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Wolbachia infection affects female fecundity in *Drosophila suzukii*

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Abstract

Bacteria in the genus *Wolbachia* are intracellular symbionts widespread in several arthropod species that majorly cause reproductive alterations in their hosts, but may also display a variety of other beneficial or negative interactions. In this study, an antibiotic-treated *Wolbachia*-free line of *Drosophila suzukii* Matsumura (Diptera Drosophilidae) was created in the laboratory for mating with the naturally *Wolbachia*-infected flies. Crossing experiments on two consecutive fly generations between antibiotic-treated and untreated individuals were carried out to evaluate the effect of *Wolbachia* on *D. suzukii*. The results obtained showed no difference in the vitality of parental individuals; nevertheless a reduction of 30-50% in emerging progeny abundance was recorded in all crosses when females were cured of *Wolbachia*. These results suggest a mutualistic association between *Wolbachia* and *D. suzukii*, resulting in increased female fecundity. Further research on this symbiotic interaction could have promising implications for developing symbiotic strategies for the containment of *D. suzukii*.

Key words: reproductive manipulator, spotted-wing drosophila, crossing experiments, progeny, symbiosis.

Introduction

In recent years, many kinds of symbiotic relationships between insects and bacteria residing in their body have been uncovered and characterised. One of the most common among insects is the interaction with reproductive manipulators, and especially with the α-Proteobacterium *Wolbachia* (Bourtzis, 2008). *Wolbachia* is transmitted through the egg cytoplasm and commonly causes reproductive modifications in its hosts such as parthenogenesis, feminisation, male killing and cytoplasmic incompatibility (CI) (Coscrato *et al.*, 2009; Sarakatsanou *et al.*, 2011). Moreover, many other roles have been reported to be played by *Wolbachia*. Facultative or obligate mutualism, including increased fecundity and longevity, nutritional supply, or protection against pathogens were recently reviewed by Zug and Hammerstein (2015). Detrimental effects have been recorded as well, such as the lifespan reduction observed for the *w*MelPop strain (Chrostek and Teixeira, 2015).

Host-*Wolbachia* interactions have been deeply investigated in the classic insect models of the genus *Drosophila*. A number of different effects caused by *Wolbachia* were documented in *Drosophila* species, including the induction of CI, male killing, increased fecundity, protection from viral infection, behaviour manipulations (Weeks *et al.*, 2007; Werren *et al.*, 2008), and virulence (Chrostek and Teixeira, 2015).

Among the more than 1,500 *Drosophila* species which are currently known in the world (Walsh *et al*., 2011), *Drosophila suzukii* Matsumura (Diptera Drosophilidae) is one of the few species causing economic damage to crops (Lee *et al.*, 2011), as it can oviposit in healthy fruits (Grassi and Pallaoro, 2012). The long, serrated ovipositor of females, containing many sclerotised teeth, enables females to lay eggs on a range of stone, pome and wild fruit plants (Hauser, 2011; Harris *et al*., 2014; Lee *et al*., 2015). *D. suzukii* is native to South-East Asia and was first introduced into North America in 2008 (Beers *et al.*, 2011) and subsequently into Europe (Calabria *et al.*, 2012). Within a few years, this fly rapidly spread into many countries of both continents (CABI, 2015). Especially in the USA, Canada, Italy and France, the presence of this pest has caused sensible economic losses of cherries, strawberries and other soft fruit crops (Lee *et al*., 2011; Cini *et al.*, 2012). The use of insecticides alone has been demonstrated to be unsuitable for the control of *D. suzukii* due to its high reproduction rate, its extreme rapidity of infestation and the difficulty respecting the maximum residue limit on fruits (Gargani *et al.*, 2013).

Recently the genome of *Wolbachia* endosymbiont from *D. suzukii* (*w*Suzi) was sequenced, and a close relatedness was recorded with the genome of the *w*Ri strain allied to *Drosophila simulans* Sturtevant (Siozios *et al.*, 2013). However, at present, little information is available concerning the effects induced by *Wolbachia* on *D. suzukii.* In American populations, the influence of *Wolbachia* on fly fecundity was suggested by Tochen *et al*. (2014), whereas decreased fecundity of *Wolbachia*-infected individuals was reported by Hamm *et al*. (2014). Conversely, nothing is known about effects on European populations harbouring *Wolbachia*. In this study, we investigated the infection status of Italian *D. suzukii*, along with examining the role played by this α-Proteobacterium.

Materials and methods

Insect material and molecular analyses

The *Wolbachia*-infected population of *D. suzukii* was originally collected from blueberries and raspberries in orchards of the Cuneo province, Piedmont (North-West Italy) in summer 2011. *Wolbachia* prevalence in this population was assessed on 60 adults (30 males and 30 females) by specific PCR assays. DNA extraction was carried out on single whole insect bodies by modified

sodium dodecyl sulphate-proteinase K-cethyltrimethyl ammonium bromide treatment (Gonella *et al*., 2012). Detection of *Wolbachia* was performed by amplifying *Wolbachia* 16S rRNA gene with the W-Spec f/r primer pair as previously described (Werren and Windsor, 2000).

The *Wolbachia*-infected population was reared until 2012 on organic fruits (strawberries, blueberries, grapes and kiwi fruits) inside cages (30 × 30 × 30 cm) at 25 ± 1 °C, 65 ± 5% RH and 16L:8D photoperiod. At the beginning of 2012, insects were maintained for four discrete generations on a standard medium to allow their adaptation before antibiotic treatment. The medium contained 15 g l^{-1} sucrose, 10 g l^{-1} soy flour, 17 g l^{-1} dead yeast, 71 g l^{-1} maize flour and 5.6 g l^{-1} agar. Propionic acid (4.7‰) and a vitamin mixture (2.5‰) were added to the medium. *D. suzukii* reared on such medium were maintained in climatic chamber at 25 ± 1 °C, 65 ± 5% RH and 16L:8D photoperiod.

Antibiotic treatment

After insect adaptation to the feeding medium about 2,000 individuals were obtained; half of the specimens were taken for antibiotic treatment to create a *Wolbachia*-cured line, whereas the rest of the flies were continually reared on antibiotic-free medium to preserve *Wolbachia*-infected specimens.

To obtain the *Wolbachia*-cured line, flies were maintained on a medium provided with tetracycline [final concentration 0.3 mg ml^{-1} (0.03%)] for five consecutive generations. Successively, the new descendants were reared for two discrete generations on the standard medium without antibiotic before the beginning of crossing experiments, to avoid any possible effect of the antibiotic on the flies' fitness (Fry *et al*., 2004). Removal of *Wolbachia* was confirmed by means of specific PCR on DNA extracted from 20 flies (10 males and 10 females).

Crossing experiments for fecundity evaluation

At the end of artificial medium adaptation, individuals from the tetracycline treatment as well as untreated flies were taken to be used for crossing experiments. Pupae from each of the rearings were singly isolated until adult emergence in order to obtain virgin females. Newly emerged adults (<5 days old) were collected for crossing trials.

An initial experiment (experiment 1) was performed to evaluate the vitality and fecundity of flies in the eighth generation, through crosses between tetracycline-treated and *Wolbachia*-infected specimens (treated male × treated female, T × T; untreated male × untreated female, U × U; untreated male × treated female, U × T; treated male × untreated female, T × U). For each cross, six individuals (three males and three virgin females) were kept for 12 days in vials containing 15 ml of medium. After this period, parental flies were removed and their mortality rates were recorded; vials were left for up to 40 days for offspring emergence. The mean number of newly-emerged flies per *D. suzukii* female was calculated and the sex ratio of the offspring were counted. Each cross was replicated 30 times.

A second experiment (experiment 2) was carried out to study the vitality and fecundity of insects in the following generation, in order to assess the viability of the progeny obtained from experiment 1. Therefore, the same four cross trials were applied to the newly-emerged flies from T × T and U × U lines. Six adults (three males and three virgin females) that emerged from each cross were reared on 15 ml of medium for 12 days. Afterwards, specimens found alive were removed and the mortality rates registered. The mean number of flies per *D. suzukii* female and sex ratio of the new generation were counted. Each cross was replicated 12 times.

In both experiments, mortality data were analysed by one-way ANOVA. Moreover, as progeny counts often had high variability, a non-parametric test (Kruskal-Wallis test) was performed on these records in both experiments, and individual groups were compared using the Mann-Whitney U test (Hoffman, 1988). Finally, sex ratios were analysed by χ^2 test. Statistical analyses were carried out with SPSS version 20 (Chicago, Illinois, USA).

Results

Wolbachia was proven to consistently infect our field collected *D. suzukii* population. PCR assays showed that more than 90% of flies were positive for this bacterium (average infection rate of 91.67%). The percentage of positive specimens was similar for males and females: the average infection rates were 93.33% and 90.00% for males and females, respectively. Nevertheless, *Wolbachia* was never detected in cured specimens, confirming the efficacy of antibiotic treatment. Hence, these two lines were used for the crossing trials.

Insects used in the two cross experiments were first checked for an evaluation of mortality rates. Table 1 shows the mortality rate in the two cross experiments. In the first trial the mortality of parental individuals after 12 days was about 35%. Only for T × U crosses a higher mortality was recorded (51.67%). However, no significant differences were observed among treatments (one-way ANOVA: df = 3, 116; F = 2.081, P > 0.05). In the

Table 1. Mortality at the end of 12-day long oviposition trials of *Wolbachia*-infected and tetracycline treated *D. suzukii* used in the two crossing experiments. There were no significant differences between the treatments in both experiments (one-way ANOVA; P > 0.05).

Exp.	Treatment (male × female)	% mortality	P value
1	U × U	38.89 ± 6.26 (70/180)	0.107
1	T × T	32.22 ± 6.19 (58/180)	
1	U × T	34.44 ± 6.03 (62/180)	
1	T × U	51.67 ± 5.61 (93/180)	
2	U × U (progeny)	38.89 ± 9.25 (28/72)	0.133
2	T × T (progeny)	38.89 ± 10.33 (28/72)	
2	U × T (progeny)	59.72 ± 10.35 (43/72)	
2	T × U (progeny)	66.67 ± 10.86 (48/72)	

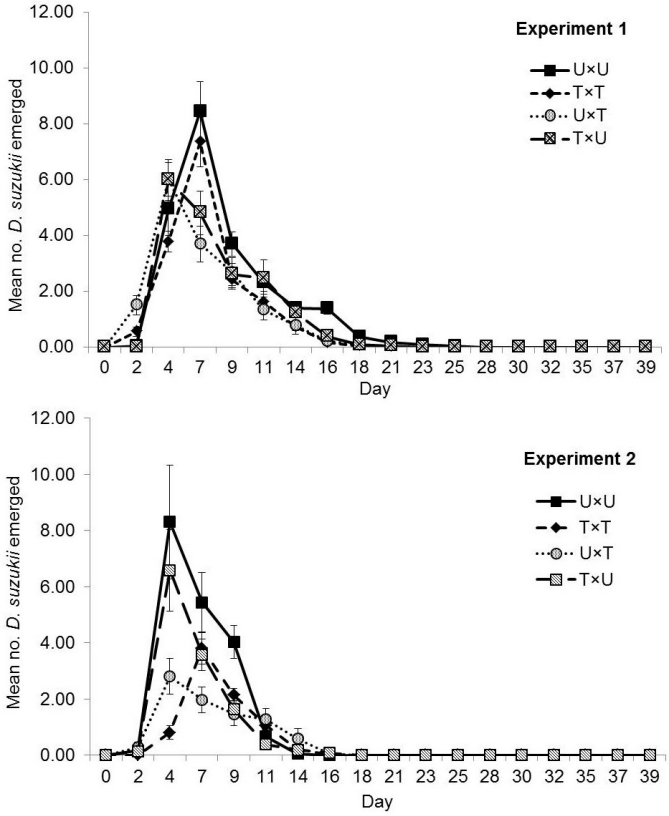

Figure 1. Trend of emergence of *D. suzukii* offspring for 40 days after the removal of parentals in the four treatments during experiment 1 and experiment 2. The average number of emerged adult per female was calculated for each day; standard errors are provided. U × U: untreated male × untreated female; T × T: treated male × treated female; U × T untreated male × treated female; T × U: treated male × untreated female.

second experiment, an equal mortality rate was found for the progeny derived from T × T and U × U (38.89%). Instead, the mortalities of progenies derived from U × T and T × U were higher (59.72% and 66.67%, respectively). Yet, no significant differences were registered (one-way ANOVA: df = 3, 44; F = 1.965, P > 0.05).

The prolificacy of flies involved in each crossing experiments was checked as well. In each treatment, the first specimens emerged two days after the removal of parental individuals (14 days after the beginning of ovipositions). Subsequently a high peak of emergences was recorded at 4-7 days after the removal of parentals (16-19 days after the beginning of ovipositions). Finally the number of emergences decreased, and stopped after 25 and 18 days in experiment 1 and 2, respectively (37-30 days after the beginning of ovipositions) (figure 1). Striking differences were found in the number of emerged flies in both experiments (figure 2). In experiment 1, a significantly higher number of offspring was recorded in the U × U control (with a mean of 22.87 ± 1.66 emerged flies) than in crosses T × T and U × T, where the mean numbers of emerged flies were 16.69 ± 1.34 and 16.49 ± 2.01, respectively. Conversely, in the T × U cross (with a mean of 19.03 ± 2.27 emerged flies) no significant difference with the other treatments was observed (Kruskal-Wallis test: df = 3; χ^2 = 7.904; P < 0.05). No deviations from a 1:1 sex ratio were detected, indicating that *Wolbachia* does not cause male-killing in *D. suzukii* [χ^2 test: df = 1; χ^2 = 0.136; P > 0.05 (U × U); df = 1; χ^2 = 0.001; P > 0.05 (T × T); df = 1; χ^2 = 0.001; P > 0.05 (U × T); df = 1; χ^2 = 0.405; P > 0.05 (T × U)], (table 2).

Similar results were also found in experiment 2. The highest number of adults emerged from insects derived from the U × U control (mean of 18.67 ± 2.77 flies emerged) followed by the T × U progeny (mean 12.61 ± 1.54 flies). A lower number of specimens was detected in the T × T and U × T progeny (mean of 7.75 ± 0.58 and 8.44 ± 1.85 flies emerged, respectively), with a highly significant difference between these two crosses and the control and between T × T and T × U progeny. Instead, no significant difference was found between T × U progeny and the remaining two crosses (Kruskal-Wallis test: df = 3; χ^2 = 14.655; P < 0.01). The sex ratio obtained in each cross can be considered 1:1 [χ^2 test: df = 1; χ^2 = 0.673; P > 0.05 (U × U); df = 1; χ^2 = 0.032; P > 0.05 (T × T); df = 1; χ^2 = 0.000; P > 0.05 (U × T); df = 1; χ^2 = 0.235; P > 0.05 (T × U)] (table 2).

Discussion and conclusions

This study aimed to investigate the influence of *Wolbachia* on *D. suzukii*, considering the high infection incidence found in our population of this fly. Experiments performed on two consecutive generations showed similar values of offspring emergence compared to previous observations on flies reared in similar conditions

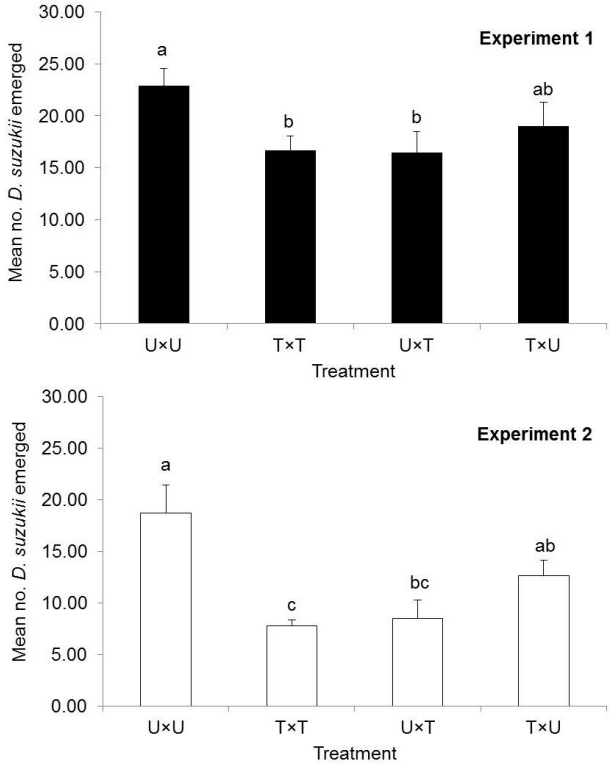

Figure 2. Mean number of *D. suzukii* emerged in the four treatments for experiment 1 and experiment 2. U × U: untreated male × untreated female; T × T: treated male × treated female; U × T untreated male × treated female; T × U: treated male × untreated female. Different letters above histogram bars indicate significant differences [Mann-Whitney U test following Kruskal-Wallis test, $P < 0.05$ (Experiment 1); Mann-Whitney U test following Kruskal-Wallis test, $P < 0.01$ (Experiment 2)].

(Tochen *et al.*, 2014); furthermore we demonstrated that crosses involving females treated with tetracycline showed a reduction of 30-50% in progeny abundance. These data suggest that female fecundity is beneficially influenced by *Wolbachia*. Mutualistic associations where *Wolbachia* positively affects host lifespan and fecundity have been largely documented in insects (Vavre *et al.*, 1999; Dobson *et al.*, 2001; Wade and Chang, 1995) and in filarial nematodes (Bandi *et al.*, 1999). Within the genus *Drosophila*, such interactions are also common. Weeks *et al.* (2007) recorded a reduction of 10% in *D. simulans* fecundity on uninfected females than in infected specimens under laboratory conditions. On the other hand, studies on American populations of *D. suzukii* demonstrated a *Wolbachia*-induced reproductive disadvantage, probably balanced by other beneficial functions (Hamm *et al.*, 2014). Moreover that study highlighted large heterogeneity of infection frequencies, always below 60%, whereas in our population *Wolbachia* prevalence was close to 100%; different host and symbiont genotypes may be related to variable effects, as observed elsewhere. For example, some infected lines of *D. simulans*, where *Wolbachia*-induced CI was detected, do not express this effect (Turelli and Hoffmann, 1995); furthermore it has been shown that

Table 2. Sex ratio of *D. suzukii* (±SE) emerged in both experiments. Results of χ^2 statistics are indicated (χ^2 test, $P > 0.05$).

Exp.	Treatment (male × female)	% Males	% Females	P-value
1	U×U	48.15 ± 1.98	51.85 ± 2.06	0.712
1	T×T	49.87 ± 1.43	50.13 ± 1.43	0.979
1	U×T	49.87 ± 3.22	50.13 ± 3.34	0.978
1	T×U	53.18 ± 3.03	46.82 ± 2.80	0.524
2	U×U	45.90 ± 3.33	54.10 ± 3.33	0.412
2	T×T	49.10 ± 3.59	50.90 ± 3.59	0.858
2	U×T	50.00 ± 5.03	50.00 ± 4.49	1.000
2	T×U	52.42 ± 2.54	47.58 ± 2.54	0.628

different *Wolbachia* strains display dissimilar distribution, correlated with different CI levels, in *Drosphila* embryos (Veneti *et al.*, 2004).

In many strains of *Drosophila melanogaster* Meigen, *Wolbachia* was demonstrated to improve fly fitness and contribute to prolonged host survival (Fry *et al.*, 2004). In our experiments, no differences were found in the vitality of parental individuals at the end of the experiments, suggesting a similar lifespan of infected and uninfected flies (figure 1); however, this aspect may require further investigations. In *D. melanogaster*, Fry and Rand (2002) demonstrated that fly survival depends on several factors, such as the interaction between *Wolbachia* and the host genotype, sex and reproductive status. Considering the numerous aspects involved in the relationship between the symbionts and the host, deep investigations are necessary to understand how *D. suzukii* and *Wolbachia* interact, establishing a mutualistic symbiosis.

Besides the evaluation of *D. suzukii* fecundity in infected and uninfected specimens, the sex ratio of emerged flies was also recorded to better understand the possible implication of *Wolbachia* in different reproductive alterations. *Wolbachia*-borne male-killing, feminisation and parthenogenesis are mainly well known and common in Coleoptera and Lepidoptera, but such effects were also detected in Diptera and within the genus *Drosophila* (Hurst *et al.,* 2000; Weeks *et al.*, 2002). However, our crossing experiments did not show any sex-biasing effect (table 2) on *D. suzukii* from different treatments, suggesting that *Wolbachia* does not imbalance the sex ratio in this fly.

In summary, this study on the biological effects of *Wolbachia* on *D. suzukii* provided evidence for a beneficial influence on female fecundity. Further investigations are required to clarify the nature of this association, which our current results suggest to be mutualistic. Understanding the possible implication of *Wolbachia* on *D. suzukii* fecundity could be useful for the development of symbiont-based management measures against the spread of this pest.

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