 4, and 6 days after emergence, the Wolbachia densities in singly infected females were significantly higher in doubly infected females (Figure 5A, p < .01). A similar pattern was observed for Spiroplasma, which revealed that Spiroplasma density dynamics were significantly affected by coinfecting Wolbachia (Figure 5B, p < .001).

4 | DISCUSSION

In this study, we found that double infections of Wolbachia and Spiroplasma within the same individuals are frequent in natural T. truncatus populations. Among the six examined populations, 79.5% of mites were doubly infected, and double infection was fixed in three populations. Wolbachia and Spiroplasma are both maternally transmitted symbionts and act as reproductive manipulators in many arthropods. Symbiont infection frequency is theoretically determined by several factors, including reproductive manipulation, infection costs, vertical transmission efficiency, and horizontal transmission rate (Hoffmann, Hercus, & Dagher, 1998; Werren, 1997). In this respect, empirical studies have revealed that facultative symbionts can spread quickly within the host population when they provide a large fitness benefit at no or relatively little cost (Himler et al., 2011; Jaenike et al., 2010; Oliver, Campos, Moran, & Hunter, 2008). In such cases, reproductive manipulations and/or providing fitness benefits of double infections would be predicted in T. truncatus.

Wolbachia-induced CI phenotypes have been observed in some Tetranychus species (Gotoh, Noda, & Hong, 2003; Zhao, Chen, Ge, Gotoh, & Hong, 2013; Zhu et al., 2012). However, there was no evidence of Wolbachia-mediated CI in T. truncatus, which is consistent with a previous finding (Zhao, Chen, et al., 2013). Singly Wolbachia-infected females may suffer fitness costs, such as lower hatch percent, but they also have a higher female offspring proportion. Wolbachia strains that infect T. truncatus are phylogenetically diverse (Zhang et al., 2013), and non-CI Wolbachia were present in this study. Some likely reasons have been explained for the lack of CI (Ros & Breeuwer, 2009). Although Spiroplasma carried by T. truncatus has a closer phylogenetic relationship with the male-killing strain in small brown planthopper, Laodelphax striatellus (Sanada-Morimura et al., 2013), it is not a male killer, because Spiroplasma did not cause significant differences in offspring sex ratio, and its incidence in male hosts was relatively high.

It is worth noting that doubly infected males were incompatible with both singly infected and uninfected females, which was inferred based on significant reduction in the egg hatchability. The female offspring proportions of uninfected females were significantly lower than those of other strains, and symbionts carried by females might have

**FIGURE 3** Comparison of Wolbachia and Spiroplasma effects on female Tetranychus truncatus survival. Survival curves for individual hosts were compared using the Kaplan–Meier method and log-rank test

**FIGURE 4** Development times of different female (a) and male (b) Tetranychus truncatus strains. Differences were analyzed using Mann–Whitney test (*p < .05; ***p < .001; NS, not significant)
Density dynamics of *Wolbachia* and *Spiroplasma* during host development in singly and doubly infected female *Tetranychus truncatus*. Copy numbers per ml were determined by quantitative PCR. Asterisks indicate statistically significant differences (Mann-Whitney U test, *p* < .05; **p** < .01; ***p*** < .001; NS, not significant).

In this study, double infections appeared to enhance female and male mites' developmental speed. Whatever the mechanism, it is evident that more generations will be produced within the doubly infected mite strains under certain circumstances. As a result, *T. truncatus* occurrence will directly benefit from these manipulations, and such manipulations will also maintain higher frequencies of double infection in nature. Similarly, Himler et al. (2011) found that *Rickettsia*-infected whiteflies developed faster, and this observed increased performance can partly be explained by the rapid spread of *Rickettsia* across the southwestern United States within 8 years. Moreover, Jaenike et al. (2010) found that the frequent positive association between *Wolbachia* and *Spiroplasma* within *D. neotestacea* is a consequence of emerging symbiotic mutualism. Together, these observations strongly highlight symbionts' substantial influences on their hosts' ecology and population dynamics. Furthermore, these impacts are often influenced by symbionts (strains or infection patterns) and hosts (genetic background). For example, the fitness benefits to *T. truncatus* are mainly associated with double infections, which raises the possibility that *Wolbachia* and *Spiroplasma* coordinately regulate host biological traits.

Symbionts' complex effects on their hosts result from long-term interactions and coevolution. Symbionts may manipulate their host physiological process and affect their fitness traits. wMelPop *Wolbachia* is well known for shortening the life of its *Drosophila* host (Min & Benzer, 1997). In *Drosophila hydei*, *Spiroplasma* showed enhanced survival when attacked by *Leptopilina heterotoma* wasps (Xie, Vilchez, & Mateos, 2010). Negative effects of symbionts on host survival were also observed (Costopoulos, Kovacs, Kamins, & Gerardo, 2014).

In this study, symbionts did not influence *T. truncatus* survival, but significantly regulated female fecundity. Relative to singly infected and uninfected females, doubly infected females produced more eggs. Fecundity advantage provided by symbionts is not surprising, and other studies report a similar phenomenon (Dobson et al., 2004; Himler et al., 2011; Zug & Hammerstein, 2015). These findings are concordant with a hypothesis that vertically transmitted symbionts cause their hosts to produce more infected daughters than are produced by uninfected females to facilitate their own spread (Werren & O'Neill, 1997). The infection density of symbionts is among the most important factors that influence their biological effects, such as intensity of reproductive phenotypes, level of fitness effects, and fidelity of vertical transmission. Oliver et al. (2006) proposed that over-proliferation of symbionts may be harmful to the aphid host. In *T. truncatus*, the high density of symbionts may result in fecundity cost to singly infected females. Additionally, QPCR estimates also indicated that numbers of each of the two symbionts in singly infected females are significantly higher than those in doubly infected females.

In summary, double infections of *Wolbachia* and *Spiroplasma* are beneficial to their mite host by inducing CI, and increasing fecundity and developmental rate. These benefits can help maintain stable double infections and also drive *T. truncatus* outbreaks in combination with other environmental factors.
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AUTHOR CONTRIBUTIONS

YKZ and XYH conceived and designed experiments. YKZ, KY, YXZ performed the experiments. YKZ and KY analyzed the data. YKZ and XYH wrote the paper. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

None declared.

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