Wolbachia dominate Spiroplasma in the co-infected spider mite Tetranychus truncatus

Kun Yang¹, Kang Xie¹, Yu-Xi Zhu¹, Shi-Mei Huo¹, Ary Hoffmann², Xiao-Yue Hong¹*

¹ Department of Entomology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

²Bio21 Institute, School of BioSciences, The University of Melbourne, Victoria 3010, Australia

Running Head: Wolbachia dominate Spiroplasma in spider mite

*Corresponding author: Xiao-Yue Hong, Tel: +86-25-84395339. E-mail: xyhong@njau.edu.cn

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/imb.12607

This article is protected by copyright. All rights reserved.
Abstract

_Wolbachia_ and _Spiroplasma_ are both maternally inherited endosymbionts in arthropods, and they can co-infect the same species. However, how they interact with each other in the same host is not clear. Here we investigate a co-infected _Tetranychus truncatus_ spider mite strain that shares the same genetic background with singly-infected and uninfected strains to detect the impacts of the two symbionts on their host. We found that _Wolbachia_-infected and _Spiroplasma_-infected mites can suffer significant fitness costs involving decreased fecundity, although with no effect on lifespan or development. _Wolbachia_ induced incomplete cytoplasmic incompatibility (CI) in _T. truncatus_ both in singly-infected and doubly-infected strains, resulting in female killing. In both females and males of the co-infected spider mite strain, _Wolbachia_ density was higher than _Spiroplasma_ density. Transcriptome analysis of female adults showed that the most differently expressed genes (DEGs) were found between the co-infected strain and both the singly-infected _Spiroplasma_ strain and uninfected strain. The _Wolbachia_ strain had the fewest DEGs compared to the co-infected strain, consistent with the higher density of _Wolbachia_ in the co-infected strain. _Wolbachia_ therefore appears to have a competitive advantage in
host mites over Spiroplasma and is likely maintained in populations by cytoplasmic incompatibility despite having deleterious fitness effects.

**Key words:** Wolbachia, Spiroplasma, spider mite, co-infection, cytoplasmic incompatibility, transcriptome analysis

**Introduction**

Maternally inherited endosymbionts are widespread in arthropods, and *Wolbachia* and *Spiroplasma* are two well-known endosymbionts with impacts on their hosts. *Wolbachia* can be responsible for four types of reproductive manipulations on hosts, involving cytoplasmic incompatibility (CI), parthenogenesis, feminization and male-killing (O’Neill *et al.*, 1997). Among them, CI is considered *Wolbachia*’s most common impact on arthropod hosts (Hoffmann and Turelli 1997). These *Wolbachia* effects assist the spread of the bacterium across hosts, which can be further assisted by benefits provided by *Wolbachia* on host fitness (Zug and Hammerstein 2015).

*Spiroplasma* are descendants of Gram-positive bacteria lacking walls that maintain some of the smallest genomes known for self-replicating organisms, and
they are most often found in association with insects and ticks, including Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera, Odonata and Acari (Regassa and Gasparich 2006). These bacteria tend to be male-killers in insect hosts (Hurst and Jiggins 2000) although they also have other effects. When Drosophila melanogaster are infected with MSRO Spiroplasma (an isolated strain of Spiroplasma which naturally infects D. melanogaster), flies have increased susceptibility to Gram-negative bacterial pathogens (Herren and Lemaitre 2011). However, Spiroplasma also encode a ribosome-inactivating protein (RIP) to protect hosts against parasites and pathogens (Hamilton et al., 2016, Ballinger and Perlman 2017), potentially enhancing host fitness.

In spider mites, Wolbachia are widely distributed, and can be found in many Tetranychidae species such as Tetranychus truncatus, Tetranychus kanzawai, Tetranychus urticae (Zhang et al., 2016). When Wolbachia infects two-spotted mites (Tetranychus urticae), it can induce incomplete CI, with CI strength of the same Wolbachia strain influenced by host genetic backgrounds (Sun et al., 2016, Zhao et al., 2013). In T. urticae, transcriptome analysis shows that Wolbachia affects numerous biological processes, including oxidation-reduction, digestion and detoxification, and processes involving lipocalins (Zhang et al., 2015). However, Spiroplasma infections are not common in spider mites. To date, Spiroplasma has
only been found in *T. truncates* and *T. urticae* in the Tetranychidae (Staudacher *et al.*, 2017, Zhu *et al.*, 2018). No reproductive manipulations induced by *Spiroplasma* in spider mites have been found.

Symbiont co-infections are common in arthropods such as spider mites, aphids and grasshoppers (Machtelinckx *et al.*, 2012, Lukasik *et al.*, 2013, Wamwiri *et al.*, 2013, Funkhouser-Jones *et al.*, 2015, Moutailler *et al.*, 2016, Zhang *et al.*, 2016, Zytynska *et al.*, 2016, Nguyen *et al.*, 2017). The presence of co-infections in mites and other arthropods raises questions about how such infections are generally maintained. Do multiple infections increase or decrease the extent and type of reproductive effects associated with single infections? Does the presence of multiple infections lead to additional costs on hosts? What is the nature of competitive interactions among symbionts?

Where coinfections have been investigated, they have a range of phenotypic effects; some have no additional impact over single infections, whereas others appear to enhance the effects of single infections or lead to novel phenotypic effects. In two-spotted mites (*Tetranychus urticae*), *Wolbachia* induce weak CI, while *Cardinium*-infected and doubly-infected males cause severe CI (Xie *et al.*, 2016). In pea aphids, protection against natural enemies provided by symbionts is the same in single infections of *Rickettsiella* and co-infections with *Rickettsiella* and *Regiella*.
symbionts (McLean et al., 2018). Also in aphids, the pathogen-protective phenotype conferred by coinfections with the symbionts *Rickettsia* and *Spiroplasma* varies among host genotypes, but is not influenced by co-infection with *Hamiltonella* (Lukasik et al., 2013). Haplo-diploid thrips populations co-infected by *Wolbachia* and *Cardinium* induce complete CI. While *Wolbachia* density in these thrips co-infections is higher than *Cardinium* density, *Wolbachia* removal does not impact *Cardinium* density, suggesting a lack of competition within hosts (Nguyen et al., 2017).

In the spider mite *Tetranychus truncatus*, *Spiroplasma* and *Wolbachia* can co-infect the same individual. To investigate phenotypic effects associated with co-infections, we previously established 4 strains in this species, which involved a co-infected strain, two singly-infected strains and one uninfected strain (Zhang et al., 2018). We found mites doubly-infected with *Wolbachia* and *Spiroplasma* gained benefits over singly-infected and uninfected *T. truncatus*. However, spider mites tested in that study were collected from different sites and shared different genetic backgrounds. *Drosophila* data show that *Wolbachia*-associated fitness effects can depend on genetic background (Dean 2006; Olsen et al., 2001). To conclusively demonstrate effects of co-infection by *Wolbachia* and *Spiroplasma*, a controlled genetic background should therefore be used.
To control genetic background effects, we here performed backcrossing between the three symbiont-infected strains and one uninfected strain. We obtained four different symbiont-infected strains that shared similar genetic backgrounds and compared symbiont effects on hosts by crossing female adults with 1-day-old and 5-day-old male adults. We considered the following questions: 1) Do Wolbachia induce CI in *T. truncatus*? 2) Do Spiroplasma influence the CI induced by Wolbachia? 3) Do the two endosymbionts affect the longevity and developmental rate of hosts? 4) Does Wolbachia density or male age influence CI strength? 5) Do Wolbachia and Spiroplasma affect the gene expression of *T. truncatus*? 6) Finally, which endosymbiont is in the dominant position, Wolbachia or Spiroplasma?

**Results**

**Wolbachia and Spiroplasma decrease fecundity of host spider mites**

Using the four new spider mite strains sharing a similar genetic background, we made 16 crosses in all combinations (Fig. 1). In order to clarify effects of endosymbionts on host mite fecundity, we scored egg laying for each mite strain, and found that the uninfected strain had the most eggs, regardless of whether 1-day-old or 5-day-old males were used in crosses (Fig. 1A, 1D). For 1-day-old male crosses, fecundity of the strains differed $[F_{(3,21)} =7.512, P<0.01$, one-way ANOVA] and
fecundity of the W-S- strain (33.33±0.65) was significantly higher than that of the other three strains, while egg numbers for W-S+ (Wolbachia negative, Spiroplasma positive) differed significantly from W+S- (Wolbachia positive, Spiroplasma negative) ($P<0.05$, posthoc test); and the coinfect ed W+S+ strain had an intermediate egg number that was not significantly different compared with W-S+ and W+S- (Fig. S1B, S1C). For fecundity of crosses with the 5-day-old males (Fig. 1D), we found a similar pattern, with strain differences overall [$F(3,226) = 27.857$, $P<0.001$, one-way ANOVA], and the W-S- strain having the highest fecundity (36.28±0.92) which was significantly different to that of W-S+ (31.29±1.05) and W+S- (26.02±0.88) ($P<0.001$, posthoc test). The W+S+ strain (25.64±0.90) and W-S+ also differed significantly ($P<0.001$, posthoc test), a different pattern compared to that observed in crosses with 1-day-old males. The results indicate that both Wolbachia and Spiroplasma decrease fecundity of host mites, with Wolbachia having a relatively larger fitness cost.

Wolbachia induces strong female-killing CI in T. truncates, while Spiroplasma has little effect on reproduction

To test whether symbionts had an effect on reproduction, we compared the hatch rate of each crossing combination (Fig. 1B, 1E), and found that when Wolbachia-infected males ($\delta$W+S+ and $\delta$W+S-) were crossed with Wolbachia-uninfected females ($\varphi$W-S+ and $\varphi$W-S-), there was a significant decline
in the hatch rate of offspring $[F_{(15,204)}=7.228, P<0.05, \text{one-way ANOVA}]$. This result showed that *Wolbachia* induced CI in *T. truncates*. Furthermore, we found the female offspring number declined in incompatible combinations (Fig. 1C, 1F), pointing to female mortality. There was no impact on reproduction associated with the *Spiroplasma* infection.

**Male age and infected status do not influence Wolbachia-induced CI strength**

To test the impact of male age on inducing CI, we compared hatch rate in crosses involving *Wolbachia*-infected 1-day-old (Fig. 1B) and 5-day-old (Fig. 1E) males. For 1-day-old male adults, hatchability was similar in the incompatible cross regardless of whether males carried only the *Wolbachia* infection (68.73±4.76%), or were co-infected (72.99±2.99%) ($P=0.449$, Mann–Whitney U-test). In 5-day-old male incompatible crosses, hatchability was also similar for singly-infected (61.32±5.55%) and co-infected (64.02±3.39%) males ($P=0.671$, Mann–Whitney U-test). These results also suggest that CI strength induced by *Wolbachia* is similar regardless of male age (Fig. 1B, 1E), with Mann Whitney U tests indicating no difference in hatch rates for the singly-infected ($P=0.319$) or coinfected ($P=0.058$) males.

**In females, both endosymbiont densities are higher in the singly-infected strain than in the co-infected strain**
We found that in each developmental stage tested, titer of the symbiont in the singly-infected strain was higher than the same symbiont in the doubly-infected strain ($P<0.001$, Mann–Whitney U-test) (Fig. 2A, B). Both symbionts increased their titer from 1-day-old to 7-day-old adult females, except that Wolbachia in the 3-day-old females from the singly-infected strain had a lower titer than 1-day-old adult females (Fig. 2A). However, in 9-day-old females, the titer in singly-infected strain decreased compared to the titer in 7-day-old adult females, a pattern not seen in the doubly-infected strain.

In the co-infected strain, the titer of Wolbachia was significantly higher than the titer of Spiroplasma in 1-, 3- and 7-day-old females (Figure. 2C); these results indicate that Wolbachia density is higher than Spiroplasma density generally. However, when singly-infected, Wolbachia only had a higher titer than Spiroplasma in 1-day-old and 9-day-old females (Fig. 2D).

**In male adults, Wolbachia density and Spiroplasma density are different in singly-infected and co-infected strains**

Spiroplasma in male adults had a significantly higher titer in the singly-infected strain than in the co-infected strain. Except for 1-day-old male adults, the titer of Spiroplasma was significantly higher in the singly-infected strain ($P<0.01$, Mann-Whitney U-tests) (Fig. 2F). In contrast, Wolbachia density in the co-infected
strain was higher than in the singly-infected strain in four developmental periods ($P<0.01$, Mann-Whitney $U$-tests). Except in 5-day-old male adults, the singly-infected and co-infected strains had a similar *Wolbachia* density ($P=0.49$, Mann-Whitney $U$-tests) (Fig. 2E). When *Wolbachia* and *Spiroplasma* co-infected a male adult, *Wolbachia* density was significantly higher than *Spiroplasma* density ($P<0.001$, Mann-Whitney U-test) (Fig. 2G).

**Neither *Wolbachia* nor *Spiroplasma* affects longevity and developmental rate**

To further clarify the symbionts’ effects on host mite fitness, we measured the lifespan (Fig. 3A) and developmental rate of each infected strain (Fig. 3B and C). No significant difference was found in longevity ($W+S$ vs $W-S$, $P=0.113$; $W+S$ vs $W+S$, $P=0.984$; $W+S$ vs $W-S$, $P=0.979$; $W+S$ vs $W+S$, $P=0.892$; $W+S$ vs $W-S$, $P=0.190$; $W-S$ vs $W-S$, $P=0.154$, Kaplan Meier tests) or developmental rate (male and female) of the strains (see Fig. 3B and 3C).

**RNA sequencing and de novo transcriptome assembly**

Eleven *T. truncatus* transcriptome libraries were sequenced, 3 involving hosts singly-infected by *Wolbachia* ($W+S$), 2 singly-infected with *Spiroplasma* ($W-S$), 3 co-infected with both symbionts ($W+S$) and 3 without symbiont infections ($W-S$). After the trimming of adaptors and filtering for low quality reads, we obtained a total of 568,372,164 clean reads from all 11 libraries (Q30%>92%), with an average of
51,670,197 reads per library. Since there was no published reference genome for T. truncatus, we used the complete set of reads to make a de novo transcriptome assembly with Trinity (see methods). After that, Corset (Davidson and Oshlack, 2014) was used to hierarchically cluster the transcripts by reads mapped to the assembled de novo transcriptome and expression pattern (https://code.google.com/p/corset-project/). A total of 57,067 transcripts were assembled, including 47,034 unigenes, encompassing 139 million base pairs (bp). The median length of unigenes was 2276 bp with an N50 of 4681 bp (Table 1). Over 34,231 unigenes were larger than 1 kb in size. To better evaluate the transcriptional differences between libraries, gene expression levels were divided into six levels according to their fragments per kilobase of exon model per million mapped reads (FPKM) values. Most genes were expressed at low levels in all 11 libraries (Fig. S2).

**Gene functional annotation**

In this analysis, 7 databases of gene functional annotation were used. A total of 33,097 genes could be annotated in the NR database (NCBI non-redundant protein sequences) by using Diamond v. 0.8.22 (with an e-value of 1e-5), covering 70.36% of the total assembled unigenes (47034). Part of our assembly of genes couldn’t be mapped to the NR database, likely linked to misassembly or a lack of representation in the database (Caragata et al., 2017).
Among the genes mapped to the NR database, 85.5% of total mapped genes (28215 unigenes) could be matched to the *Tetranychus urticae* genome (Fig. S3), which showed a high degree of similarity between our assembled *T. truncatus* transcriptome and the *T. urticae* genome, which is the only genome published for species from the Tetranychidae. *T. truncatus* and *T. urticae* are closely related species.

Our assembled unigenes were also annotated to KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) databases (Fig. 4), which were respectively annotated by using KEGG Automatic Annotation Server (with an e-value of 1e-10) and Blast2 GO v2.5. In total, 28498 unigenes could be annotated to the GO database, encompassing 60.59% of our total assembled unigenes. We identified genes associated with a large range of biological processes, molecular functions and cellular components. From the whole set of contigs, 28,498 were assigned to at least one GO term. The majority of contigs in the biological process category were associated with cellular or metabolic processes. Cell and cellular part were the most frequent classes for genes in cellular component terms, while binding and catalytic activity were the most common terms in the molecular category. The most common KO terms were related to signal transduction.

*Transcriptional response to symbionts infection*
We used a hierarchical clustering heat map to give an overall view of gene expressions of all 4 spider mite lines (Fig. 5). As shown in the map, all 4 groups differed in gene expression patterns, suggesting effects of Wolbachia and/or Spiroplasma infections on expression patterns. Differences were particularly evident when Wolbachia-infected lines were compared to Wolbachia-uninfected lines, suggesting that Wolbachia had larger effects than Spiroplasma on host spider mite expression patterns.

Venn diagrams provide information on differently expressed genes (DEGs) which are particularly evident when W+S+ is compared to W-S+ and W-S-, but less so when W+S+ is compared to W+S- (Fig. 6). In other words, when Wolbachia and Spiroplasma co-infected a single spider mite, Wolbachia effects on gene expression patterns tended to predominate.

DEGs showed differences linked to gene ontology (GO) categories (Fig. 7). When the W+S- strain was compared to W-S+, only genes related to molecular function (MF) categories were upregulated, including genes with scavenger receptor activity and cargo receptor activity (Fig. 7A). These DEGs in the same MF categories were also identified in the comparison of W+S- and W-S-, and in W+S+ compared to W-S-; DEGs related to scavenger receptor activity and cargo receptor activity were the most abundant in two combinations (Fig. 7C, 7D). However, DEGs in W+S+
compared to W-S+ showed different ontogenies, involving oxidation-reduction in biological process (BP) (Fig. 7B). Changes in the immunoglobulin complex were uniquely involved in this comparison. Some GO terms were found in both W+S+ compared to W-S- and W+S+ compared to W-S+, such as cellulose biosynthetic process and UDP-glucose metabolic process in BP categories, cellular synthase activity and cellulose synthase (UDP-forming) activity in MF categories (Fig. 7B and 7D). Very few genes related to cell component (CC) terms were found in all 4 combinations in GO categories.

**Quantitative real-time PCR validation**

With RT-qPCR detection, we confirmed expression differences in 7 random genes, including 10 differently expressed combinations (Fig. 8) and 6 similarly expressed combinations (Fig. 9). For example, two contigs, cluster-2804.19525 and cluster-2804.21232, were predicted to be downregulated by W+S+ compared to W-S-, and further analysis confirmed that expression was significantly lower in W+S+ (Mann Whitney U tests: 19525-\(P < 0.0001\); 21232-\(P < 0.001\)). In total, changes in expression were in good agreement with the RNA-seq results, except for cluster-2804.21764 in W+S- compared to W-S-. Cluster-2804.21764 was predicted to be similarly expressed between W+S- and W-S--; however, our results showed that 21764 was higher in W-S- (Fig. 8K) (Mann Whitney U test: \(P < 0.0001\)).
Interested differently expressed genes between every 4 spider mite strains

We selected DEGs terms of interest to investigate symbiont-infection interactions in *T. truncates*, including detoxification, immune response, and lipocalins. Most DEGs were detected between the doubly-infected strain and *Spiroplasma* singly-infected strain or uninfected strain; however, gene expression patterns of the *Wolbachia* singly-infected strain were similar to the doubly-infected strain (Table 2). Almost all detected genes were homologous to *T. urticae* genes.

Many spider mites express detoxification genes, such as cytochrome P450 monooxygenases (CYPs), glutathione S-transferases and ABC transporters. In the present study, detoxification gene expression was largely affected by co-infection of *Wolbachia* and *Spiroplasma*, when compared to the uninfected strain or *Spiroplasma* singly-infected strain. Most CYPs were upregulated in W+S+; most glutathione S-transferases and ABC transporters were also upregulated in this strain compared to the uninfected strain or the *Spiroplasma* singly-infected strain.

The lipocalins are a family of proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids, and lipids (Cowan *et al.*, 1990). Contrary to the pattern for detoxification genes, lipocalin genes were downregulated in W+S+ compared to W-S+. A few differently expressed lipocalin genes were evident in the other comparisons. Two autophagy protein genes were downregulated, and one gene was upregulated in W+S+ compared to the uninfected strain. Among genes related to
reproduction, 5 histone-related genes and 3 vitellogenin genes were found differently expressed. We also detected 24 putative immune-related genes affected by symbiont infection, especially in the coinfected strain when compared to the un-infected or singly-infected strain. Lastly there were 33 genes related to lipid metabolic process differently expressed between the strains.

**Discussion**

In our experiment, we firstly used a backcrossing experiment to obtain spider mite strains with four similar genetic backgrounds, but differing in symbiont infection status. In comparing the strains, we found different effects of *Wolbachia* and *Spiroplasma* on host spider mites. *Wolbachia* could induce CI in spider mites both in the *Wolbachia* singly-infected strain and co-infected strains. At the same time, symbiont-infected spider mite strains suffered fitness costs, which lowered fecundity.

In our previous study (Zhang *et al.*, 2018), we found that the spider mites doubly-infected with *Wolbachia* and *Spiroplasma* have reproductive and fitness advantages, like higher egg deposition and faster developmental rate, compared to singly-infected and uninfected spider mite strains. Doubly-infected mites could induce incomplete CI, while *Wolbachia* singly-infected mites could not induce CI. As a result, doubly-infected mites were potentially thought to have a competitive advantage over
the other mites, helping symbionts spread in the field. However these findings were confounded by the fact that the three symbiont-infected strains were collected from different fields in Shenyang, while uninfected strain was obtained from a tetracycline-treated W-S+ strain. The contrasting findings highlight the importance of controlling genetic background, which has been raised in other systems, such as in the whitefly Bemisia tabaci infected with Rickettsia (Hunter et al., 2016) and in Drosophila melanogaster infected by Wolbachia (Fry et al., 2004; Olsen et al., 2001).

Based on the new results presented here, it is clear that both Wolbachia-infected and Spiroplasma-infected mites have fitness costs, which mainly results in a lower fecundity (Fig. 1A and 1D). These results contrast with the previous study (Zhang et al., 2018), where co-infection was considered to confer host benefits, and the symbiont infections did not have fitness costs. Similar results have been obtained in Nasonia vitripennis, when wasps infected with two Wolbachia strains (wAv and wBv) appeared to produce more offspring when in different genetic backgrounds to the strains used for comparison, whereas no positive fitness effects were found once host genetic background was controlled (Bordenstein and Werren, 2000). Fitness variation, including fecundity and survival, will often largely represent a function of host genetic background (Russell et al., 2018). The costs we have detected for the Wolbachia and Spiroplasma contrast with the effects of a Wolbachia and Spiroplasma
co-infection in *Drosophila melanogaster*, where there is no evidence of any benefits or costs when compared to single infections (Montenegro *et al.*, 2006).

Although both endosymbionts are maternally inherited, it is not necessary for them to be beneficial to hosts to spread when they cause cytoplasmic incompatibility (Caspari and Watson, 1959, Hoffmann and Turelli, 1997). *Wolbachia* induces CI in *Tetranychus truncates*, although the strength of CI is relatively weak (Fig. 1B and 1E). In a species related to *Tetranychus truncates* studied here, the two-spotted mite *Tetranychus urticae*, *Wolbachia* can also induce incomplete CI (Zhao *et al.*, 2013, Xie *et al.*, 2016). The CI in that species was not affected by age (Sun *et al.*, 2016), which is also the case in the present study, as the hatchability of incompatible crosses with 5-day-old males and 1-day-old males was similar (Fig. 1B and 1E). *Spiroplasma* had little effect on CI, whereas in *Drosophila melanogaster* the male-killing ability of *Spiroplasma* (MSRO strain) was lowered in the presence of *Wolbachia* (Silva *et al.*, 2012), although different *Spiroplasma* (NSRO strain) may be unaffected by co-infecting *Wolbachia* (Goto *et al.*, 2006). To date, cytoplasmic incompatibility is the only *Wolbachia*-induced reproductive manipulation reported in the Tetranychidae (Breeuwer 1997, Gotoh *et al.*, 2003, Zhu *et al.*, 2012).

Through Q-PCR, we found that *Wolbachia* and *Spiroplasma* titers were higher in singly-infected strains, and that *Wolbachia* titer was significantly higher than
Spiroplasma in the co-infected strain, except for Wolbachia in male *T. truncates* (Fig. 3). These results were similar to those found in our former study (Zhang et al., 2018). There was no direct association between CI and Wolbachia density, with Wolbachia density significantly higher in 1-day-old male adults from the co-infected strain (Fig. 2E), but the CI strength being similar between singly and co-infected strains (Fig. 1B). Data from other studies show an inconsistent association between CI strength and Wolbachia density (Duron et al., 2006, Sun et al., 2016) although this relationship may hold in comparisons of the same infection and host species. In mosquitoes, high densities of Wolbachia do not increase the strength of CI or maternal transmission efficiency relative to low Wolbachia densities (Duron et al., 2006). A threshold density of Wolbachia might nevertheless be required to induce CI.

We used transcriptome analysis to compare the DEGs (differently expressed gene) associated with the endosymbionts, which may in turn impact fitness costs and CI for Wolbachia and fitness costs for Spiroplasma (Fig. 1). Previous transcriptome analysis of *T. urticae* indicated that the Wolbachia infection affects numerous biological processes (Zhang et al., 2015) which provides a point of comparison. We matched 85.5% of expressed genes to mapped *T. truncates* genes. As for *T. urticae, T. truncates* is a polyphagous herbivore feeding on more than 86 plants species (Migeon, 2015), with the expectation that there would be numerous detoxification genes which
are connected to diet at least in insects (Rane et al., 2019). In T. urticae, genes related to spider mite feeding and detoxification may be influenced by Wolbachia (Zhang et al., 2015), which may indicate a role of the symbiont in host use. In many insects, microbial symbionts can promote the pest status of certain insect species by detoxifying plant allelochemicals (van den Bosch and Welte, 2017). Here we found DEGs involving a set of genes related to detoxification, particularly in W+S+ (Table 2). This raises the issue of whether Wolbachia might assist in detoxification. Unlike Wolbachia in T. urticae, Wolbachia in T. truncates only influenced two ABC transporter genes (Cluster-2804.16024, Cluster-2804.10578), which suggests a contrasting pattern in these species particularly as W+S- of T. truncates showed a similar pattern of expression effects to the doubly-infected strain.

Although Wolbachia and Spiroplasma both reduced the fecundity of T. truncates, no DEGs related to oogenesis were found between the Wolbachia infected strain and uninfected strain. This was also the case in T. urticae (Zhang et al., 2015). However in the current study some DEGs related to spider mite reproduction, including histones, highly alkaline proteins found in eukaryotic cell nuclei that package and order the DNA into structural units called nucleosomes (Cox et al., 2005). Many histone variants are related to reproduction, and H3.3 is a conserved histone variant that is structurally very close to its canonical counterpart also related
to reproduction (Orsi et al., 2009). We observed five histone genes related to reproduction with expressions affected by symbiont infection, most of them were found differentially expressed between doubly-infected strain compared to uninfected strain or *Spiroplasma* singly-infected strain (Table 2). Reproductive effects may also stem from vitellogenins, proteins that are synthesized extra-ovarially and become the major egg yolk protein, vitellin (Hagedorn and Kunkel, 1979). In *T. truncatus*, three vitellogenin genes were differentially expressed between symbiont-infected strains, although these involved both upregulation and downregulation.

In *T. urticae*, very few genes related to humoral immune and autophagy pathway were differentially expressed by the *Wolbachia* infection (Zhang et al., 2015), while in present study, only one gene (cluter-2804.3054) related to immune response was induced by *Wolbachia*. However, co-infection of both *Wolbachia* and *Spiroplasma* influenced more putative immune related genes, and most of genes were upregulated, suggesting that it may be worth exploring the role of co-infection of *Wolbachia* and *Spiroplasma* on protection from pathogenic bacterial infection which may help explain why co-infection of *Wolbachia* and *Spiroplasma* are widespread in nature (Zhang et al., 2018).

*Wolbachia* dominate *Spiroplasma* in *Tetranychus truncatus*, as *Wolbachia* induced reproductive manipulation (CI) and *Wolbachia* density was significantly
higher than Spiroplasma density in female and male co-infected adults. Moreover, gene expression patterns of the Wolbachia singly-infected strain were similar to the doubly-infected strain, which showed a stronger effect of Wolbachia in the co-infected strain (Table 2). While co-infection of Wolbachia and Spiroplasma could protect host mites from pathogenic bacteria, it is still unclear why Spiroplasma persist in natural populations when their effects are dominated by Wolbachia.

Experimental Procedures

Collection and cultivation of Tetranychus truncatus

All four tested spider mite lines used in this experiment were derived from different sites of Shenyang, Liaoning Province, China. When spider mites transited into the laboratory, isofemale lines of each single strain were established. As spider mites are haplo-diploid, all individuals of one strain are offspring of a single mother. All spider mites in the laboratory were reared on leaves of kidney bean (Phaseolus vulgaris L.). Bean leaves were placed on a water-saturated sponge mat in Petri dishes (dia. 9) at 25±1℃, 60% humidity and under L16D8 conditions.

Detection of Wolbachia and Spiroplasma in T. truncatus

To detect infection of Wolbachia and Spiroplasma in laboratorial mites, we amplified the Wolbachia surface protein (wsp) gene of Wolbachia with primers
wsp-F1 and wsp-R1 (Baldo et al., 2006) and the 16s rRNA gene of Spiroplasma with primers of Spits-J04 and Spits-N55 (Jaenike et al., 2007), respectively. Genomic DNA was extracted using TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver. 5.0 (TaKaRa BIO INC, Japan). Each reaction was carried out on a Veriti Thermal Cycler (ABI Biosystems, CA, USA) in a 25 μl volume containing 12.5 μl 2× Taq Master Mix (Thermo Scientific, CA, USA), 1 μl primer (20 μmol/L each), and 2 μl DNA extract. Positive (W+S+ female adult DNA template) and negative controls (ddH2O) were included in PCRs.

Establishment of different symbiont-infected T. truncatus strains with similar genetic backgrounds

We tested all the T. truncatus strains in the laboratory and found three different types of symbiont-infected mite strains: 1) doubly-infected strain (mites infected with both Spiroplasma and Wolbachia, named as W+S+), 2) Wolbachia singly-infected strain (named as W+S-), and 3) Spiroplasma singly-infected strain (named as W-S+). Then we generated a mite strain infected with neither Wolbachia nor Spiroplasma (W-S-) by tetracycline treatment of the W-S+ strain for three generations. To eliminate potential side effects of tetracycline, we cultivated the W-S-strain in the lab for seven generations. To reduce genetic background differences among the strains, we then mated three infected female adults (W+S+, W+S- and W-S+) with the male
uninfected adults (W-S-) and backcrossed female offspring for seven consecutive
generations to generate introgressed lines. After that, we cultured the four strains for
five generations before experiments. Prior to every experiment, the infection status of
symbionts in each strain was checked with PCR.

_Crossing experiments between different spider mite strains_

To test how symbionts influence host mite reproduction, such as cytoplasmic
incompatibility (CI) or male-killing (MK), we undertook a crossing experiment,
which involved mating 1-day-old female virgins of one strain with 1-day-old and
5-day-old unmated male of all other spider mite strains. In total, this experiment
includes 16 crossing combinations, and each combination is named as ♀ (infected
status) crossed with ♂ (infected status) (Fig. 1). The male adults were allowed to mate
with females for 2 days, and the mated females were then allowed to lay eggs for 5
days. Eggs on leaf disks were checked daily to calculate hatchability. After emergence,
we assessed the female proportion (male adults divide female adults) of offspring.
Fecundity was measured by the number of eggs laid in 5 days. Fecundity and
hatchability data were first tested for normality (Kolmogorov–Smirnov test) and
homogeneity of group variances (Levene’s test). When the data followed normal
distribution, they were analyzed with a one-way ANOVA with post-hoc Tukey HSD
analysis. Since female proportion data does not follow a normal distribution, female
proportion data were analyzed by a Kruskal-Wallis test and Dunn’s test with Bonferroni correction for multiple comparisons. SPSS 19 was used to carry out all statistical tests.

**Survival assay**

The impacts of symbionts on host mite longevity were tested. Since male spider mites often died accidentally (mite male adults would reach the margin of leaves and drown), we only measured the longevity of female mites. Firstly, 100 third stationary phase (last larval phase before eclosion) female spider mites were selected from the four spider mite strains, and were collected on a single leaf. After 12 hours, all emerged female adults were removed from the leaves. Eight hours later, 24 spider mites of each strain were divided equally across three new leaves, each leaf containing 8 spider mites. The number of dead spider mites was recorded daily. All females were transferred to new leaves every 3 days, to provide fresh food. Survival curves were compared using the Kaplan–Meier method (Bland and Altman 1998).

**Developmental rate assay**

The effects of symbionts on host mite development were assessed. Thirty virgins (which produced males) or thirty mated female adults (which produced male and female offspring, respectively) from each strain were placed on a leaf disk and left to
lay eggs for 3 hours. Eggs were moved to new small leaf disks, with 24 female eggs and 24 male eggs per disk. The disks were monitored every 8 hours, and the stage of mite was recorded until adulthood. The developmental rate of each stage was then calculated. The total development time of spider mite strains were compared with Mann Whitney tests.

**Densities of Wolbachia and Spiroplasma**

The *Wolbachia* density and *Spiroplasma* density were measured by quantitative PCR (Q-PCR), carried out on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, CA, USA). The primers used for quantifying *Wolbachia* target a 141-bp fragment of *wsp* (wQF1, 5′-GAGCAGCAGATGTAAGCAATC-3′, and wQR1, 5′-AATAACGAGCACCAGCATAAAAG-3′). The primers used for quantifying *Spiroplasma* target a 141-bp fragment of *Spiroplasma16S rRNA* gene (sQF1, 5′-TGTAGTTTCTCAGGGAGTTGTGCACCTCTC-3′, and sQR1, 5′-CGCTTTCCAACCATCGCTCTTT-3′) (Zhang et al., 2018). Primers utilized to amplify mite host single copy gene target a 99-bp fragment of *rps18* (ribosome protein S18, RPS18-F, 5′-ACGTGCTGTTGAACTTACCGAAGAGTGC-3′, and rps18-R, 5′-TGCCCTATTTCAAGAACCATAAAGTCGG-3′) (Sun et al., 2010). The 20 μl Q-PCR reaction mixture consisted of 10 μl 2× SYBRP remix Ex Taq (Vazyme, China), 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, CA, USA). The Q-PCR cycling conditions included one cycle (5 min at 95°C) followed by 40 cycles (10 s at 95°C and 34 s at 60°C), and finally one cycle for a melt curve (15 s at 95°C, 1 min at 60°C and 15 s at 95°C). Each DNA sample was run as three replicates. Standard curves were plotted using a 10-fold dilution series of the DNA samples prepared from plasmid DNA. The plasmid DNA was obtained with the pEASY-T3 vector (TransGen Biotech, Beijing, China). The quality and concentration of all purified standard DNA were measured on a Nanodrop 2000 (Thermo Scientific, MA, USA). Densities of straits were compared with Mann-Whitney U-tests.

**Sample preparation for Transcriptome analysis**

**RNA quantification and qualification**

Twelve groups of samples were prepared for sequencing, with each group going on to form an independent library. Three groups each of 80 7-day old female adults from four strains were collected, except that the W-S+ strain only had two groups. Total RNA was extracted by using Trizol® protocol according to manufacturer’s instructions (Invitrogen, CA, USA), for a total of 6 independent samples, with 3 biological replicates per treatment, except for W-S+. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life
Technologies, CA, USA). Sample degradation and contamination were checked by running a portion of the samples on a standard non-denaturing agarose gel containing bleach (Aranda et al., 2012). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

**Library preparation for Transcriptome sequencing**

A total amount of 1.5 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer’s recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase). Second strand cDNA synthesis was subsequently performed with DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3’ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 250–300 bp in length, the library fragments were purified with AMPure XP system.
(Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

**Clustering and sequencing**

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and paired-end reads were generated.

**Transcriptome analysis**

**Quality control**

Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality. All eleven Illumina RNAseq datasets were combined in order to assemble a
more reliable transcriptome, with a total of 568,372,164 paired-end, high quality reads, with an average of 51,670,196 clean reads per replicate.

**Transcriptome assembly and gene functional annotation**

Since no reference genome was available for *T. truncates*, a de novo transcriptome assembly was built with Trinity using default parameters. The left files (read1 files) from all libraries/samples were pooled into one big left.fq file, and right files (read2 files) into one large right.fq file. Transcriptome assembly was accomplished based on the left.fq and right.fq with min_kmer_cov set to 2 by default and all other parameters set default (Grabherr et al, 2011). The assembled contigs were annotated with Diamond v0.8.22 ([http://github.com/bbuchfink/diamond/](http://github.com/bbuchfink/diamond/)) to the NCBI non-redundant (NR), Clusters of Orthologous Groups of proteins (COG), euKaryotic Ortholog Groups (KOG) and Swiss-Prot Protein Sequence database. Diamond v0.8.22 parameters were set with an -e value of 1e-5, 1e-3, 1e-3 and 1e-5, respectively. HMMER 3.0 package was used to annotate contigs with Protein family (Pform) database; parameters were set with an –e value of 0.01. In addition, assembled contigs were annotated to KEGG Ortholog database (KO) with KEGG Automatic Annotation Server, parameters were set with an –e value of 1e-10. Blast2GO v2.5 (Götz et al., 2008) was used to retrieve Gene Ontologies to annotated transcripts; parameters were set with an -e value of 1e-6.
Quantification of gene expression levels

Gene expression levels were estimated by RSEM (Li and Dewey, 2011) for each sample, clean data were mapped back onto the assembled transcriptome obtained by Trinity as a reference sequence and read count for each gene was obtained from the mapping results. Parameters of bowtie 2 used in RSEM were mismatch 0.

Differential expression analysis

Differential expression analysis of two groups was performed using the DESeq R package 1.10.1 (Kvam et al., 2012). Significantly differently expressed contigs of six combinations (W+S- vs W-S-, W-S+ vs W+S-, W+ vs S-, W-S vs W+S, W+S vs W+S+ and W+S vs W-S-) were listed, in order to clarify the influence of symbiont infections in *T. truncates*. DESeq provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq were considered differentially expressed.

GO enrichment analysis and KEGG pathway enrichment analysis

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution (Young et al., 2010), which can adjust for gene length
bias in DEGs. GO terms with a corrected P value less than 0.05 were considered significantly enriched. KEGG (Kanehisa et al., 2008) is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used KOBAS (Mao et al., 2005) software to test the statistical enrichment of differential expression genes in KEGG pathways.

Confirmation of differential expression by quantitative real-time PCR

To confirm the results of RNA-Seq analysis, the expression levels of randomly selected genes were measured by quantitative real time qRT-PCR. The qRT-PCR reactions were performed on the Applied Bio-systems 7500 Real-Time PCR System with the SYBR Premix Ex Taq (Takara Bio, Kyoto, Japan). Primer sequences were designed using Primer Premier 5.0 (Lalitha, 2000).

Conflicts of Interest

The authors have no conflict among themselves in relation to the article.

Acknowledgments

We sincerely thank Xue Xia, Ya-Ting Chen and Yue-Ling Song of Nanjing
Agricultural University, China for their help with the sample collection and species identification. We also thank Dr. Jing-Tao Sun of Nanjing Agricultural University, China for his helpful comments on the manuscript. This study was supported in part by a grant-in-aid for Scientific Research (31672035, 31871976) from the National Natural Science Foundation of China.

References


This article is protected by copyright. All rights reserved.


Table 1. Information on *de novo* assembled genes and genes annotated to different databases for female *Tetranychus truncates*.

<table>
<thead>
<tr>
<th>Main terms</th>
<th>Gene information and databases</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>De novo</em> assembly</td>
<td>Total Nucleotides</td>
<td>139357125</td>
</tr>
<tr>
<td>genes</td>
<td>N50</td>
<td>4681</td>
</tr>
<tr>
<td></td>
<td>N90</td>
<td>1646</td>
</tr>
<tr>
<td></td>
<td>Median Length (bp)</td>
<td>2276</td>
</tr>
<tr>
<td></td>
<td>Unigenes larger than 2kb</td>
<td>25714</td>
</tr>
<tr>
<td></td>
<td>Total number of unigenes</td>
<td>47034</td>
</tr>
<tr>
<td>Annotated unigenes</td>
<td>NR</td>
<td>33097</td>
</tr>
<tr>
<td></td>
<td>GO</td>
<td>28498</td>
</tr>
<tr>
<td></td>
<td>NT</td>
<td>9078</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>17598</td>
</tr>
<tr>
<td></td>
<td>SwissProt</td>
<td>25869</td>
</tr>
<tr>
<td></td>
<td>PFAM</td>
<td>28323</td>
</tr>
<tr>
<td></td>
<td>KOG</td>
<td>18301</td>
</tr>
<tr>
<td>Mapping rate (%)</td>
<td>Annotated in all Databases</td>
<td>10.22</td>
</tr>
<tr>
<td></td>
<td>Annotated in at least one Database</td>
<td>78.03</td>
</tr>
</tbody>
</table>

This article is protected by copyright. All rights reserved.
Table 2. Genes differently expressed by *Wolbachia* or *Spiroplasma* infection in *Tetranychus truncates*

<table>
<thead>
<tr>
<th>Gene id</th>
<th>Description</th>
<th>Log2 Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>W+S+ vs W-S+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W-S+ vs W-S+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W+S+ vs W+S-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W+S- vs W-S-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster-2804.16063</td>
<td>Cytochrome P450 2C31-like</td>
<td>9.1068</td>
</tr>
<tr>
<td>Cluster-2804.15218</td>
<td>Cytochrome P450 315s1, mitochondrial-like</td>
<td>8.0879</td>
</tr>
<tr>
<td>Cluster-2804.9989</td>
<td>Cytochrome P450 2C31-like</td>
<td>-1.4485</td>
</tr>
<tr>
<td>Cluster-2804.19836</td>
<td>Cytochrome P450 302a1, mitochondrial-like</td>
<td>7.0011</td>
</tr>
<tr>
<td>Cluster-2804.29491</td>
<td>Cytochrome P450 3A2-like</td>
<td>-1.8117</td>
</tr>
<tr>
<td>Cluster-2804.2693</td>
<td>Cytochrome P450 2J2-like</td>
<td>-2.9173</td>
</tr>
<tr>
<td>Cluster-2804.14230</td>
<td>Cytochrome P450 2C31-like</td>
<td>10.816</td>
</tr>
<tr>
<td>Cluster-2804.1870</td>
<td>Cytochrome P450 3A16-like</td>
<td>1.4818</td>
</tr>
<tr>
<td>Cluster-2804.32745</td>
<td>Cytochrome P450 2B6-like</td>
<td>-1.6737</td>
</tr>
<tr>
<td>Cluster-2804.17326</td>
<td>Cytochrome P450 2C15-like</td>
<td>1.6068</td>
</tr>
<tr>
<td>Cluster-2804.24125</td>
<td>Probable Cytochrome P450 4d14</td>
<td>-3.5442</td>
</tr>
<tr>
<td>Cluster-2804.30119</td>
<td>NADPH--Cytochrome P450 reductase-like</td>
<td>1.8559</td>
</tr>
<tr>
<td>Cluster-2804.12797</td>
<td>NADPH--Cytochrome P450 reductase-like</td>
<td>-8.0893</td>
</tr>
<tr>
<td>Cluster-2804.23253</td>
<td>ABC transporter G family member 23-like</td>
<td>12.606</td>
</tr>
<tr>
<td>Cluster-2804.22450</td>
<td>ABC transporter G family member 23-like</td>
<td>9.8928</td>
</tr>
<tr>
<td>Cluster-2804.33188</td>
<td>ABC transporter G family member 23-like</td>
<td>-9.2676</td>
</tr>
<tr>
<td>Cluster-2804.23533</td>
<td>ABC transporter G family member 23-like</td>
<td>8.381</td>
</tr>
<tr>
<td>Cluster-2804.33190</td>
<td>ABC transporter G family member 23-like</td>
<td>7.0003</td>
</tr>
<tr>
<td>Cluster-2804.24123</td>
<td>ABC transporter G family member 23-like</td>
<td>6.4392</td>
</tr>
<tr>
<td>Cluster-2804.25596</td>
<td>ABC transporter G family member 23-like</td>
<td>7.6067</td>
</tr>
<tr>
<td>Cluster-2804.18356</td>
<td>ABC transporter G family member 23-like</td>
<td>6.4037</td>
</tr>
<tr>
<td>Cluster-2804.16024</td>
<td>ABC transporter G family member 23-like</td>
<td>8.424</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Cluster-2804.10578</td>
<td>ABC transporter G family member 23-like</td>
<td>-5.0014</td>
</tr>
<tr>
<td>Cluster-2804.34355</td>
<td>ABC transporter G family member 22, partial</td>
<td>-8.2131</td>
</tr>
<tr>
<td>Cluster-2804.26034</td>
<td>Glutathione S-transferase Mu 1-like</td>
<td>8.2286</td>
</tr>
<tr>
<td>Cluster-2804.10405</td>
<td>Glutathione S-transferase Mu 1-like</td>
<td>1.301</td>
</tr>
<tr>
<td>Cluster-2804.19645</td>
<td>Glutathione S-transferase Mu 1-like</td>
<td>-1.1569</td>
</tr>
<tr>
<td>Cluster-2804.25925</td>
<td>Glutathione S-transferase Mu 1-like</td>
<td>-1.3416</td>
</tr>
<tr>
<td>Cluster-2804.22715</td>
<td>Glutathione S-transferase 1, isoform C-like isoform</td>
<td>-1.4679</td>
</tr>
<tr>
<td>Cluster-2804.11227</td>
<td>Glutathione S-transferase omega-1-like</td>
<td>1.1409</td>
</tr>
<tr>
<td>Cluster-2804.23234</td>
<td>Glutathione peroxidase 1-like</td>
<td>-7.0922</td>
</tr>
<tr>
<td>Cluster-2804.36697</td>
<td>S-formylGlutathione hydrolase-like</td>
<td>7.3805</td>
</tr>
<tr>
<td>Cluster-2804.13409</td>
<td>Probable phospholipid hydroperoxide Glutathione peroxidase isoform X1</td>
<td>6.0209</td>
</tr>
</tbody>
</table>

2. Lipocalins
| Cluster-2804.10646 | Apolipoprotein D-like | -1.5618 | 2.3315 |
| Cluster-2804.16506 | Apolipoprotein D-like | -2.5738 | 2.6478 |
| Cluster-2804.23994 | Apolipoprotein D-like | -4.2138 |
| Cluster-2804.39826 | Apolipoprotein D-like | 3.7107 | 4.4563 |
| Cluster-2804.14284 | Apolipoprotein D-like | -2.6151 |
| Cluster-2804.4955 | Apolipoprotein D-like | -2.9746 | -1.7954 |
| Cluster-2804.12557 | Apolipoprotein D-like | -2.4702 |
| Cluster-2804.8135 | Apolipoprotein D-like | -1.3026 |
| Cluster-2804.12428 | Apolipoprotein D-like | -2.2161 |
| Cluster-2804.18399 | Apolipoprotein D-like | -1.8603 |
| Cluster-2804.30100 | Apolipoprotein D-like | 1.4812 | 1.5823 |

3. Autophagy pathway
| Cluster-2804.29327 | Autophagy protein 5-like | -10.565 | -1.581 | -12.134 |
| Cluster-2804.28939 | Autophagy protein 5-like | 1.2863 |
| Cluster-2804.33629 | Autophagy protein 5-like | -7.2686 |

4. Potential in reproduction
| Cluster-2804.18107 | Histone H3.3 | -2.2522 |
| Cluster-2804.22671 | Histone acetyltransferase KAT6B-like | 2.2414 | -1.7415 |
| Cluster-2804.8385 | Histone-lysine N-methyltransferase | -4.3867 | -4.565 | -5.3383 |
| Cluster-2804.10103 | Sin3 Histone deacetylase corepressor complex component SDS3-like isoform X2 | 7.883 | 4.7831 |
| Cluster-2804.24230 | Probable Histone deacetylase 1-B | 1.2223 | 1.2738 |
| Cluster-2804.19842 | Vitellogenin 4 | 1.8927 |
| Cluster-2804.19359 | Vitellogenin-5-like | -1.6396 | 1.8591 |
**5. Immune response**

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Description</th>
<th>Log2 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster-2804.32406</td>
<td>F-box only protein 47-like</td>
<td>-11.077</td>
</tr>
<tr>
<td>Cluster-2804.32405</td>
<td>F-box only protein 47-like</td>
<td>-8.6428</td>
</tr>
<tr>
<td>Cluster-2804.30751</td>
<td>F-box only protein 47-like</td>
<td>8.8988</td>
</tr>
<tr>
<td>Cluster-2804.26502</td>
<td>Septin-7-like isoform X2</td>
<td>-21.137</td>
</tr>
<tr>
<td>Cluster-2804.10580</td>
<td>Palmitoyltransferase ZDHHC17-like</td>
<td>-20.387</td>
</tr>
<tr>
<td>Cluster-2804.13527</td>
<td>Ubiquitin-like modifier-activating enzyme 1</td>
<td>1.3481</td>
</tr>
<tr>
<td>Cluster-2804.13876</td>
<td>ATPase family AAA domain-containing protein 3-B-like</td>
<td>1.0738</td>
</tr>
<tr>
<td>Cluster-2804.16953</td>
<td>Palmitoyltransferase ZDHHC17-like</td>
<td>7.8188</td>
</tr>
<tr>
<td>Cluster-2804.27797</td>
<td>Prostatic acid phosphatase-like</td>
<td>-1.6016</td>
</tr>
<tr>
<td>Cluster-2804.19655</td>
<td>Serine/threonine-protein kinase</td>
<td>6.4425</td>
</tr>
<tr>
<td>Cluster-2804.36482</td>
<td>GABA gated chloride channel RDL1</td>
<td>-4.1971</td>
</tr>
<tr>
<td>Cluster-2804.2713</td>
<td>MHC class I antigen, partial</td>
<td>-6.4742</td>
</tr>
<tr>
<td>Cluster-2804.7370</td>
<td>Uncharacterized protein LOC107364615</td>
<td>2.9532</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. Results of crosses between different *Tetranychus truncates* strains. Egg amount of all combinations involving 1-day-old males (A) and 5-day-old males (D), hatchability of crosses involving 1-day-old males (B) and 5-day-old males (E), female proportion of each combination involving 1-day-old males (C) and 5-day-old males (F). Results are shown as box and whiskers, the same letters above columns represent homogeneous groups in post-hoc tests ($P>0.05$) following an ANOVA, except for
female proportion which was analyzed by Kruskal-Wallis tests and Dunn’s tests with Bonferroni correction for multiple comparisons.

**Figure 2. Wolbachia and Spiroplasma density in Tetranychus truncates adults.** For female spider mites, symbiont density in singly-infected and co-infected strains is given for *Wolbachia* (A) and *Spiroplasma* (B) relative to a host gene. A comparison of *Wolbachia* and *Spiroplasma* density is provided for singly-infected (C) and co-infected (D) strains. In male spider mites, symbiont density in singly- and co-infected male adults is presented for *Wolbachia* (E) and *Spiroplasma* (F) as well as a comparison of the infections in co-infected male adults (G). Individual data points of the single copy genes *16S rRNA* (*Spiroplasma*), and *wsp* (*Wolbachia*) were normalized to the *RPS-18* (host) reference gene. Asterisks above points indicate statistically significant differences (Mann-Whitney U test, * P<0.05, ** P<0.01, ***P<0.001, NS, not significant).

**Figure 3. Influence of Wolbachia and Spiroplasma on Tetranychus truncates longevity and developmental rate.** Survival curves for individual hosts were compared using the Kaplan-Meier method and log-rank test (A). Each strain’s developmental rate is displayed, including for males (B) and females (C). Differences were analyzed using Mann Whitney test (ns, not significant).
Figure 4. GO terms (A) and KEGG terms (B) of female *Tetranychus truncates*. A. 56 most enriched gene ontology terms, including biological process (red), cellular component (green) and molecular function (blue) categories. B. 32 most enriched KEGG terms.

Figure 5. Hierarchical clustering heat map of the gene abundance in the four spider mite strains. Red color means genes highly expressed, blue color means genes weakly expression. Color from red to blue mean Log10 (FPKM+1) from high to low.

Figure 6. Venn diagram of DEGs (differently expressed genes) between every comparison combination. A. DEGs of three cytoplasmic incompatibility combinations. B. DEGs of all combinations except for W+S- strain compared to W-S+ strain.

Figure 7. Significantly different enriched GO terms. (A) W+S- compared to W-S+; (B) W+S+ compared to W-S+; (C) W+S- compared to W-S-; (D) W+S+ compared to W-S-.

Figure 8. Confirmation of genes differently expression by RT-qPCR. 4 genes differently expressed in 11 different combinations of strains are shown in the graphs. (Mann Whitney U tests, * P < 0.05; ** P < 0.01; *** P < 0.001).
Figure 9. Confirmation of genes not differently expressed by RT-qPCR. 4 genes not differently expressed in 6 combinations of spider strains are shown in the graphs (NS = not significant).

Supplement File

Figure S1. Design of introgression of spider mites (A), total egg number for each strain in crosses with 1-day-old males (B) and 5-day-old males (C). Columns in different colors stand for different female spider mite strains.

Figure S2. Distribution of FPKM in each Tetanychus truncates sample. The y-axis represents for FPKM number; the x-axis represents different T. truncates samples. FPKM means fragments per kilobase of transcripts per million fragments mapped, different colors represent different FPKM intervals.

Figure S3. Breakdown of gene annotations by organism of origin (comparison by Nr database).
IMB_12607_Figure7.tif
Author/s:
Yang, K; Xie, K; Zhu, Y-X; Huo, S-M; Hoffmann, A; Hong, X-Y

Title:
Wolbachia dominate Spiroplasma in the co-infected spider mite Tetranychus truncatus

Date:
2020-02

Citation:

Persistent Link:
http://hdl.handle.net/11343/286167