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ORIGINAL RESEARCH ARTICLE

Demographic evidence showing that the removal of *Wolbachia* decreases the fitness of the brown planthopper

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Abstract

Wolbachia is a maternally inherited intracellular bacterium (Rickettsiaceae) that infects a wide range of arthropods and nematodes, plays an influential role in host development and triggers various reproductive abnormalities. In this study it was investigated whether *Wolbachia* infection of the *Nilaparvata lugens* (Stål) species of Guizhou brown planthopper (BPH) induces cytoplasmic incompatibility (CI) and alters BPH developmental timing, longevity, and fecundity. It was shown that although *Wolbachia* does not induce CI in the species of BPH, its absence decreases BPH fitness. Furthermore, the cross of a *Wolbachia*-free female BPH and an infected male ($F^{\triangledown} \times M$) resulted in a significantly shorter pre-adult developmental period (11.15 ± 0.22), significantly reduced ovi-days (5.80 ± 0.2438), significantly shorter longevity (22.9 ± 0.38), and lower fecundity (176.28 ± 5.79b). This cross also affected a series of population parameters by increasing the finite rate (λ) (1.2903 ± 0.02) and intrinsic rate value (*r*) (0.2548 ± 0.01), reducing the net reproductive rate (R_0) (88.12 ± 19.9) and decreasing longevity (22.9 ± 0.38).

Keywords Brown planthopper · *Wolbachia* · Developmental time · Fecundity · Life history

Introduction

Wolbachia spp. is a common intracellular bacteria found in many species, including nematodes (Luck et al. 2014; Voronin et al. 2015), crustaceans (Sicard et al. 2014), insects (Li et al. 2015), and acarids (Glowska et al. 2015). *Wolbachia* acts as a reproductive manipulator and can spread through host populations via feminization (Hosseinali et al. 2014; Kern et al. 2015), male killing (Hornett et al. 2014; Zeh et al. 2005), parthenogenesis (Goryacheva and Andrianov 2015; He et al. 2015), and cytoplasmic incompatibility (Gerardo and Parker 2014; Carrington et al. 2015; Ming et al. 2015; Zhang et al. 2015).

Rice planthoppers (Hemiptera: Delphacidae) are among the most devastating pests of rice fields in Asian countries. Three of the most important planthopper species in China and the surrounding areas are the brown planthopper (BPH) *Nilaparvata lugens* (Stål), the white-backed planthopper (WBPH) *Sogatella furcifera* (Horváth), and the small brown planthopper (SBPH) *Laodelphax striatellus* (Fallén). These planthoppers feed on the stems of rice, which causes physiological stress and "hopper burn", a noncontagious disease caused by damage due to direct feeding by certain planthoppers and leafhoppers (Hayashi and Chino 1990; Backus et al. 2005). Furthermore, planthoppers can transmit plant-pathogenic viruses that substantially decrease rice yields (Sōgawa 1982; Hibino et al. 1988). The brown planthopper *Nilaparvata lugens* is an insect pest prevalent in the paddy fields of East and South China. BPHs harbor yeast-like symbiotes (YLS), particularly in mycetocytes formed by the gathering of YLS cells in the fat cells of the abdomen (Chen et al. 1981).

Wolbachia is a well-known arthropod reproductive endosymbiont and infects at least 20% of all arthropods and certain nematode species (Werren et al. 1995a, 1995b; Bandi et al. 1998; West et al. 1998; Werren and Windsor 2000; Kikuchi

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and Fukatsu 2003; Tagami and Miura 2004; Hilgenboecker et al. 2008). Bacterial endosymbionts might provide essential nutrients that are lacking in the diet or aid in food digestion and detoxification (Cardoza et al. 2006; Adams and Celniker 2000). Previous studies have indicated that symbiotic bacteria play roles in insect defense systems by enhancing pathogen and parasitoid resistance (Oliver et al. 2005, 2008; Currie et al. 2006; Hedges et al. 2008; Teixeira et al. 2008; Brownlie and Johnson 2009) and might aid in prey preservation and nest hygiene (Kaltenpoth et al. 2009). In addition, endosymbionts have been shown to mediate the thermal tolerance of their hosts (Dunbar et al. 2007) and facilitate the use of novel hosts (Tsuchida et al. 2011). Studies conducted on *Liposcelis tricolor* have demonstrated that the removal of *Wolbachia* can reduce egg production (Dong et al. 2006). Previous research has demonstrated that *Wolbachia* is capable of inducing CI and enhances the fitness of the host. The current study investigated whether *Wolbachia* induces CI in the Guizhou BPH species and how *Wolbachia* affects the developmental timing, longevity and reproduction of *N. lugens*.

Materials and methods

Insect gathering and rearing

In this study, a *Wolbachia*-harboring strain of BPH was collected from a paddy field near Guizhou University in June 2011. The BPH strain was reared in a greenhouse for 10 generations and subsequently used for experimentation.

Preparation of infected and uninfected strains

Other BPHs used in this study originated from a paddy field near Guizhou University and were collected in June 2014. The BPHs obtained from rice were reared in a greenhouse for 10 generations and subsequently used for experimentation. The presence of BPHs harboring a *Wolbachia* strain was confirmed by polymerase chain reaction (PCR) amplification of a partial sequence of the *wsp* gene. To obtain *Wolbachia*-infected and *Wolbachia*-free lines, pairs of male and female adults from naturally occurring populations were placed in individual plastic cups (120 mm in height and 80 mm in diameter) that contained rice seedlings. The seedling root was wrapped in cotton and then dipped in an artificial Espino culture (Liu and Wu 2010) in a test tube (50 cm length × 38 cm diameter), which was maintained at 25 ± 1 °C with 85 ± 5% humidity and a 16 h light:8 h dark cycle in an air-conditioned room. The pairs were allowed to lay eggs into the rice seedlings for 1 week and were removed from the cup. The infection status of these pairs were assessed by PCR amplification. Only offspring from parents that tested either positive or negative for *Wolbachia* by PCR screening were continually reared and used as parental stock. This selection regime was maintained for several successive generations until *Wolbachia*-infected populations and *Wolbachia*-free populations were obtained.

Immature developmental traits of BPHs

Within three to 5 days of hatching, 250–300 of the *Wolbachia*-harboring BPH adults were aspirated into 15–20 plastic bottles (1 cm in height × 4 cm in diameter) with a nylon screen top and containing a piece of black filter paper. The bottles were then placed in a 5000 ml desiccator with 75–80% humidity and a saturated NaCl solution, and the desiccator was maintained at 28 °C. The BPHs oviposited eggs for 12 h and were then removed. Pieces of filter paper containing a single egg were cut and transferred separately into plastic vials (1 cm in height, 2.4 cm in diameter, and a nylon screen top), and a small amount of food (10 mg) was added. Between 25 and 60 eggs were placed in each humidified desiccator. The desiccators were placed in growth chambers (Chongqing Electronic, Chongqing, China) maintained at a temperature of 28 °C.

Mature longevity and reproduction

For these experiments, adults were reared from eggs in growth chambers. After maturation, male and female adults were paired and reared separately in plastic bottles. After the onset of reproduction, the number of adult BPHs and the number of offspring produced each day were counted. The offspring were removed after counting so that they could not mature to adulthood and reproduce. After a 12 h period of BPH egg laying, the adults were removed. Pieces of filter paper containing a single egg were cut, transferred separately into plastic vials (1 cm in height, 2.4 cm in diameter with a nylon screen top) and provided a small amount of food (10 mg). The total number of eggs laid was recorded daily until all of the released BPH females had died. The eggs were counted using a magnifying glass. Food (10 mg) (Liu and Wu 2010) was added every 2 days.

PCR amplification detection

For DNA extraction, individual BPHs were homogenized in 40 μl of STE (100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA [pH 8.0]) in a 1.5 ml Eppendorf tube and incubated with proteinase K (10 mg/ml, 2.5 μl) at 37 °C for 30 min and then at 95 °C for 5 min. The samples were briefly centrifuged and either used immediately for PCR or stored at −20 °C for later use. The presence of *Wolbachia* in the BPHs was determined by PCR amplification of a partial sequence of the *wsp*

gene (the expected size for the PCR product ranged from 590 to 632 bp depending on the individual *Wolbachia* strain). The *wsp* gene was amplified using the specific primers *wsp* 81F (5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′) and *wsp* 691R (5′-AAAAATTA AACGCTACTCCA-3′) (Zhou et al. 1998). The temperature program used for PCR was 95 °C for 10 min, 30 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, and a final extension of 72 °C for 7 min. A 10 μl sample of the PCR product was electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized by ultraviolet illumination.

Estimating *Wolbachia* density

To estimate the abundance of *Wolbachia*, the copy numbers of the *wsp* gene were determined using an Applied Biosystems 7300 real-time PCR system. Based on the sequences of the *wsp* gene in the sequenced *Wolbachia* strain (wLug), new specific primers (WSP/F 5′-TGAGT AAAGACGG AGATGTGGC-3′ and WSP/R5'-TTTTGTCGCTAAAG GGTTGC-3′) were designed using Primer Premier 5.0 and used for the quantitative PCR (qPCR) amplification of a 170 bp DNA fragment.

To estimate the copy number of the *wsp* gene, a standard curve was plotted using dilutions of a pGEM-T vector containing part of the *wsp* sequence. A SYBR Premix Ex Taq PCR Reagent Kit (Takara Shuzo) was used to measure the copy number of the *Wolbachia* genes. Based on the assumption that each genome harbors one copy of the *wsp* gene, the *wsp* copy number was considered an estimate of the number of *Wolbachia* bacteria. The temperature program used for PCR was 30 s at 95 °C, 40 cycles of 95 °C for 5 s and 60 °C for 31 s, and a dissociation stage of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 5 s. The 20 μl PCR mixture included 10 μl of SYBR Premix Ex Taq, 0.2 μM of each primer, 7.2 μl of double-distilled water (ddH_2O), and 2 μl of template DNA. Each DNA template was analyzed in triplicate.

DNA sequence analysis

The *wsp* amplicons from several individuals were cloned using the TIANamp Genomic DNA Kit according to the manufacturer's recommended protocol. Multiple clones of each individual were submitted to the Beijing Genomics Institute (Beijing, China) for DNA sequencing. The length of the cloned *wsp* gene ranged from 590 to 611 bp. The sequences were aligned using the BioEdit software package, and a BLAST search of GenBank was performed to identify identical or highly similar sequences reported in other organisms. All sequences have been submitted to GenBank under the accession number KU933913.

Data analysis

An age-stage, two-sex life table model was used for the data analysis (Chi and Liu 1985) based on the method described by Chi (1988). This model includes the following parameters: R_0 (net reproduction rate), r (intrinsic rate of increase), λ (finite rate of increase), and T (mean generation time). The intrinsic rate of increase was determined using the iterative bisection method based on the following equation:

$$\sum_{x=0}^{\infty} e^{-r(x+1)} l_x m_x = 1 \quad (1)$$

The mean generation time was defined as the length of time required for a R_0-fold increase in the population size (i.e., $\lambda T = R_0$) at the stable age-stage distribution. The mean generation time (T) and the gross reproductive rate (GRR) were calculated using the following equations:

$$T = \frac{\ln R_0}{r} \quad (2)$$

$$\sum m_x = GRR \quad (3)$$

The age-stage life expectancy (e_{xj}) was calculated according to Chi and Su (2006). The means, variances and standard errors of the life table parameters were estimated using the jackknife method (Sokal and Rohlf 1995). To facilitate the tedious process of raw data analysis, the computer program TWOSEX-MSCHART was used for the age-stage, two-sex life table analysis (Chi 2010; Farhadi et al. 2011). This program was developed in VISUAL BASIC for Windows and is available at http://140.120.197.173/Ecology/prod02.htm (Chung Hsing University) and http://nhsbig.inhs.uiuc.edu/wes/chi.html (Illinois Natural History Survey).

Results

PCR amplification-based detection of the *wsp* gene

PCR was used to detect *Wolbachia* infection in BPHs. Fig. 6a shows a 574 bp DNA fragment amplified from 15 BPHs, which represents *Wolbachia* infection, and Fig. 6b shows eight BPHs that did not contain *Wolbachia* and therefore failed to produce a PCR product.

Estimation of *Wolbachia* density

The *Wolbachia* density of two different populations was tested: one population collected from Southern Guizhou Province, which yielded uninfected female BPHs after several generations, and one population from Northern Guizhou Province. *Wolbachia* was detected in greater numbers in females than in males of the Southern population, whereas the

opposite was found for the Northern population, which is consistent with previous reports (Bourtzis and O'Neill 1998; Sugimoto and Tsuchida 2015). However, the *Wolbachia* number significantly decreased during pre-adult development in both BPH populations. The *wsp* gene copy number significantly increased from 32 to 37 days of age in the Northern BPH population, whereas the copy number slightly increased from 25 to 32 days of age and then significantly increased from 33 to 35 days of age in the Southern BPH population (Fig. 5).

Developmental time, longevity and fecundity of the brown planthopper

The survival and fecundity of BPHs are shown in Fig. 1 and Table 1. The longest pre-adult developmental time was 20.15 ± 0.31 days for the F × M treatment, whereas the shortest pre-adult stage observed among all of the experiments was 11.15 ± 0.22 days for the F♂ × M treatment (Table 1). The oviposition period of the F♂ × M treatment was 5.80 ± 0.244 days, which was significantly lower than other treatments. The longevity of the adult BPHs was also affected by *Wolbachia* infection: the longevity of the F × M treatment (43.95 ± 0.049) was greater than those of other three treatments, whereas the longevity of the F♂ × M cross (22.9 ± 0.387) was significantly shorter than those of the other treatments (Table 1).

Females began to lay eggs on day 10 (F♂ × M treatment) or day 15 (other treatments; Fig. 4). The total fecundities were 674.61 ± 24.21 eggs/female for the F × M cross, 176.28 ± 5.79 eggs/female for the F♂ × M cross, 393.76 ± 18.43 eggs/female for the F × M♂ cross and 438.7 ± 24.78 eggs/female for the F♂ × M♂ cross (Table 2). These data indicated that the number of BPH eggs laid by uninfected females was significantly deceased. Together, these results showed that all parameters, including the developmental time, longevity and fecundity, of the F♂ × M cross were lower than those of the other treatments. Therefore, it was concluded that *Wolbachia* could increase the fitness of BPHs and that this effect is more pronounced in infected females.

The average reproductive value for age *x* and stage *j* per day is presented in Fig. 2, and the *lx* and *mx* values are presented in Fig. 1. The *lx* curve, which reflects changes in the survival rate of a population over time, revealed significant differences among the four experiments. The higher survival rate of the F × M cross (greater than 0.6) was apparent from days 24 to 28, and the highest survival rate of the F♂ × M cross (slightly greater than 0.4) was observed between days 14 and 16.

The detailed age-stage survival rates (S_{xj}, which is the probability that a newborn will survive to age *x* and stage *j*) of *N. lugens* under different experimental conditions are plotted in Figs. 3, 4, 5, and 6. The survival rate curves of the *N. lugens* cohorts were significantly different between the experiments. However, for the F♂ × M cross, the female survival rate was higher than the male survival rate.

Population parameters

The means and standard errors of the population parameters of *N. lugens* subjected to the different treatments are listed in Table 2. The intrinsic rates (*r*) obtained with the different treatments were 0.2056 ± 0.009, 0.2548 ± 0.01, 0.1858 ± 0.09, and 0.2052 ± 0.01, and these values did not differ significantly. However, the net reproductive rate (R_0) was different than the intrinsic rate. The greatest R_0 value (337.18 ± 76.03) was more than four-fold greater than the lowest value

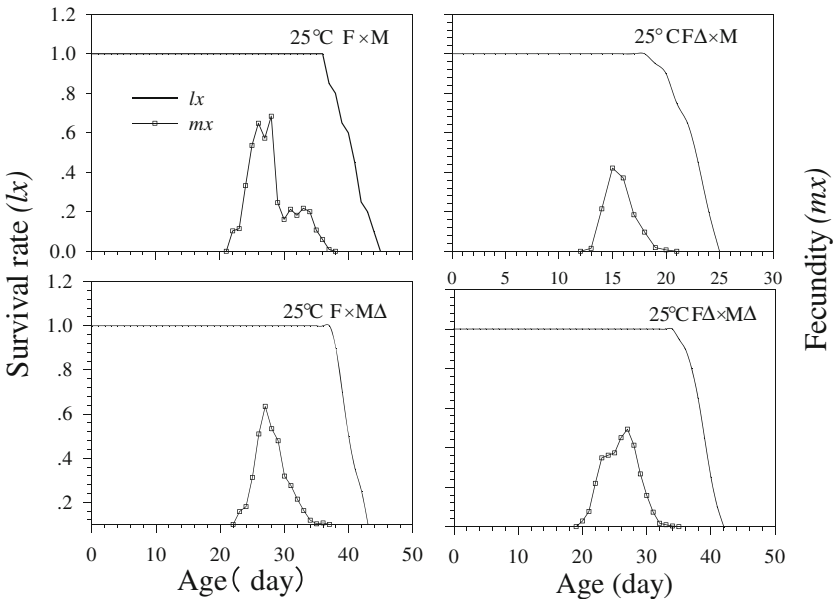

Fig. 1 Age-specific survival rate (*lx*) and age-specific fecundity (*mx*) of *N. lugens* under different treatment conditions: *Wolbachia*-infected males were mated with infected females (F × M), uninfected females were mated with infected males (F♂ × M), infected females were mated with uninfected males (F × M♂), infected females were mated with uninfected males

Table 1 Pre-adult developmental time and adult longevity (mean ± SE) of BPHs under different treatment conditions. The symbol ▽ represents uninfected with *Wolbachia*. SEs were estimated via 100,000 bootstrap resamplings

Statistics	25 °C			
	F × M	F▽ × M	F × M▽	F▽ × M▽
Pre-adult developmental time (d)	20.15 ± 0.31a	11.15 ± 0.22b	13.09 ± 0.26b	18.8 ± 0.34a
APOP (d)	43.95 ± 0.05c	3.50 ± 0.21b	6.90 ± 0.45 cd	5.20 ± 0.20a
TPOP (d)	12.45 ± 1.35c	14.89 ± 0.17b	26.19 ± 0.60c	23.5 ± 0.65a
Ovi-days (d)	7.902 ± .3979a	5.80 ± 0.2438b	6.60 ± 0.298a	7.1 ± .39a
Longevity	43.95 ± 0.049a	22.9 ± 0.387b	40.8 ± 0.416a	39.15 ± 0.437a

There were four different treatment conditions in this study: F × M represents *Wolbachia*-infected males were mated with infected females, F▽ × M represents uninfected females were mated with infected males, F × M▽represents infected females were mated with uninfected males, F▽ × M▽and infected females were mated with uninfected males, the same as bellow

(88.12 ± 19.9). The longevity of the F▽ × M cross (22.9 ± 0.38) was half the value obtained for the other treatments, and the mean generation time (*T*) was markedly decreased to 17.469 ± 0.19 days for the F▽ × M cross.

Discussion

Intracellular rickettsial bacteria of the genus *Wolbachia* are found in numerous invertebrates, including insects and nematodes. *Wolbachia* often manipulate the reproductive mode of their insect hosts, resulting in cytoplasmic incompatibility (CI). To obtain *Wolbachia*-free insect strains, most researchers use antibiotics to remove *Wolbachia*. In this work, we obtained *N. lugens* populations that were uninfected and infected with *Wolbachia* and used PCR technology to screen the natural populations, which enables us to measure the strength of CI induced by *Wolbachia* in BPHs from Guizhou Province without the negative effects of antibiotics (Bandi et al. 1999).

Wolbachia-induced CI occurs when males infected by one *Wolbachia* strain produce sperm that appears to be "toxic" to oocytes of uninfected females or females infected with another *Wolbachia* strain. In this study, we did not observe the occurrence of CI (the F▽ × M cross is viable, although the number of eggs was significantly decreased). Instead, it was found that *Wolbachia* exerts a positive effect on the fitness of the host and observed enhanced fecundity and shortened longevity only when infected males mated with uninfected females or females infected with a *Wolbachia* strain. *Wolbachia* induces CI in a density-dependent manner. In a related example, the bacterial density was found to be correlated with compatibility differences among male and female parasitoid *Nasonia vitripennis* wasps, with males from strains with high bacterial numbers presenting incompatibility with females from strains with lower numbers (Breeuwer and Werren 1993). Among *Drosophila* species, *Drosophila simulans* Hawaii, *Drosophila sechellia*, and *Drosophila auraria* exhibit high levels of CI relative to the level of infection, whereas all other species, including *D. simulans* Riverside, exhibit significantly lower levels of CI relative to the level of infection (Bourtzis et al. 1996). The symbiont density is also a major factor in host-symbiont

Table 2 Population parameters of brown planthoppers subjected to different treatments: mean fecundity (*F*), finite rate of increase (λ), intrinsic rate of increase (*r*), net reproductive rate (R_0), mean generation time (*T*) and GRR. The symbol ▽ represents the removal of *Wolbachia*. SEs were estimated via 100,000 bootstrap resamplings

Parameters	25 °C			
	F × M	F▽ × M	F × M▽	F▽ × M▽
Fecundity (*F*) (eggs/female)	674.61 ± 24.21bc	176.28 ± 5.79bc	393.76 ± 18.43b	438.7 ± 24.78a
Finite rate (λ) (d^{-1})	1.2283 ± .011a	1.2903 ± 0.02a	1.2042 ± .011a	1.2278 ± 0.01a
Intrinsic rate (*r*) (d^{-1})	0.2056 ± 0.009a	0.2548 ± 0.01a	0.1858 ± 0.09a	0.2052 ± 0.01a
Net reproductive rate (R_0)	337.18 ± 76.03a	88.12 ± 19.9a	196.85 ± 44.85a	219.35 ± 50.57a
Longevity (d^{-1})	43.95 ± 0.04	22.9 ± 0.38b	40.8 ± 0.42a	39.15 ± 0.44a
Mean generation time (*T*)(d)	28.18 ± .394c	17.469 ± .19c	28.42 ± 0.59b	26.26 ± 0.72c
GRR	337.3 ± 76.034a	88.71 ± 19.936c	196.85 ± 44.85b	219.35 ± 50.57b

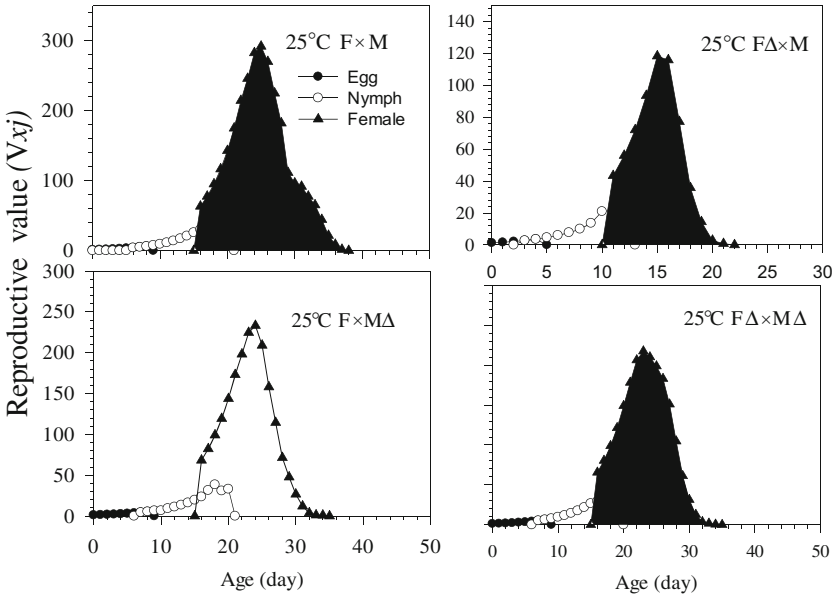

Fig. 2 Age-stage-specific reproductive value (v_{xj}) of *N. lugens* under different treatment conditions: *Wolbachia*-infected males were mated with infected females (F × M), uninfected females were mated with infected males (F▽ × M), infected females were mated with uninfected males (F × M▽), infected females were mated with uninfected males

relationships because it can influence both the efficiency of transmission and the expression of symbiont functions. The *wsp* gene copy number (represents the symbiont density) significantly increased from 32 to 37 days of age in the northern BPH population, and a similar result was found for the southern BPH population, with the copy number increasing slightly from 14 to 18 days of age and then increasing significantly from 33 to 36 days of age. As shown in Fig. 1, the highest fecundity for the F▽ × M cross occurred from 14 to 18 days of age, whereas the peak fecundity of the other treatments occurred from 22 to 28 days of age. Therefore, the greatest bacterial density was found for the F▽ × M cross, and this high bacterial density decreased the fitness of the BPHs. This result illustrates how protective symbionts affect their hosts: by increasing their hosts' fitness, symbionts increase the proportion of hosts in populations that harbor the symbiont, thereby furthering their own distribution (Gerardo and Parker 2014).

The developmental time (11.15 ± 0.22d) and adult longevity of the F▽ × M cross were shorter (22.9 ± 0.387d) than those of the other treatments (developmental time 13.09~20.15d;

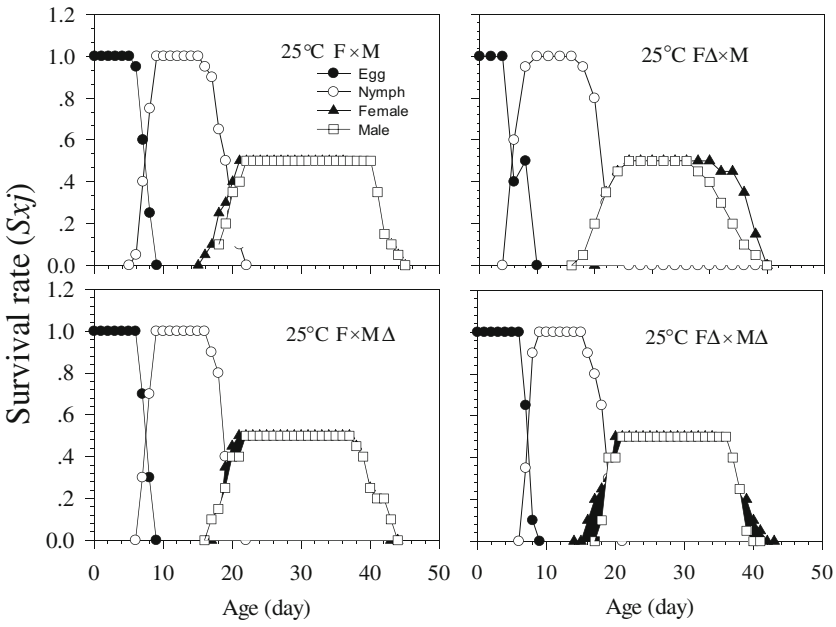

Fig. 3 Age-stage-specific survival rate (S_{xj}) of *N. lugens* under different treatment conditions. *Wolbachia*-infected males were mated with infected females (F × M), uninfected females were mated with infected males (F▽ × M), infected females were mated with uninfected males (F × M▽), infected females were mated with uninfected males

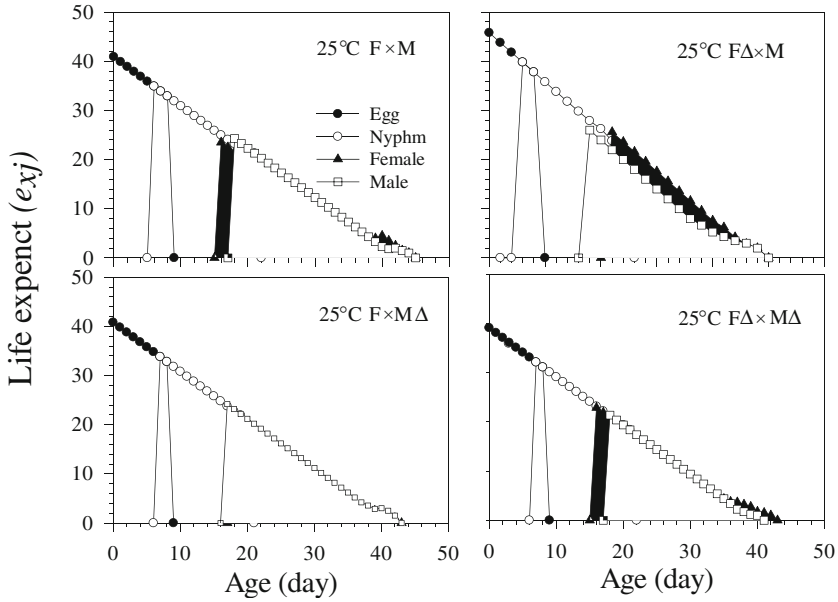

Fig. 4 Age-stage-specific life expectancy (e_{xj}) of *N. lugens* under different treatment conditions: *Wolbachia*-infected males were mated with infected females (F × M), uninfected females were mated with infected males (F▽ × M), infected females were mated with uninfected males (F × M▽), infected females were mated with uninfected males

longevity 39.15~43.95d). More importantly, the developmental time of the F▽ × M cross was shorter than that of the F × M▽ cross.

The population parameters calculated from the life table studies reveal the life-long effects of a variety of factors on the population and summarize the joint effect of the age-specific survival rate and fecundity on population growth. However, the tedious calculations inherent in life table analyses might yield errors in population parameters, which would alter their interpretation. Four treatments were used in this work: *Wolbachia*-infected males were mated with infected females (F × M) at 25 °C in the lab, uninfected females were mated with infected males (F▽ × M), infected females were mated with uninfected males (F × M▽), and infected females were mated with uninfected males (F▽ × M▽). All of the treatments in this study revealed that $R_0 > 1$ and $r > 0$. These results are consistent with the life table theory, which states that $R_0 > 1$ must always be accompanied by $r > 0$. In addition to the R_0 and r parameters, GRR values of the four treatments were also calculated. The largest GRR value was 337.3 ± 76.034 eggs/female and was observed with the F × M cross (Table 2). Because the GRR is a simple summation of the age-specific fecundity (mx) over all ages, it is easily estimated from the fecundity curve. Based on the curve shown in Fig. 1, it was found that the GRR of the F × M cross was approximately 116 eggs/female between days 11 and 23. The five values obtained for the intrinsic rate did not differ significantly.

The obligate reliance of many insects on their symbionts reveals a potential target for the biological control of devastating agricultural pests. Therefore, this study examined the importance of *Wolbachia* for host fitness with the goal of manipulating these partnerships and rendering insect pests more vulnerable to broad-scale measures of population control by targeting bacterial symbionts. A large number of manipulations have been administered to hinder the development and survival of insect pests

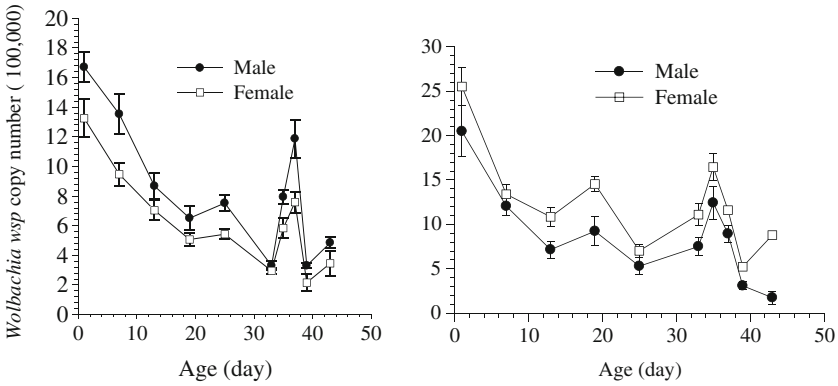

Fig. 5 *Wolbachia* density (*wsp* gene copy number) changes in two BPH strains with their age

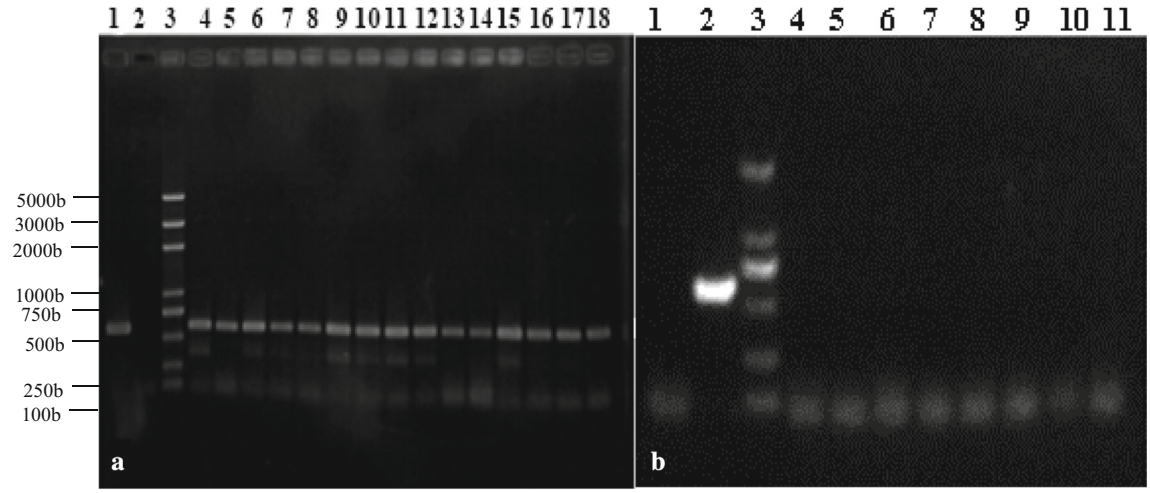

Fig. 6 PCR-based detection of infected (**a**) and uninfected (**b**) *Wolbachia* BPHs

by targeting their bacterial partners primarily through the application of antibiotics (Brownlie and Johnson 2009) and/or the disruption of the symbiont's route of transmission to the next host generation (Salem et al. 2015).

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