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Background-dependent *Wolbachia*-mediated insecticide resistance in *Laodelphax striatellus*

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Summary

Although facultative endosymbionts are now known to protect insect hosts against pathogens and parasitoids, the effects of endosymbionts on insecticide resistance are still unclear. Here we show that *Wolbachia* are associated with increased resistance to the commonly used insecticide, buprofezin, in the small brown planthopper (*Laodelphax striatellus*) in some genetic backgrounds while having no effect in other backgrounds. In three *Wolbachia*-infected lines from experimental buprofezin-resistant strains and one line from a buprofezin-susceptible line established from Chuxiong, Yunnan province, China, susceptibility to buprofezin increased after removal of *Wolbachia*. An increase in susceptibility was also evident in a *Wolbachia*-infected line established from a field population in Rugao, Jiangsu province. However, no increase was evident in two field populations from Nanjing and Fengxian, Jiangsu province, China. When *Wolbachia* was introgressed into different genetic backgrounds, followed by *Wolbachia* removal, the data pointed to *Wolbachia* effects that depend on the nuclear background as well as on the *Wolbachia* strain. However, there was no relationship between *Wolbachia* density and the component of buprofezin resistance associated with the symbiont. The results suggest that *Wolbachia* effects associated with chemical resistance are complex and unpredictable, but also that they can be substantial.

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Introduction

Wolbachia are extremely common facultative symbionts notably found in insect pests, and well known for their effects on host reproduction involving cytoplasmic incompatibility, parthenogenesis, feminization or male killing (O'Neill *et al*., 1997; Werren *et al*., 2008). In recent years, effects of *Wolbachia* other than reproductive effects have started to receive increasing attention, particularly through data collected from flies and mosquitoes. These studies have shown that some *Wolbachia* strains protect against some RNA viruses (Hedges *et al*., 2008; Teixeira *et al*., 2008; Bian *et al*., 2010; Walker *et al*., 2011; Martinez *et al*., 2014), and that some *Wolbachia* strains provide fitness benefits in terms of life history traits, nutrition and other effects (Weeks *et al*., 2007; Moriyama *et al*., 2015; Zug and Hammerstein, 2015). However, *Wolbachia* can also have the opposite effects on hosts both in terms of protection and life history benefits; for instance, a greater susceptibility to viruses has been noted for *Wolbachia* in *Spodoptera exempta* (Graham *et al*., 2012), and *Wolbachia* can have a range of deleterious effects on the life history of their hosts (Fleury *et al*., 2000; Ross *et al*., 2019).

The relationship between insecticide resistance and symbiont presence has been investigated in some studies. In *Culex pipiens*, there was a higher *Wolbachia* load in mosquitoes carrying an organophosphate resistant gene than in susceptible individuals lacking the gene, but the presence of *Wolbachia* did not affect the strength of resistance (Berticat *et al*., 2002; Duron *et al*., 2006). And in *Aedes aegypti*, the *w*Mel strain of *Wolbachia* had no impact on resistance to several insecticides, namely, the organophosphate temephos, the insect growth regulator s-methoprene, the pyrethroid bifenthrin, and the entomopathogen *Bacillus thuringiensis* var. *israelensis* (Endersby and Hoffmann, 2012). However, another endosymbiont, *Rickettsia* was associated with increased susceptibility to some insecticides in *Bemisia tabaci* (Ghanim and Kontsedalov, 2009), and a bacterial symbiont of the genus *Burkholderia* from soil, acquired by the

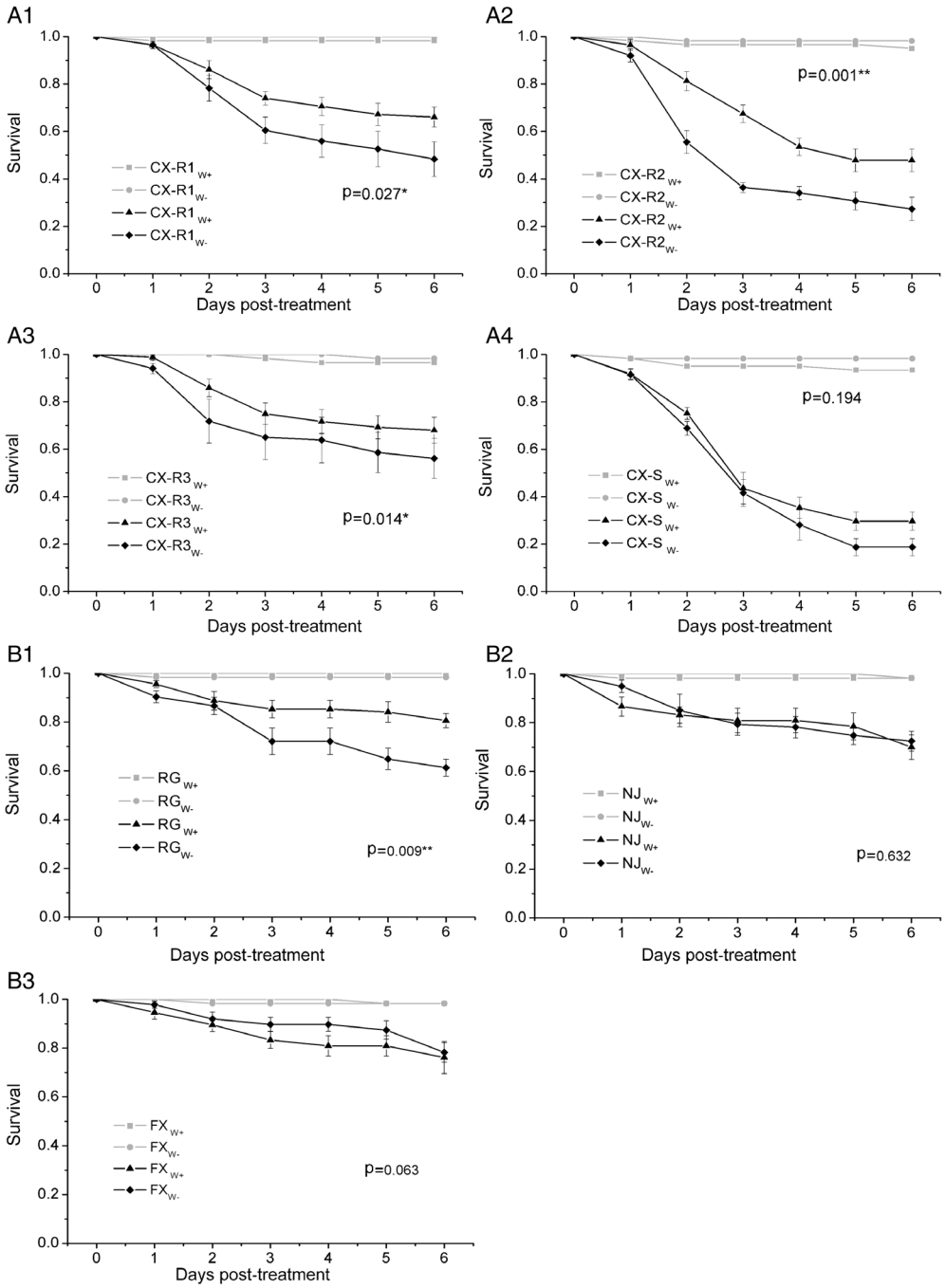

Fig. 1. Survivorship of *Wolbachia*-infected and *Wolbachia*-cured *Laodelphax striatellus* lines exposed to buprofezin (black – buprofezin; grey – water). Error bars represent standard error of the mean calculated from six (chemical) or four (water control) replicates of 15 nymphs scored for survival at each concentration. Black lines refer to survival of *L. striatellus* exposed to buprofezin, grey lines refer to survival of *L. striatellus* exposed to water.

A1–A4. Comparison of the survival of four *L. striatellus* lines from selected and control strains exposed to 200 mg l^{-1} buprofezin.

B1–B4. Comparison of the survival of lines derived from field populations from NJ, FX and RG exposed to 1000 mg l^{-1} buprofezin.

Log rank test on Kaplan–Meier curves show that the survival of *Wolbachia*-infected and *Wolbachia*-cured *L. striatellus* is significantly different among paired R1 lines ($p < 0.05$), R2 lines ($p < 0.01$), R3 lines ($p < 0.05$) and RG lines ($p < 0.01$) and is not significantly different among paired S, NJ and FX lines (all $p > 0.05$) (*$p < 0.05$, **$p < 0.01$).

bean bug *Riptortus pedestris*, degraded the pesticide fenitrothion and conferred resistance when present in the insect gut (Kikuchi *et al*., 2012).

The small brown planthopper (SBPH), *Laodelphax striatellus* (Fallén), is a notorious pest of rice crops, which not only causes direct damage by feeding, but also acts

Table 1. Effects of *Wolbachia* infection on buprofezin resistance of *Laodelphax striatellus* in Chuxiong (CX) resistance-selected populations and field populations as assessed by survival

Factors	df	Mean square	*F*	*p*
CX selected populations				
Wolbachia	1	0.20	15.22	**0.001**
Host origin	2	0.25	20.09	**0.002**
Wolbachia * host origin	2	0.01	0.35	0.766
Deviation	30	0.02		
Field populations				
Wolbachia	1	0.02	1.79	0.191
Host origin	2	0.02	1.23	0.308
Wolbachia * host origin	2	0.05	3.81	**0.034**
Deviation	30	0.01		

The degrees of freedom and the mean square are given along with the *F*-statistic and its associated probability (in bold when less than 0.05).

Table 2. Designation of lines in bioassay experiments.

Expected nuclear genetic background	Infection status	*Wolbachia* source	Line designation
Chuxiong (S strain)	+	Chuxiong	CX-S_{w+}
Chuxiong (S strain)	–		CX-S_{w-}
Fengxian	+	Fengxian	FX_{w+}
Fengxian	–		FX_{w-}
Nanjing	+	Nanjing	NJ_{w+}
Nanjing	–		NJ_{w-}
Rugao	+	Rugao	RG_{w+}
Rugao	–		RG_{w-}
Fengxian 96.9%, Chuxiong (S strain) 3.1%	+	Chuxiong	FX_W^{CX}
Nanjing 96.9%, Chuxiong (S strain) 3.1%	+	Chuxiong	NJ_W^{CX}
Fengxian 96.9%, Rugao 3.1%	+	Rugao	FX_W^{RG}
Nanjing 96.9%, Rugao 3.1%	+	Rugao	NJ_W^{RG}
Chuxiong (S strain) 96.9%, Fengxian 3.1%	+	Fengxian	CX_W^{FX}
Rugao 96.9%, Fengxian 3.1%	+	Fengxian	RG_W^{FX}
Chuxiong (S strain) 96.9%, Nanjing 3.1%	+	Nanjing	CX_W^{NJ}
Rugao 96.9%, Nanjing 3.1%	+	Nanjing	RG_W^{NJ}

as a vector of plant viruses such as the rice stripe virus (Kisimoto, 1967). Buprofezin has been one of the most used insecticides to control SBPH in China for many years, but its frequent application has resulted in resistance problems (Gao *et al*., 2007; Wang *et al*., 2008a). *Wolbachia* is a common symbiont in *L. striatellus* (Noda *et al*., 2001; Li *et al*., 2018) and we have found that susceptibility of a *Wolbachia*-infected line originating from the buprofezin-resistant strain increased when *Wolbachia* was cleared (Li *et al*., 2018; Liu and Guo, 2019). Here we tested whether *Wolbachia* in fact played a role in mediating resistance to buprofezin.

To test for resistance effects associated with *Wolbachia*, we established pairs of *Wolbachia*-infected and *Wolbachia*-cured lines with the same genetic background from insecticide-resistant and insectide-susceptible strains and field populations, and compared buprofezin susceptibility of *Wolbachia*-infected and *Wolbachia*-cured lines. We then investigated the density of *Wolbachia* in all the *Wolbachia*-infected lines. Because effects of genetic background were evident in the results, a series of backcrosses was undertaken to separate host nuclear background and *Wolbachia* strain effects on variation in resistance.

Results

Wolbachia *infection partly associates with buprofezin resistance*

To analyse whether the *Wolbachia* strain from the Chuxiong (CX) population of *L. striatellus* could protect hosts from insecticide-induced mortality, buprofezin susceptibility of the three *L. striatellus* lines infected with *Wolbachia* established from the selected strain (CX-R1, CX-R2 and CX-R3) were characterized, along with one *L. striatellus* line from the experimental buprofezin-susceptible strain (CX-S) and their paired lines without *Wolbachia* infection. In all three paired lines from the selected strain with the same genetic background from CX, there was a significant increase in susceptibility to 200 mg l^{-1} buprofezin after *Wolbachia* were removed from the *Wolbachia*-infected line (R1: $\chi_1^2 = 4.86$, $p = 0.027$, Fig. 1A1; R2: $\chi_1^2 = 11.307$, $p = 0.001$, Fig. 1A2; R3: $\chi_1^2 = 6.009$, $p = 0.014$, Fig. 1A3), whereas in paired lines from the control strain, there was no difference in susceptibility to 200 mg l^{-1} buprofezin after the removal of *Wolbachia* (*S*: $\chi_1^2 = 1.69$, $p = 0.194$; Fig. 1A4).

We next investigated resistance across *Wolbachia*-infected lines derived from field populations. SPBH with and without *Wolbachia* were challenged by spraying with a 1000 mg l^{-1} buprofezin suspension. The Rugao (RG) line (estalished from RG population of *L. striatellus*, RG line) infected with *Wolbachia* showed high survival, and the insects of the same genetic background without *Wolbachia* exhibited a significantly lower survival ($\chi_1^2 = 6.77$, $p = 0.009$, Fig. 1B1), consistent with the above results for *Wolbachia*. However, protection was not observed in the Nanjing (NJ) line (estalished from the NJ population of *L. striatellus*, NJ line) and Fengxian (FX) line (estalished from the FX population of *L. striatellus*, FX line), with no significant difference in survival between the *Wolbachia*-infected and *Wolbachia*-cured lines (NJ: $\chi_1^2 = 0.241$, $p = 0.632$, Fig. 1B2; FX: $\chi_1^2 = 3.468$, $p = 0.063$, Fig. 1B3). Comparing the susceptibility among different field populations, we found that there was no significant

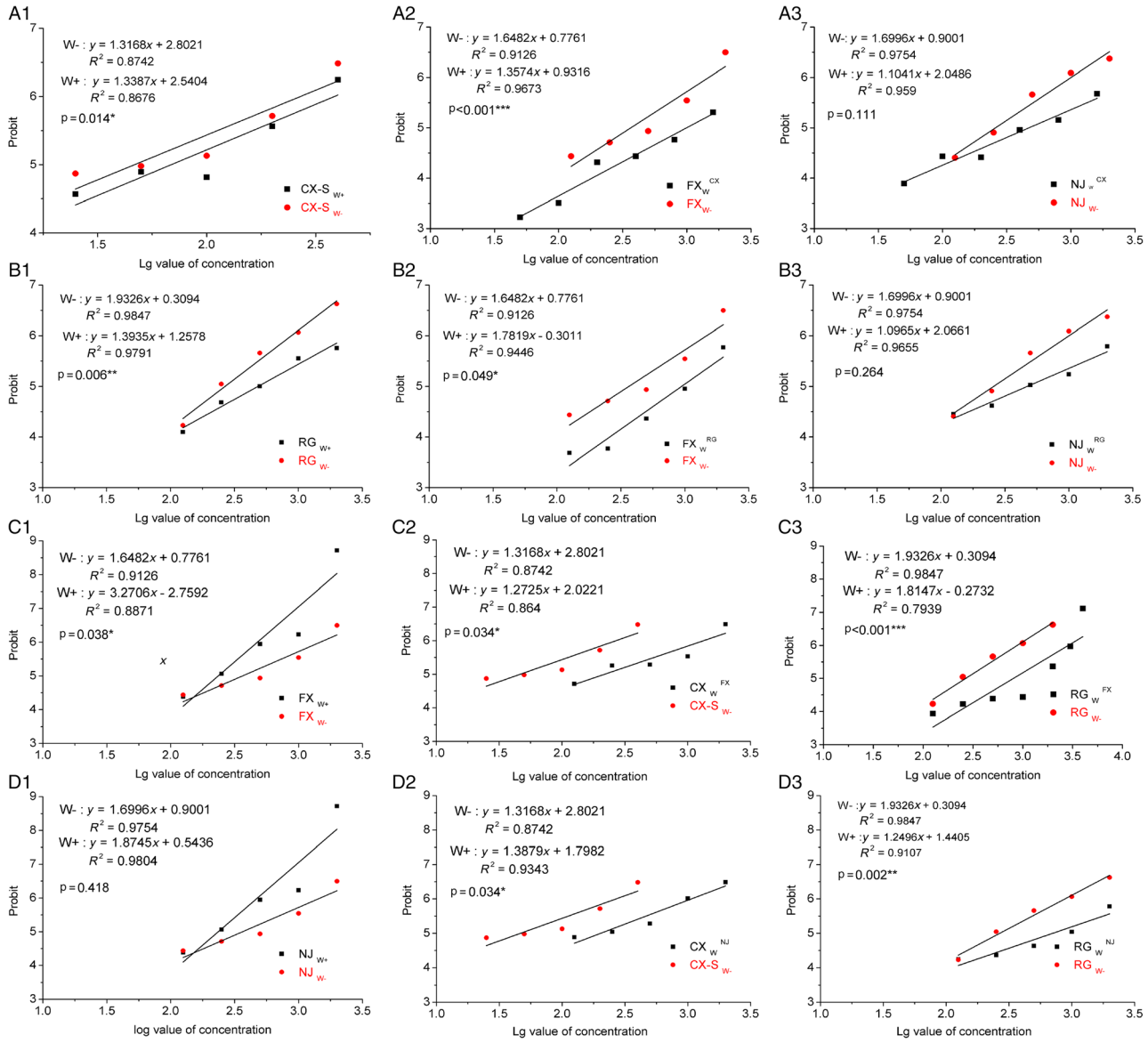

Fig. 2. Relationship between mortality (expressed as probits) and log concentration of buprofezin (mg l^{-1}) in *Wolbachia*-introgressed and *Wolbachia*-cured *Laodelphax striatellus* lines with different genetic backgrounds. Values are based on four replicates of 15 nymphs per concentration and LC_{50} values (probit = 5) were computed separately for each replicate. Lines represent linear regressions based on average data points.

A. FX and NJ lines introgressed with *Wolbachia* from CX.

B. FX and NJ lines introgressed with *Wolbachia* from RG.

C. CX and RG lines introgressed with *Wolbachia* from FX.

D. CX and RG lines introgressed with *Wolbachia* from NJ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). [Color figure can be viewed at wileyonlinelibrary.com]

differences in survival among *Wolbachia*-infected field populations ($F_{2,15} = 1.083$, $p = 0.346$), but significant differences in survival among *Wolbachia*-cured field populations ($F_{2,15} = 4.905$, $p = 0.022$).

Based on the survival rate, we further tested the interaction of host origin and infectious status in an overall analysis of data from selected populations or field populations. For selected populations from CX, there was no significant interaction, with buprofezin resistance only affected by main effects of *Wolbachia* or host origin (Table 1). For field populations, both *Wolbachia* and host origin did not affect buprofezin resistance, but a significant interaction between *Wolbachia* and host origin was found (Table 1).

Table 3. Effects of *Wolbachia* infection on buprofezin resistance of *Laodelphax striatellus* in backcrosses as assessed by LC_{50} values (log transformed for analysis).

Factors	df	Mean square	*F*	*p*
CX *Wolbachia*				
Wolbachia	1	0.56	38.94	**<0.001**
Genetic background	2	2.41	168.13	**<0.001**
Wolbachia * genetic background	2	0.02	1.68	0.214
Deviation	18	0.01		
RG *Wolbachia*				
Wolbachia	1	0.04	11.57	**0.001**
Genetic background	2	0.27	1.70	0.209
Wolbachia * genetic background	2	0.30	13.01	**<0.001**
Deviation	18	0.02		
FX *Wolbachia*				
Wolbachia	1	0.63	32.55	**<0.001**
Genetic background	2	1.14	58.67	**<0.001**
Wolbachia * genetic background	2	0.47	24.25	**<0.001**
Deviation	18	0.02		
NJ *Wolbachia*				
Wolbachia	1	0.72	48.13	**<0.001**
Genetic background	2	0.91	60.99	**<0.001**
Wolbachia * genetic background	2	0.27	17.87	**<0.001**
Deviation	18	0.02		

The degrees of freedom and the mean square are given along with the F-statistic and its associated probability (in bold when less than 0.05).

Introgression suggests a role for Wolbachia *variation and nuclear background variation in resistance*

To investigate whether the variable *Wolbachia* effects described above reflect the nuclear background or uncharacterized *Wolbachia* differences, the infection from each line was introgressed into two different genetic backgrounds (Table 2). Comparisons of lethal concentration of 50 percent individuals (LC_{50}) values showed that *Wolbachia* infections from CX and RG increased buprofezin resistance in their original hosts (CX: $t = 3.44$, df = 6, $p = 0.014$; RG: $t = 4.23$, df = 6, $p = 0.006$; Figs. 2A1 and B1, S2A and B), and also when *Wolbachia* was introgressed into a FX background (FX_W^{CX}: $t = 9.31$, df = 6, $p < 0.001$; FX_W^{RG}: $t = 2.47$, df = 6, $p = 0.049$; Fig. 2A2 and B2) even though native *Wolbachia* decreased resistance in this background (FX: $t = 2.65$, df = 6, $p = 0.038$; Figs. 2C1 and S2C). Although *Wolbachia* infections from NJ did not affect resistance in their original hosts ($t = 0.86$, df = 6, $p = 0.418$; Figs. 2D1 and S2D), they did when *Wolbachia* was introgressed into other host backgrounds (CX: $t = 2.74$, df = 6, $p = 0.034$; RG: $t = 5.28$, df = 6, $p = 0.002$; Figs. 2D2 and D3, S2D) which suggested resistance mediated by native *Wolbachia*. In contrast, the *Wolbachia* infections from FX decreased the LC_{50} of buprofezin in their original hosts (Figs. 2C1 and S2C), but increased it following introgression of *Wolbachia* from FX to CX and RG hosts (CX: $t = 2.75$, df = 6, $p = 0.034$; RG: $t = 12.55$, df = 6, $p < 0.001$; Figs. 2C2 and C3, S2C).

To further test whether *Wolbachia* affected insecticide resistance differently on the genetic background of the hosts, we tested the interaction of genetic background and infection status in an overall analysis of data from each backcross comparison (Table 2). For *Wolbachia* from CX, no significant interaction was found (Table 3), with buprofezin resistance only affected by main effects of *Wolbachia* or genetic background (Fig. 2A). For *Wolbachia* from RG, a significant interaction between symbiont and genetic background was found (Fig. 2B, Table 3). For the other two comparisons, there were significant main effects as well as interaction effects (Table 3). Overall LC_{50} data from the parental strains are consistent with the mortality data, highlighting differences in *Wolbachia* effects among strains, and the LC_{50} data highlight that strain differences are both related to the *Wolbachia* (or other maternal effect) and also to the nuclear background of the strain (Table 3).

Variation in Wolbachia *density is not related to resistance*

When *Wolbachia* from CX was introduced to FX and NJ populations of *L. striatellus*, line differences were evident ($F_{2,11} = 33.760$, $p < 0.0001$; Fig. 3A), with *Wolbachia* density significantly increasing in the NJ_W^{CX} line, but not changing in the FX_W^{CX} line. When *Wolbachia* from RG was introduced to FX and NJ populations, line differences were marginally non-significant ($F_{2,11} = 3.900$, $p = 0.06$; Fig. 3B), with *Wolbachia* density significantly decreasing in the FX_W^{RG} line, and not being affected in the NJ_W^{RG} line. Line differences were also detected ($F_{2,11} = 19.794$, $p = 0.001$; Fig. 3C) when *Wolbachia* from

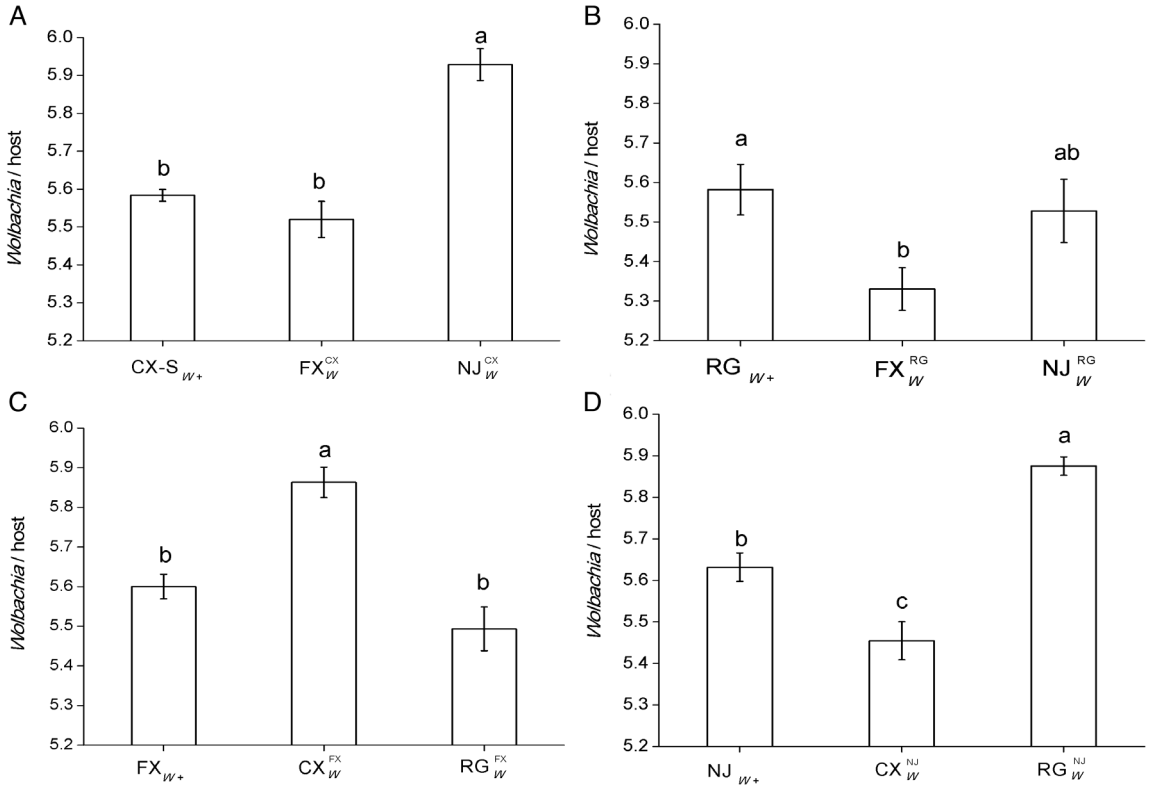

Fig. 3. Comparison of *Wolbachia* density in the *Wolbachia*-introgressed and original lines of *Laodelphax striatellus*.
A. CX line and FX, NJ introgressed lines.
B. RG line and FX, NJ introgressed lines.
C. FX line and CX, RG introgressed lines.
D, NJ line and CX, RG introgressed lines.
The *Wolbachia* density was expressed as copy number of *wsp* gene per invidividual.

FX was introduced to the CX and RG populations, with *Wolbachia* density increasing in the CX_W^{FX} line. Finally, there were line differences ($F_{2,11} = 35.710$, $p < 0.0001$; Fig. 3D) when *Wolbachia* from NJ was introduced to the CX and RG populations of *L. striatellus*, with *Wolbachia* density decreasing in the CX_W^{NJ} line, and increasing in the RG_W^{NJ} line.

These differences in *Wolbachia* density did not associate clearly with changes in buprofezin resistance (Fig. 4); in several cases such as in the *Wolbachia* from FX in the FX, CX and RG backgrounds, there was no direct connection between density in the crosses and the extent to which *Wolbachia* increased resistance (Fig. 3C). *Wolbachia* increased resistance even when present at a relatively low density, and vice versa.

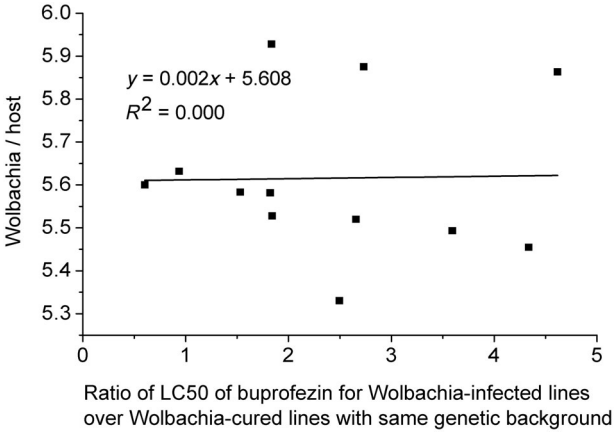

Fig. 4. Relationship between density and protection of *Wolbachia* in *Laodelphax striatellus*.
No significant relationship was found between *Wolbachia* density and the ratio of LC_{50} values for infected individuals over uninfected individuals.

Discussion

While *Wolbachia* has been shown to provide insects with protection from some entomopathogens including viruses and fungi (Panteleev *et al*., 2007; Teixeira *et al*., 2008; Osborne *et al*., 2009), this is one of the few cases where *Wolbachia* has been implicated in providing protection to a chemical insecticide. In mosquitoes, no obvious effects of *Wolbachia* on resistance were found (Endersby and

Hoffmann, 2012). Results from other endosymbionts seem to point to greater susceptibility to insecticides rather than greater resistance, such as in whiteflies where the presence of *Rickettsia* increased susceptibility to four out of six insecticides (Kontsedalov *et al*., 2008), and where double infections of *Rickettsia–Arsenophonus* and *Wolbachia–Arsenophonus* which carried higher amounts of symbionts overall also showed increased susceptibility to three out of the six insecticides when compared to strains infected with *Arsenophonus* alone (Ghanim and Kontsedalov, 2009). Finally, in brown planthoppers a distinct strain of *Arsenophonus* decreased insecticide resistance (Pang *et al*., 2018).

In our study we found that *Wolbachia* was associated with resistance to buprofezin in some lines of SBPH, but also that this association was not always evident and depended on the combination of *Wolbachia* and nuclear backgrounds tested. Although the pattern appeared clear in lines from CX regardless of their selection history, we only established this pattern in one of three *Wolbachia*-infected lines from Jiangsu province. This line difference had a complicated basis involving either the nuclear or *Wolbachia* backgrounds. We could not distinguish between *Wolbachia* strains based on a standard gene set (Fig. S1), but the different behavior of the *Wolbachia* strains may reflect other genomic differences that were not detected here and that likely will require a full genomic comparison of the *Wolbachia* which may reveal other DNA-based differences (Chrostek and Teixeira, 2015). In addition, there might be an interaction between *Wolbachia* and other maternally inherited components, notably mitochondria, which hitchhike along with *Wolbachia* in populations as they spread (Hale and Hoffmann, 1990) and can interact with *Wolbachia* in influencing life history traits (Dean, 2006).

Buprofezin has been developed as an insecticide for more than 30 years (Nagata, 1986), and it has been intensively used against rice planthoppers in agricultural fields in China for more than 20 years. Buprofezin resistance in SBPH is serious in China in recent years, and mechanisms have been attributed to evolutionary changes in pest insect genomes such as alteration of drug target sites, up-regulation of degrading enzymes, and enhancement of drug excretion (Gao *et al*., 2007; Wang *et al*., 2008a; Wang *et al*., 2008b). A single P450 gene, *CYP6CW1* plays a role in the resistance of *L. striatellus* to buprofezin (Zhang *et al*., 2012). We have found that the RG population, which had the longest history of buprofezin exposure, showed significantly higher survival in *Wolbachia*-infected individuals, and it was also the only population to show an effect of the *Wolbachia* infection and P450 expression levels. However, we have not found this gene to be consistently upregulated in comparisons of cured and *Wolbachia*-infected lines that expressed increased resistance versus no effect (Fig. S3). We also failed here to show an association between *Wolbachia* density and resistance effects, even though density has to some extent been previously associated with fitness-related phenomena in *Wolbachia* (Hoffmann *et al*., 2015), including a correlation between the density of *Wolbachia* and the extent of antiviral protection in *Drosophila*: the three *Wolbachia* strains of *w*Mel, *w*Ri and *w*Au, which provide strong antiviral protection, were significantly more abundant than the strains of *w*Ha and *w*No which did not provide protection (Osborne *et al*., 2009).

The mechanism involved in insecticide resistance related to *Wolbachia* remains unclear; it might be related to changes in metabolism, expression changes particularly in genes involved in detoxification, or *Wolbachia*-related triggering of immune responses in the insect hosts. The association between resistance and *Wolbachia* appears complex given the line differences in *Wolbachia* effects. Although these line differences may be mediated by other symbionts, we did not find differences among lines for the presence of several symbiont groups previously described from planthoppers. *Wolbachia*-associated resistance adds yet another relevant factor when considering the distribution of *Wolbachia* within and across pest populations. Chemical insecticides are stressors that insect pests often face, and there are likely to be other cases where endosymbionts are associated with resistance. Such effects may need to be considered when introducing *Wolbachia* into natural populations for pest and disease control.

Conclusions

We found that *Wolbachia*, common facultative symbionts found in insect pests, can be associated with increased resistance to the widely used insecticide buprofezin in the SBPH in some genetic backgrounds while having no effect in others. The data supported the notion that *Wolbachia* effects on insecticide resistance could depend on the nuclear background as well as on the *Wolbachia* strain or other maternal factors, which suggest that *Wolbachia* effects on chemical resistance are complex and unpredictable, but also that they can be substantial.

Methods

SBPHs and Wolbachia

Wolbachia-infected SBPHs were obtained from field populations in RG, FX, NJ, Jiangsu province, China and CX, Yunnan province, China. The CX population had been collected from Yunnan in July 2001 (at that time, buprofezin was rarely used), and reared in the laboratory

without contact with any insecticides. A buprofezin-resistant strain was developed from this susceptible strain through 32 generations of selection (Zhang *et al*., 2012). Both the buprofezin-resistant strain and buprofezin-susceptible strain from the CX population were used in experiments. The other three populations were collected in May, 2014. The insecticide exposure history was different among the three regions, buprofezin has been widely used in RG since 1990 and in NJ since 2001, while it is rarely used in FX. All populations were infected by *Wolbachia*.

All the strains and lines were reared at the same density on rice seedlings hosts under the same environmental conditions. For maintaining each line, about 2000 7-day-old seedlings were randomly assigned to lines in a cage (length 29.5 cm × width 20 cm × height 18 cm), and there were 1800 *L. striatellus* individuals in each cage. At the time of the experiment, we set up multiple cages per line to rear the insects, so the experimental subjects were combined across more than one cage. Seedlings were replaced by new seedlings every week. The insects were reared at a constant temperature of 27 (±1)°C with a photoperiod of 14 : 10 h light : dark.

Preparation of Wolbachia*-infected and Wolbachia-cured lines*

Before the establishment of lines, three isofemale lines of *L. striatellus* with different levels of resistance from the buprofezin-selected strain were established. Each line was developed from a pair of newly emerged female and male adults randomly selected from the resistant strain to minimize variation in genetic background within each strain. The same procedure was followed to establish lines from one buprofezin-susceptible strain from the Yunnan population and the three field populations from Jiangsu.

In each line, the presence of all symbionts known to be potentially present in planthoppers, including *Wolbachia*, *Arsenophonus*, *Cardinium hertigii*, *Acinetobacter*, *Chryseobaterium*, *Serratia*, *Arthrobacter*, and *Spiroplasma*, was checked in adult insects as previously described (*Wolbachia*: Zhou *et al*., 1998; *Arsenophonus* Thao and Baumann, 2004; *Cardinium*: Nakamura *et al*., 2009; *Acinetobacter*: Vanbroekhoven *et al*., 2004; *Chryseobaterium*: Alonso *et al*., 2007; *Serratia*: Zhu *et al*., 2008; *Arthrobacter*: Koch *et al*., 1994; *Spiroplasma*: Sanada-Morimura *et al*., 2013). Both the females and males used to establish lines were only infected with *Wolbachia*. Their offspring were used to identify the *Wolbachia* infection through molecular markers (see further).

To generate SBPH lines free of *Wolbachia*, each *Wolbachia*-infected planthopper line was treated with 0.2% tetracycline by oral ingestion (Noda *et al*., 2001). One hundred newly hatched SBPH were fed rice seedlings sprayed with a 0.2% tetracycline solution. The rice seedlings were treated with antibiotics every 3 days, and new seedlings were supplied at these times. SBPH were treated with antibiotics over the entire nymphal stage for two generations, and newly emerged SBPH were then paired. After egg production, the presence of *Wolbachia* was checked in adult insects. Only female and male offspring without *Wolbachia* were kept. Before bioassays were performed, insects were reared for more than five generations without exposure to antibiotics. The symbiont infection status of the *Wolbachia*-infected and *Wolbachia*-cured lines was reconfirmed prior to experiments by diagnostic polymerase chain reaction (PCR; as specified above) and quantitative PCR (as specified below).

A further eight massbred lines were created by introgressing *Wolbachia* infections from each of the three locations into the genetic background of the other locations, by mating 50+ virgin infected females with an equivalent number of *Wolbachia*-cured males, and then using repeated backcrossing for five generations to ensure that the nuclear background was (a predicted) 96.9% of the one from which insects were sourced for backcrossing (Kriesner *et al*., 2016). The backcrosses were only done with males that were from a stock treated with antibiotics. Males only transfer the nuclear genome, not the endosymbionts or mitochondrial genomes. Thus the 'native' components remain intact with respect to the *Wolbachia* and associated backgrounds. All mass-bred lines are listed in Table 2.

Evaluation of buprofezin resistance

Wolbachia-cured and *Wolbachia*-infected lines of *L. striatellus* were used to compare susceptibility to buprofezin (98%, Jiangsu Anpon Electrochemical, China) using the rice seedling dipping method (Wang *et al*., 2008a). Before the bioassay, to validate the *Wolbachia* infection of the lines, at least 20 individuals were randomly collected from each tested line for diagnostic detection of *Wolbachia*. To test whether there was an effect of tetracycline treatment on gut bacteria of *L. striatellus*, 10 individuals were randomly collected from each tested line, and diagnostic detection of the gut bacteria including *Enterobacter* sp. and *Enterococcus* sp. was conducted using previously described methods (Xia *et al*., 2018).

In an initial assay to compare susceptibility of lines to buprofezin, all paired *Wolbachia*-cured and *Wolbachia*-infected lines prior to introgression were treated with one diagnostic concentration of buprofezin to compare mortality. In a later assay, the LC_{50} values of all introgressed

lines were estimated based on a series of five to six concentrations of buprofezin. In the bioassay procedure, five rice seedlings were immersed in buprofezin solution for 10 s, before the plants were dried at 25 (±1)°C. The seedlings were placed in a plastic cup, and about 15 third instar nymphs were added. For each line, nymphs treated with distilled water were used as controls. All experiments were carried out at 27 (±1)°C and with a photoperiod of 14 : 10 h light : dark. Insect mortality was recorded every 24 h over a period of 144 h. For survivorship comparisons based on a single concentration (lines from CX: 200 mg l^{-1}, lines from other sites: 1000 mg l^{-1}), each line treated with buprofezin was replicated six times (six cups), while each line treated with water was replicated four times (four cups). For LC_{50} comparisons, there were four replicates (four cups) at each concentration and four LC_{50} estimates associated with *Wolbachia* source were obtained from linear regressions of log concentration against mortality (expressed as probits).

Analysis of Wolbachia *density*

Real-time quantitative PCR was performed with an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) to measure the density of *Wolbachia*. For each line, a total of 30 third instar nymphs of *L. striatellus* were collected as one sample, and the DNA was extracted with a Wizard® Genomic DNA Purification Kit (Promega). The primers for the reaction were the sense primer (*Wsp*-F) 5′-ATGTAACTCCAGAAATCAAACTC-3′ and the antisense primer (*Wsp*-R) 5′-GATACCAGCATCATCCTTAGC-3′. The 20 μl reverse transcription PCR (RT-PCR) reaction system consisted of 10 μl of SYBR® Premix Ex Taq (Tli RNaseH Plus) (2×) (Takara, Japan), 0.4 μl of forward and 0.4 μl reverse primers, 0.4 μl of ROX Reference Dye, 2 μl of DNA and 6.8 μl of ddH_2O. The RT-PCR conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s, and then 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The standard curve of real-time fluorescent quantitative PCR of the *Wolbachia wsp* gene was built to determinate *Wolbachia wsp* gene copy number as described previously for *L. striatellus* (Zhou *et al*., 2010). Note that no host gene was included but individuals were compared at the same life stage and had been reared under the same conditions. Three technical replicates were carried out for each sample, and four biological replicates were run for each line.

Statistics

All data were checked for normality and homogeneity of variances by Shapiro–Wilk tests before analysis, and LC_{50} values were log transformed to reduce differences in variance between treatments. Survival curves between *Wolbachia*-infected and *Wolbachia*-cured lines in the presence of buprofezin were compared using Kaplan–Meier analyses and log-rank tests. LC_{50} of paied lines (*Wolbachia*-infected and *Wolbachia*-cured line with the same host nuclear background) were compared by *t* tests, and LC_{50} of all lines with the same *Wolbachia* source and paired *Wolbachia*-cured lines were compared by two-way analysis of variance (ANOVA), with *Wolbachia* and genetic background as the factors in the analysis. Density among different lines was also compared with ANOVAs. Pearson's correlation was computed to test the association between the ratio of LC_{50} values for infected individuals over uninfected individuals and *Wolbachia* density. IBM Statistics (SPSS 19.0) software was used for these statistical analyses.

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Author contributions

H.G. conceived the experiments. H.G. and X.L. designed the experiments. Y.L., N.W. and Y.Z. collected the data. H.G., Y.L. and A.H. analysed the data. H.G., A.H. and Y.L. wrote and reviewed the manuscript.

Data availability statement

We agree to archive the data associated with this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information

Fig. S1. A standard gene set.

Fig. S2. Relationship between mortality and log concentration of buprofezin.

Fig. S3. Relative expression of a P450 gene (CYP6CW1).