Prevalence of *Wolbachia* in 10 Tenebrionidae stored product insects and infection density dynamics in confused flour beetle *Tribolium confusum* (Jaquelin Du Val) (Coleoptera: Tenebriondae)

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Abstract

Background: Increasingly interests in the potential use of insect symbionts *Wolbachia* to control populations of pest species were focused on many vector pest species and agricultural insects. However, few pieces of researches were reported in stored product insects.

Methods: We surveyed the prevalence of *Wolbachia* using a PCR detection method in 10 Tenebrionidae stored product insects. Subsequently, *Wolbachia* infection density and spatiotemporal dynamics in *Tribolium confusum* were investigated in detail by TaqMan probe real-time quantitative PCR, and *Wolbachia* elimination patterns by antibiotic treatment and host reproductive fitness parameters were compared.

Results: Our results identified that *T. confusum* were the only infected species in the survey. *Wolbachia* infection density consistently increased with the development of *T. confusum* and plateaued at 3.7×10^7 *wsp* copies per individual insect at the young adult stage. *Wolbachia* densities in females showed significant differences to the male at the pupae stage and varied in different tissues and organs. Aposymbiotic female beetles by feeding with Tetracycline diet were completely incapable to produce mature progenies when crossing with *Wolbachia* infected males. Embryogenesis and egg hatch rate were specifically inhibited after *Wolbachia* elimination, while other traits including egg produced numbers, pupation rate and sex ratio remained unaffected after antibiotic treatment.

Discussion: All the results indicated that *Wolbachia* infection was regarded as a mutualism but not obligate symbiont and benefited the host confused flour beetle.

Keywords

Wolbachia; Tenebrionidae; Symbionts infection density; Cytoplasmic incompatibility; Stored product insects

Introduction

Endosymbiotic bacteria *Wolbachia* infect approximately 40% of arthropod species *(Floate et al., 2006; Jeyaprakash and Hoy, 2000; Zug and Hammerstein, 2012)*. In recent years, increasing reports focus on the role of *Wolbachia* on hosts of stored product insects

for symbiont-host associated relationships could be used as alternative methods to control them. There are *Wolbachia* infections in 13 of 39 Coleoptera species, 4 of 7 Lepidoptera species, 2 of 4 Hymenoptera species *(Kageyama et al., 2010; Li et al., 2015)* and 2 species in *Liposcelis spp.* *(Dong et al., 2007; Wang et al., 2008; Yusuf et al., 2000)*.

Tenebrionidae stored product insects are cosmopolitan serious pests of wheat flour, starch, and other stored products, especially in flour mills, grain processing and storage facilities *(Campbell et al., 2004)*. In previous reports, it has demonstrated that the confused flour beetle, *Tribolium confusum* (Jaquelin Du Val) (Coleoptera: Tenebrionidae) was naturally infected with single *Wolbachia* infection *(Fialho and Stevens, 1996; Fialho and Stevens, 1997; Kageyama et al., 2010; Li et al., 2015)* and complete unidirectional incompatibility (CI) of uninfected females mate to infected males crosses *(Fialho and Stevens, 1996; Li et al., 2016; Ming et al., 2015)*. Nevertheless, the spatial and temporal infection dynamics of *Wolbachia* in *T. confusum* and relationships between infection density and phenotypic effects are primarily fragmentary.

Wolbachia strains are strictly intracellular bacteria and inherited solely through the maternal lineage of the host by trans-ovaries transmission, so they are expected to be associated with reproductive tissues of the host organisms *(Ijichi et al., 2002; Werren et al., 2008)*. Recently, it has been recognized that *Wolbachia* infections were found in a variety of somatic tissues in different insects, especially in host reproductive tissues *(Ijichi et al., 2002; Werren et al., 2008)*. In contract, fragmentary descriptions of *Wolbachia* interactions with non-reproductive tissues were reported. Several studies have

suggested that the density of *Wolbachia* in a host is positively correlated with the intensity of CI expression *(Bourtzis et al., 1996; Breeuwer and Werren, 1993)*. *Wolbachia* infection in *Drosophila melanogaster*, extensive infection of somatic tissues of adult insects resulted in a striking reduction in the life span of the insects *(Min and Benzer, 1997)*. Throughout growth and development of a host insect, the density and localization of *Wolbachia* in various tissues and organs may exhibit dynamic and highly controlled changes *(Ijichi et al., 2002)*.

It is necessary to examine *Wolbachia* infection densities in different host tissues and tissue tropism for illustrating the relationships between *Wolbachia* infection and the phenotypic effects on a host *(Ijichi et al., 2002)*. Whereas, the density of *Wolbachia* was estimated for the whole body of *Drosophila spp.* *(Bourtzis et al., 1996)*, for eggs of *Nasonia vitripennis* *(Breeuwer and Werren, 1993)*, for sperm cysts of *Drosophila simulans* *(Bressac and Rousset, 1993)*, or was assessed relative qualitatively of *Tribolium castaneum* *(Ming et al., 2015)*, little study has absolute quantitatively estimated the levels of *Wolbachia* infection in different tissues and organs of *T. confusum*. The purposes of this paper were surveying the *Wolbachia* infection in different geographical stored pest populations in China and clarifying the infection density dynamics and reproductive effects of *Wolbachia* on host to obtain basic information with the potential to be used for future stored pest management strategies.

Materials and Methods

Insect strains collection and Rearing

10 species (18 geographical populations) of Tenebrionidae stored product pests were collected from flour mills and preserved in liquid nitrogen or acetone until needed *(Fukatsu, 1999)*. A long time established laboratory strain *T. confusum* (original insect strain collected from a flour mill of Zhengzhou, Henan in 2012) used in this study were taken from Stored Product Insects Ecology Laboratory, Henan University of Technology (Zhengzhou, Henan). Insects were reared in jars with a feed medium contained a small proportion of brewer yeast (5% w/w) in whole wheat flour and maintained at 30±1 ℃ and 75±5%RH respectively. The diet moisture content of the flour diet was adjusted to 13~14% and froze at -20 ℃ for at least four weeks prior to commencement of the experiments to eliminate any insect infestation.

To collect accurate developmental stages of *T. confusum*, mated females were allowed to oviposit on the black filter paper with little wheat flour in petri dishes. The eggs were collected 24h after the start of oviposition. To collect first-instar larvae, eggs were gently detached and collected from the substratum and allowed them to hatch in a petri dish. Based on molting times and size, second-, third-, and fourth-instar larvae were separated. Pre-pupae were white and longer than pupae, whereas pupae that displayed the tan coloration of the adult cuticle were considered late pupae. Beetles were sexed at the pupal stage based on their genital lobe morphology *(Ming et al., 2015)*. Adult insects

were collected within 1 day after emergence as the new adults, 5~10 days after emergence as the young adults, and over 90 days after emergence as the old adults. To collect different tissues, the adult insects were broken with forceps under a dissecting microscope, head, thorax, abdomen, middle gut, Malpighian tubule, testis, ovary tissues were dissected and immediately used to DNA extraction.

DNA extraction

DNA from insect sample (whole insect body of larvae, pupae, adults and body parts and tissues) was extracted using Takara MiniBest bacteria genome DNA extraction kit (Takara Biotechnology (Dalian) Co., Ltd) according to the manufacturer' instruction. DNA was preferentially extracted from female pupa or adults when possible, since they tend to have the higher titers of *Wolbachia* *(Ijichi et al., 2002).* Extracted DNA was eluted using 100 μL of TE buffer and stored at -20 ℃ until amplified using a diagnostic PCR assay and real time quantitative PCR.

Diagnostic PCR

The primer set was *wsp* (*Wolbachia* surface protein gene) universal primers *wsp*81F(5'-TGGTCCAATAAGTGATGAAGAAAC-3') and *wsp*691R (5'-AAAAATT AAACGCTACTCCA-3') *(Zhou et al., 1998)*, The amplification reactions were initiated by incubation at 95 ℃ for 5min, followed by 30 cycles of 94 ℃ for 1min, 53 ℃ for 1min and 72 ℃ for 1min, and a final elongation step at 70 ℃ for 10min, and a final hold at 4 ℃.

Extracted DNA of *Wolbachia*-infected *Callosobruchus chinensis* Linnaeus (Coleoptera: Bruchidae) *(Kondo et al., 2002)* was used as the positive control and RNase free PCR grade water as negative control for each test. The 600bp PCR products of the *wsp* gene were electrophoresed in a 1% agarose gel, stained with Golden View II (Solarbio) and observed on a UV transilluminator, excised and purified by using a Gel Extraction Kit (Axygen, Corning Inc.). Purified PCR products were cloned into a TA cloning vector, pGMT19 and transfected into *Escherichia coli* JM109 competent cells, wherein ampicillin and X-gal and IPTG were used for blue-white plaque selection.

Real time quantitative PCR

To estimate *Wolbachia* densities, a real-time quantitative PCR assay based on a single-copy gene *wsp* encoding a surface protein of *Wolbachia* was used to determine *Wolbachia* copy number in the hosts in a Thermal cycler (Applied Biosystems 7500, Life Biotechnology Inc.). Primers were specifically designed to detect wCon strain and amplified 332- to 513-bp regions of the *wsp* gene (*wsp*F,5'-GCA GCA TAT ATC AGC AAT CCT TCA A; *wsp*R,5'-GCA TCA TCC TTA GCC GCC TTA T) *(Ming et al., 2015)*. The amplification reaction was monitored by using a set of fluorescent probes specific for the PCR product. A specific TaqMan probe (5' –FAM- TGT TAG CTA TGA TGT AAC TCC AGAA-TAMRA) for the central region of the PCR product was designed and used to measure the amount of *Wolbachia* infection density. PCR was performed under the following conditions: 30s at 95℃, then 40 cycles of 95℃ for 5s, 60℃ for 34s. Well

optimized 20 μL reaction system was as follow: 10 μL Premix Ex Taq (Probe qPCR) (Takara), 0.2 μM concentration of each primer, 0.4μM TaqMan probe, 2μL of template DNA, and 8.4μL of DNase/RNase-Free water. The 182bp PCR amplification products of the *Wolbachia wsp* gene were electrophoresed on a 1% agarose gel, excised, purified and were cloned into a TA cloning vector, pGMT19 and transfected into *Escherichia coli* JM109 competent cells. A standard curve was constructed by using wCon amplicons that had been cloned into pGEM-T vector (Takara) previously, linearized with *Hind III*, and quantified as the template. Three replicates were performed and averaged for each sample. Strain-specific primers to wCon were applied to all samples, and then total *Wolbachia* population density was calculated by integrating both numbers based on a single-copy gene *wsp* copy number *(Ruang-Areerate and Kittayapong, 2006)*.

Tetracycline treatment on *T. confusum*

A serial solution of tetracycline (0.3, 0.5, 1.5, 3.0 mg/ml) was made by diluting tetracycline solution (5mg/ml). These solutions were added to the feed medium (whole wheat flour and brewer yeast, 19:1, w/w) and redundant water was evaporated by cold air flows to keep the ultimate feed moisture content at 13~14%. The antibiotic supplement at total concentration of 0.3, 0.5, 1.5, 3.0 mg/g tetracycline per feed medium *(Zchori-Fein et al., 2000)*. Mated females were allowed to oviposit and eggs were collected 24h after the start of oviposition. The fourth-instar larvae, pupae and adult were separated to examine the density of *Wolbachia* after 2, 3 and 4 weeks' antibiotic treatment respectively. Pupae

were sexed and separated after 3 weeks' antibiotic treatment. Experiments were conducted at 30 ±1 ℃ and 70 ±5%RH.

Crossing experiments and reproductive parameters of *T. confusum*

Beetles with PCR positive for *Wolbachia* were designated w^+, while those *Wolbachia* eliminated by tetracycline treatment were designated w^-. Four crossing couples were performed in 6 well cell culture plates; every well contained a pair of virgin adults of each crossing couples and 1g diet. The total number of eggs laid was counted every two days by sieving the diet through 80 meshes, and then transferred the eggs to 24 well cell culture plates to observe and calculate the hatch percentage, and pupation rate and sex ratio was calculated. 16 replicates of each crossing in every test were conducted.

Results

Wolbachia infection rate detection in Tenebrionidae stored-product insects

Detecting and characterizing the *Wolbachia* strains in stored product insect pests were attempted to obtain basic information with the potential to be used for future pest management strategies. In 10 Tenebrionidae stored product insects, only the *Tribolium confusum* was detected *Wolbachia* infection. Positive PCR results for the *Wolbachia* specific *wsp* gene showed that females and males of all the *T. confusum* strains were

177 infected with *Wolbachia* (Table 1).

178

179 **Table 1. *Wolbachia* infection status in Tenebrionidae stored product pests in China**

Species	Strain	Collection site (latitude and longitude)	Infected females /total tested No.	Infected males /total tested No.
Tribolium castaneum	HNZZ	Zhengzhou, Henan 34.7816, 113.5593	0/10	0/10
	HNZM	Zhongmu, Henan 34.6919,113.9447	0/10	0/10
	SDYC	Yucheng, Shandong 36.9161, 116.6477	0/10	0/10
	HBWH	Wuhan, Hubei 30.417643, 114.31386	0/10	0/10
	GDGZ	Guangzhou, Guangdong 23.2516, 113.2880	0/10	0/10
	GDJM	Jiangmen, Guangdong 22.6284, 113.1073	0/10	0/10
	ZJHZ	Hangzhou, Zhejiang 30.3461, 120.1682	0/10	0/10
	FJNP	Nanping, Fujian 26.6929, 118.2262	0/10	0/10
Tribolium confusum	HNZZ	Zhengzhou, Henan 34.8344, 113.5310	32/32	32/32
	LNSY	Shenyang, Liaoning 41.8588, 123.4225	16/16	16/16
Tenebrio molitor	SXXA	Xi'an, Shaanxi 34.4538, 108.9513	0/5	0/5
	HNZM	Zhongmu, Henan 34.69196,113.94477	0/5	0/5
Tenebrio obscurus	SXXA	Xi'an, Shaanxi 34.4538，108.9513	0/5	0/5
	HNZM	Zhongmu, Henan 34.69196, 113.94477	0/6	0/6
Alphitobius diaperinus	GDJM	Jiangmen, Guangdong 22.6284, 113.1073	0/6	0/6
Alphitobius laevigatus	SXXA	Xi'an, Shaanxi 34.4538, 108.9513	0/6	0/6
Alphitobius bifasciatus	GDJM	Jiangmen, Guangdong 22.6284, 113.1073	0/6	0/6
Palorus ratzeburgi	SXXA	Xi'an, Shaanxi 34.4538, 108.9513	0/6	0/6
Palorus subdepressus	HNZM	Zhongmu, Henan 34.6919, 113.9447	0/6	0/6
Palorus cerylonoides	GDZQ	Zhaoqing, Guangdong 23.0423, 112.4331	0/5	0/5

Wolbachia density in different develop stage of *Tribolium confusum*

The *Wolbachia* infection dynamics in terms of *wsp* copies per insect were examined throughout development of *T. confusum* by using a TaqMan probe quantitative PCR technique (Fig.1). The results indicated that the population of *Wolbachia* consistently increased with the host development proceeded. The *Wolbachia* infection density reached the highest level at 3.7×10^7 *wsp* copies per insect in the young adults (5 d post-eclosion). *Wolbachia* density showed a gender difference, that females had a higher *Wolbachia* density than the males, especially in pupae stage (Fig.2, Independent samples *t*-test, $P<0.05$).

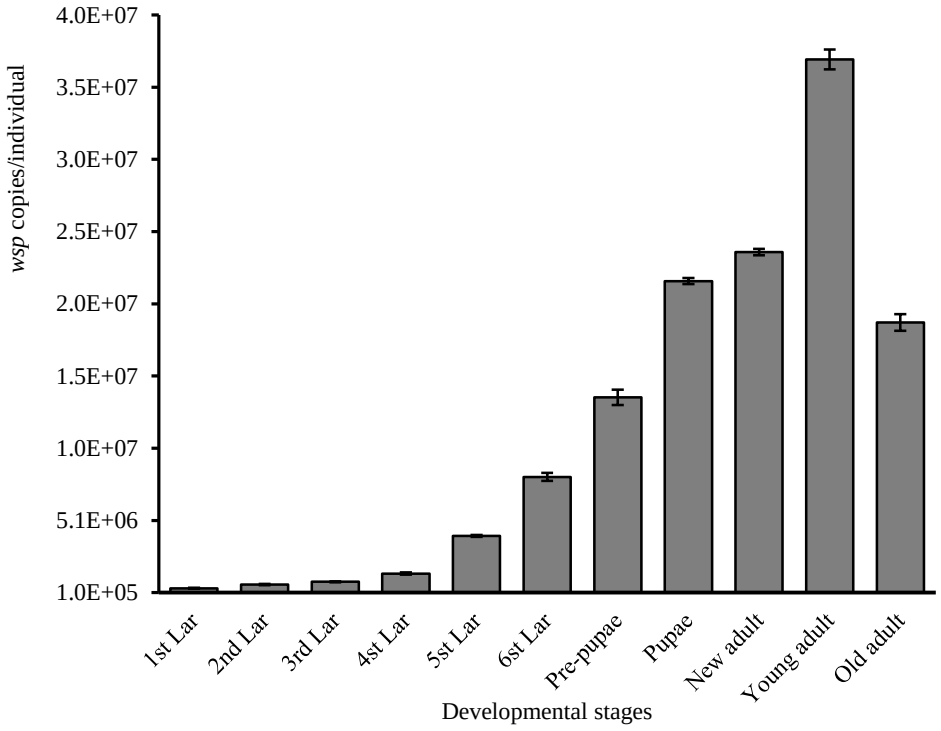


Fig.1 Dynamics of *Wolbachia* infection during the development of *Tribolium confusum*. Reported values are mean ± SD for *wsp* copies per mg insect mass (fresh weight). The error bars indicate standard deviation (SD) (n=6). Adults were females.

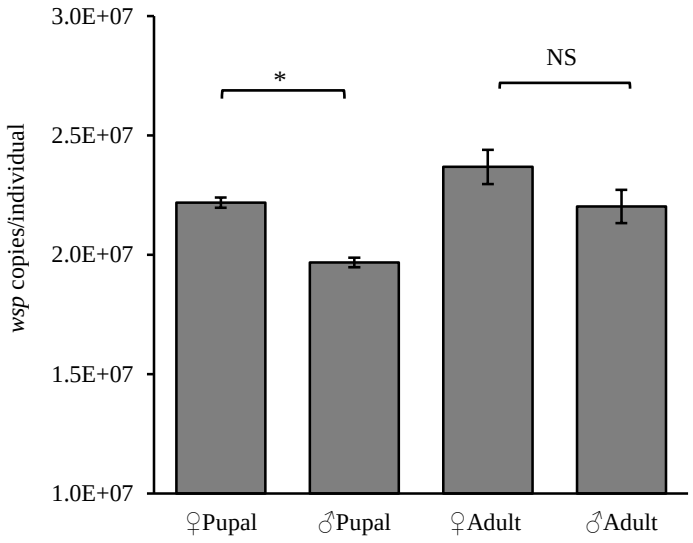


Fig.2 Comparison of *Wolbachia* infection density in *Tribolium confusum* male and female pupae and adults. (* indicates significant difference between two groups, NS indicates no significant difference, Independent samples *t*-test, $P<0.05$).

Wolbachia density in different tissues of *Tribolium confusum*

To quantitatively investigate the localization of *Wolbachia* in *T. confusum* tissues, dissected tissues and organs from adult insects were subjected to quantitative PCR analysis. The *wsp* gene copy number was quantified by using a TaqMan probe to compare localization of *Wolbachia* in different tissues and organs. In the present study, the density of *Wolbachia* was expressed as the copy number of the *wsp* gene per mass (ng) template DNA. Relative amounts of *Wolbachia* infection density were apparently higher in abdomen than in thorax and head (Fig.3).

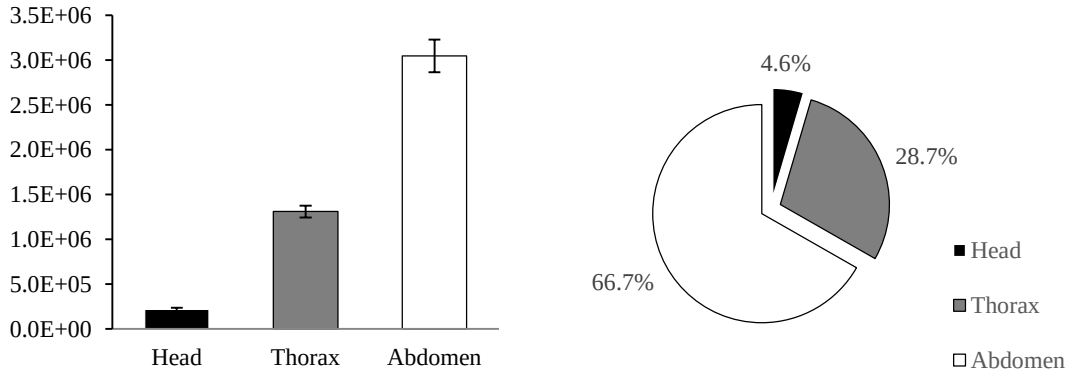


Fig.3 Relative amounts of *Wolbachia* population density in head, thorax and abdomen of *T. confusum*, expressed in terms of number of *wsp* copies per individual body part.

197 For the tissues and organs examined, the density of the *Wolbachia* differed strikingly
198 in different tissues and organs. Higher *Wolbachia* infection level was detected in the
199 reproductive tissue and fat body than in the middle gut and Malpighian tubules (Fig.4).

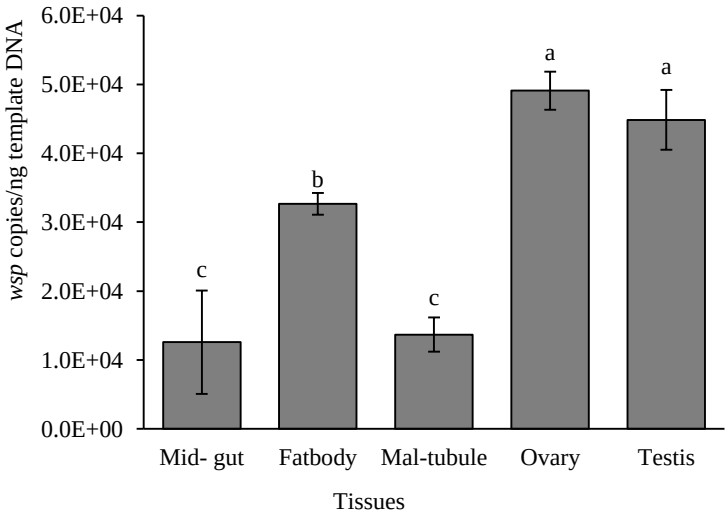
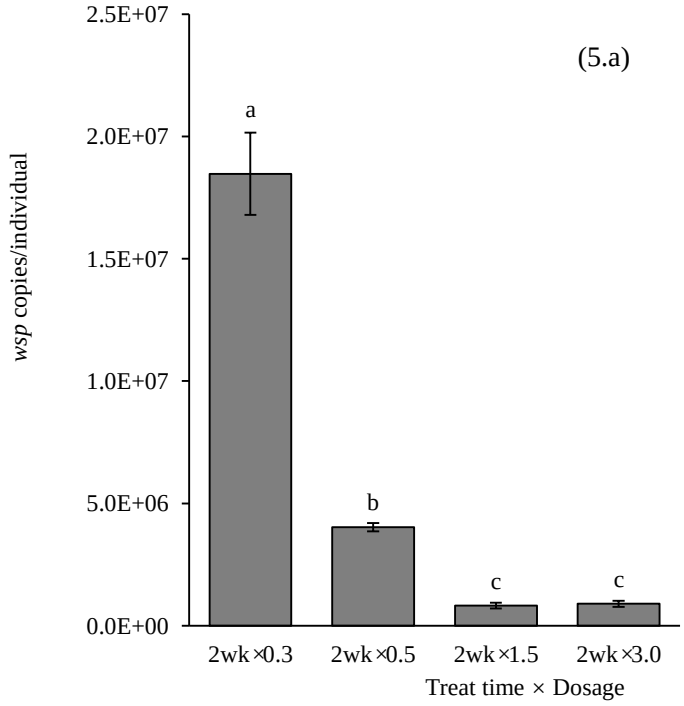
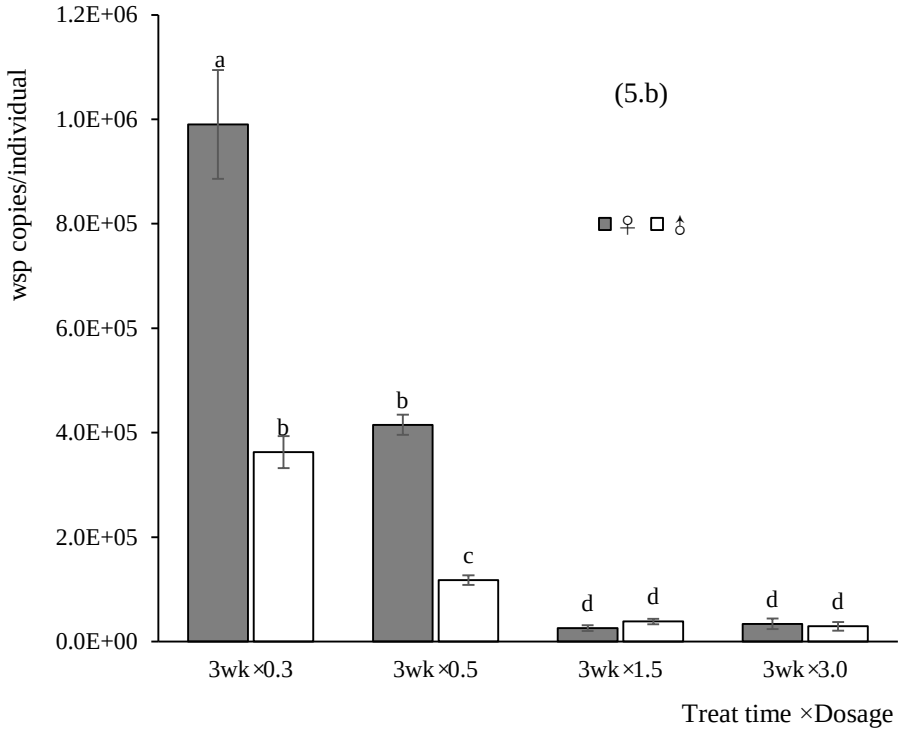


Fig.4 Comparison of the population density of *Wolbachia* in different tissues of *T. confusum*, expressed in terms of number of *wsp* copies per ng template DNA. The error bars indicate standard deviation (SD) (n=10). The different letters identify a significant difference (Turkey HSD test, $P<0.05$)

Effect of Antibiotic treatment on *Wolbachia* density in *Tribolium confusum*

The clear relationship between the total *Wolbachia* density with antibiotic treatment doses and time was showed in Fig.5. Antibiotic treatments specifically inhibit *Wolbachia* density in the flour beetle. The density elimination rate of *Wolbachia* showed sex-related differences in pupae stage (treatment for 3 weeks) the *Wolbachia* density was higher in females than in males, that means that it is easier to removal *Wolbachia* in males than in females at the lower antibiotic treatment (Fig.5c). While at the higher concentration of antibiotic treatment, it is much easier to eliminate the *Wolbachia*.





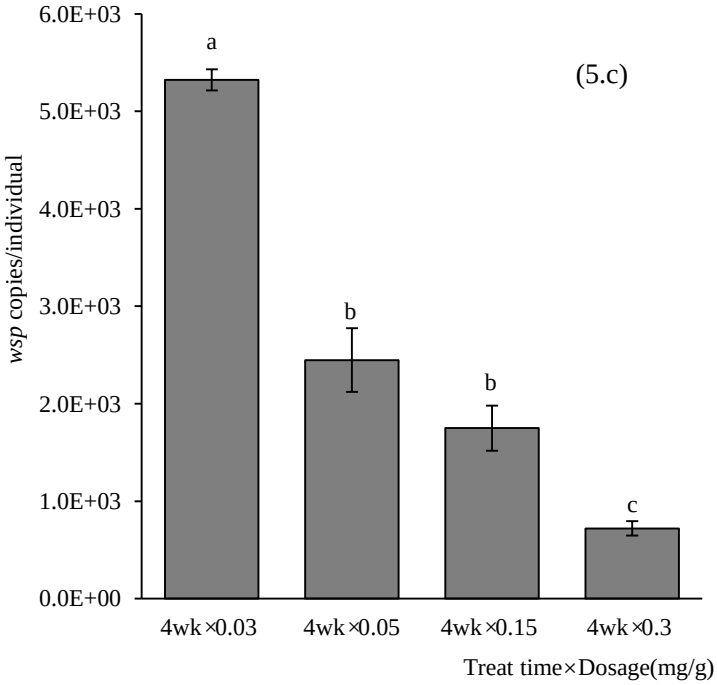


Fig.5 Comparison of the population density of *Wolbachia* in *T. confusum* by different concentration of tetracycline treatment for 2, 3 and 4 weeks. The number expressed in terms of number of *wsp* copies per individual (a: larvae CK=1.47E+07, b: pupae CK: ♀2.22E+07,

♂1.97E+07, c: adults CK=2.37E+07). The error bars indicate standard deviation (SD). The different letter indicates a significant difference (Turkey HSD test, *P<0.05*).

Effect of *Wolbachia* on fitness of *T. confusum* population

Antibiotic treatments specifically inhibit the reproductive fitness in the flour beetle *T. confusum*. Aposymbiotic (w^-) female beetles were completely incapable of producing mature embryogenesis after crossed with *Wolbachia* infected males, and therefore could not reproduce even though they can produce sterile eggs. There were no significant differences (*P>0.05*) in the total eggs number, percentage of pupal survival, and percentage of adult survival expect for significant difference (*P<0.05*) in the egg hatch rate (Table 2). In addition, the sex ratio of female/male offspring did not significantly deviate from 1:1, and no significant differences were found between them (*P>0.05*). Totally cytoplasmic incompatibility was expressed when w^+ males mated with w^- females(Table 2), resulting in few or no viable offspring produced, but the other two possible crosses (♂w^- ×♀w^+ and ♂w^+ ×♀w^-) are compatible.

Table 2. CI expression and reproduction parameters of antibiotic treated(w^-) and untreated(w^+) *Tribolium confusum*

Crosses (♂×♀)	Eggs number	Egg hatch rate （%）	Pupation rate（%）	Sex ratio（♀/♂）
$\mathbf{w^+ \times w^+}$	33.6±5.7a	90.5±3.5a	94.2±3.7a	1.054±0.04a
$\mathbf{w^- \times w^+}$	33.2±3.6a	90.9±3.4a	93.6±3.8a	1.067±0.07a
$\mathbf{w^+ \times w^-}$	31.4±4.7a	0c	-	-
$\mathbf{w^- \times w^-}$	31.7±4.2a	70.9±7.4b	85.6±5.6b	0.989±0.06a

♂, male; ♀, female; w^+, *Wolbachia*-infected beetle; w^-, *Wolbachia*-uninfected beetle (Tetracycline treatment); Eggs number is laid by one pair beetles in 30 days. Mean±SD followed by different letters in the same column indicate significant difference(Turkey HSD

test, $P<0.05$).

Discussion

In this paper, we found that *Wolbachia* infection was detected only in the *T. confusum* of 10 Tenebrionidae stored product insect strains (18 populations) from wild and laboratory strains in China. All the *T. confusum* individuals, female and male, were completely infected *Wolbachia* (Table 1). We confirmed that there was monophyletic *Wolbachia* infection in *T. confusum* in two Chinese strains (LNSY and HNZZ). This report was coincided with the conclusion of Kageyama *et al* (2010) and Li *et al* (2015) surveyed of *Wolbachia* prevalence and diversity in stored product insect species in Canada, Denmark, England, France, Germany, Greece, Italy, Portugal, Spain, USA and Japan *(Kageyama et al., 2010; Li et al., 2015)*. In addition, there were no *Wolbachia* infection in 3 *Alphitobius* species and 3 *Palorus* species, which is the first reported survey record.

T. confusum and *T. castaneum* were sibling species, as they have almost same morphological characters and close genetic distance *(Ming et al., 2014)*. It was strange that no *Wolbachia* infection *T. castaneum* strain was found in the wild, neither in the published scientific reports until now *(Goodacre et al., 2015; Kageyama et al., 2010; Li et al., 2015)*. Based on these results, we conjectured that *T. castaneum* might have an internal mechanism inhibiting the reproduction and survival of *Wolbachia*. The interspecific mating experiment indicated that horizontal and vertical transmissions of

Wolbachia didn't occur in the two closely related flour beetles *T. castaneum* and *T. confusum* (data not shown in this paper).

The absolute quantification method using TaqMan probe of real time qPCR to assay the *Wolbachia* density was conducted in this study. There were positive PCR results in the isolated heads thoraxes, middle guts, fat-bodies and Malpighian tubules (females and males) (n=10), demonstrating the presence of *Wolbachia* in digestive and immune tissues other than the reproductive tissues. This finding is consistent with studies on parasitic wasp *Asobara tabida* and adzuki bean beetle *Callosobruchus chinensis* *(Dedeine et al., 2001; Ijichi et al., 2002)*. In our study, the density of *Wolbachia* was expressed as the copy number of the *wsp* gene per ng template DNA. The data was normalized with each insect *(Ikeda et al., 2003)*, since the guts and reproductive tissues were too small to weigh and the concentration of template DNA was available to be measured.

The elimination of *Wolbachia* could be accessed by a long-time antibiotic medium treatment. It indicated that *Wolbachia* in *T. confusum* could be eliminated by tetracycline as the antibiotic treatment continued (more than 1 month), and the resultant *Wolbachia*-uninfected *T. confusum* could be obtained and used for the mating and crossing experiments. When the *Wolbachia* density was lower from 1000 copies per individual, detectable level of the *wsp* gene fragment amplified can hardly be examined by standard PCR amplification.

Wolbachia infection could generate strong reproductive incompatibilities between uninfected females and infected males (cytoplasmic incompatibility) and significantly

reduce both female and male reproductive success. The impact of *Wolbachia* on mating behavior in *Drosophila melanogaster* and *Drosophila simulans*, and it showed that infected males mated at a higher rate than uninfected males in both species *(Crespigny and Wedell, 2007)*. *D. simulans* males exhibited some preference for mating with females of the same infection status *(Awrahman et al., 2014; Crespigny and Wedell, 2007)*. According to our observation and the mate choice experiments of Ming (2015), infected and uninfected males of *T. confusum* did not have obvious preference for mating partner in mate choice tests. It may indicate that *Wolbachia* infection had no influence on mate preference in *Tribolium confusum (Ming et al., 2015)*.

It is generally accepted that vertically transmitted microorganisms should tend to evolve toward a benign state, or even to be beneficial to their hosts, for their fitness is inextricably linked to host performance *(Douglas, 1998; Douglas, 2015; Lipsitch et al., 1996)*. While in arthropods, *Wolbachia* are rarely found to be beneficial to their hosts. *Wolbachia* strains are able to maintain themselves in arthropod populations through induced modifications to host reproductive biology *(Fleury et al., 2000; Min and Benzer, 1997)*. Moreover, even if many studies have failed to detect negative effect of infection and a few studies have shown a slight enhancement of reproductive success in infected individuals *(Poinsot and Mercot, 1997)*, reproductive manipulation effects of *Wolbachia* was still not well demonstrated.

In our study, aposymbiotic lines were available and established in the confused flour beetle *T. confusum*. Even though, it seems that *Wolbachia* induced the completely CI and

decreased the fitness of host, however, a higher fecundity of w+ females than all the other crosses was found. The equality of sex ratio and the higher fecundity encourage CI expression and *Wolbachia* prevalence in flour beetle population by two beneficial outcomes: infected females increase infected offspring and infected males decrease uninfected offspring in the next generations. All these results indicated that *Wolbachia* infection was regarded as a mutualism and benefited the host confused flour beetle.

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