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Wolbachia-mediated protection against viruses in the invasive pest *Drosophila suzukii*

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Abstract

The maternally inherited bacterium *Wolbachia* is well known for spreading in natural populations by manipulating the reproduction of its arthropod hosts, but can also have mutualist effects that increase host fitness. In mosquitoes and *Drosophila* some *Wolbachia* strains can lead to an increase in survival of virus-infected insects, and in most cases this is associated with reduced accumulation of the virus in host tissues. We investigated if the *Wolbachia* strain *w*Suz, which naturally infects *Drosophila suzukii*, is able to confer protection against *Drosophila* C virus and Flock House virus in different host genetic backgrounds. We found that this strain can increase host survival upon infection with these two viruses. In some cases this effect was associated with lower viral titres, suggesting that it confers resistance to the viruses rather than allowing the flies to tolerate infection. Our results indicate that, in *D. suzukii*, the antiviral protection provided by *Wolbachia* is not correlated to its density as found in other *Drosophila* species. This study demonstrates a phenotypic effect induced by *w*Suz on its native host which could explain its maintenance in natural populations of *D. suzukii*.

Keywords: *Drosophila suzukii*, *Wolbachia*, viruses, protection.

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Introduction

Drosophila suzukii (Matsumura, 1931) (Diptera: Drosophilidae), the spotted-wing *Drosophila*, is an invasive species native to South-East Asia (Kanzawa, 1936). It was originally described in Japan in 1916 and, within the last decade, it has been observed for the first time in California (Hauser, 2011), in Spain and Italy (Calabria *et al*., 2012) in 2008, and then quickly spread throughout North America and Europe (Cini *et al*., 2012) and more recently Brazil (Deprá *et al*., 2014). In contrast to the vast majority of *Drosophila* species, *D. suzukii* is an agricultural pest because its serrated ovipositor allows it to lay eggs on healthy ripening fruits still attached to the plant (Mitsui *et al*., 2006). Damage is caused by larvae feeding on the pulp inside the fruits and berries. As a consequence *D. suzukii* can have severe economic impacts, such as in the western USA where it causes losses of up to US$500 million per year (Goodhue *et al*., 2011). Because of its remarkable invasive success and impact on agricultural production, *D. suzukii* is currently subject to intense research from both fundamental and applied perspectives.

Until now little was known about the symbiotic community of *D. suzukii*, despite maternally inherited symbionts being common and important components of arthropod biology and ecology (Zchori-Fein & Bourtzis, 2011). Some studies revealed that *D. suzukii* naturally harbours *Wolbachia* (Cordaux *et al*., 2008; Siozios *et al*., 2013; Hamm *et al*., 2014; Cattel *et al*., 2016), which is the most common endosymbiont in arthropods with an estimation of 52% of arthropod species infected (Weinert *et al*., 2015). Only one strain of *Wolbachia* has been identified in field populations of *D. suzukii* based on Multilocus Sequence Typing (MLST) markers, at least in North America and in Europe, which is closely related to *w*Ri (Siozios *et al*., 2013; Hamm *et al*., 2014; Cattel *et al*., 2016). In many associations, the spread of *Wolbachia* in the host populations is achieved through their capacity to manipulate host reproduction either by biasing the host's sex ratio towards the production of

females or, more commonly, by impeding the reproduction of uninfected females through a sterility phenomenon called cytoplasmic incompatibility (CI) (Werren *et al*., 2008). Theory predicts that the spread of CI-inducing *Wolbachia* in a population is under positive frequency-dependence and that their maintenance depends on their transmission efficiency and on the intensity of CI (Turelli & Hoffmann, 1995). *Wolbachia* can also successfully invade host populations by bringing direct fitness benefits to infected individuals such as increasing fecundity (Dobson *et al*., 2002, 2004; Fry *et al*., 2004; Weeks *et al*., 2007; Unckless & Jaenike, 2012), longevity (Gavotte *et al*., 2010; Brelsfoard & Dobson, 2011; Alexandrov *et al*., 2007; Toivonen *et al*., 2007) or provisioning nutrients (Brownlie & Johnson, 2009; Hosokawa *et al*., 2010; Unckless & Jaenike, 2012). In addition, *Wolbachia* can protect its host against viruses (Hedges *et al*., 2008; Teixeira *et al*., 2008; Osborne *et al*., 2009; Bian *et al*., 2010; Glaser & Meola, 2010; Blagrove *et al*., 2012). Such benefits could explain the presence in natural populations of *Wolbachia* strains that do not appear to rely on reproductive manipulation to spread. For example, the strain *w*Mel, which induces a very low level of CI (Hoffmann *et al*., 1994; Hoffmann *et al*., 1998), might be maintained in populations of *Drosophila melanogaster* because of positive effects such as the protection it confers against several RNA viruses (Hedges *et al*., 2008; Teixeira *et al*., 2008). Similarly, *w*Au, which naturally infects *Drosophila simulans*, does not induce CI but confers strong protection against viruses (Osborne *et al*., 2009; Martinez *et al*., 2014). This antiviral protection, which has been observed only in *Drosophila* and mosquitoes, has been shown to be highly variable according to the host species and the *Wolbachia* strain (Hedges *et al*., 2008; Teixeira *et al*., 2008; Moreira *et al*., 2009; Osborne *et al*., 2009; Mousson *et al*., 2010; Chrostek *et al*., 2013; Chrostek *et al*., 2014; Martinez *et al*., 2014).

Previous studies found that the prevalence of *w*Suz is highly variable in populations of *D. suzukii* from North America (7 to 58%) and Europe (0 to 100%) (Hamm *et al*., 2014; Cattel *et al*., 2016) and, until now, there is no indication that this strain can induce strong reproductive manipulations in *D. suzukii* such as CI or male killing (Hamm *et al*., 2014; Cattel *et al*., 2016). Moreover, in North American populations, it has been shown that *w*Suz is imperfectly vertically transmitted by wild-caught *D. suzukii* females, which would cause the bacterium to be lost from the population in the absence of any selection (Hamm *et al*., 2014). All these results suggest that *w*Suz may bring a fitness advantage to *D. suzukii* but as yet no effect has been found on fecundity, starvation tolerance or resistance to desiccation (Hamm *et al*., 2014).*w*Suz belongs to the supergroup A (Siozios *et al*., 2013), which contains several *Wolbachia* strains known to induce antiviral protection (Martinez *et al*., 2014). In the present study, we thus tested whether *w*Suz can protect *D. suzukii* against viruses. Four host lines were compared, two from France, a country that was recently invaded by *D. suzukii*, and two from Japan, its native range (Cini *et al*., 2012; Asplen *et al*., 2015). Two RNA viruses were tested, *Drosophila* C virus (DCV; highly pathogenic *Drosophila* virus) and the Flock House virus (FHV; isolated from a beetle) (Scotti *et al*., 1983; Huszar & Imler, 2008). We found that *w*Suz is able to protect *D. suzukii* against these two viruses but that the antiviral protection is very variable amongst the host lines. This beneficial effect could explain its maintenance in natural populations.

Results

Wolbachia *protects* D. suzukii *against DCV infection*

We measured the survival of French line-antibiotic treated (Fr-CP) and Japanese line-introgressed line (Jp-OGH) flies infected or uninfected, respectively, with a French and Japanese *Wolbachia* isolate after inoculation with DCV (400 flies) or saline solution (Ringer, 400 flies) (Fig. 1A). In the mock-infected flies (Ringer's control treatment), the survival of *Wolbachia*-free and *Wolbachia*-infected individuals was not significantly different, indicating that there is no intrinsic effect of *Wolbachia* on the fly survival (Cox's mixed effect model; main effect *Wolbachia*: $\chi^2 = 0.92$, df $= 1$, $P = 0.337$; host genotype $\times$ *Wolbachia* interaction: $\chi^2 = 1.57$, df $= 1$, $P = 0.210$). However, the Fr-CP line had higher survival than the Jp-OGH line (Cox's mixed effect model; $\chi^2 = 8.78$, df $= 1$, $P = 0.003$).

We found that *Wolbachia* increased the survival of flies infected with DCV (Cox's mixed effect model: $\chi^2 = 21.74$, df $= 2$, $P < 0.001$; Fig. 1A) but the effect was significant for the Fr-CP line only (Cox's mixed effect model, host genotype $\times$ *Wolbachia* interaction: $\chi^2 = 4.1$, df $= 1$, $P = 0.043$; Tukey's test, $P < 0.001$ for Fr-CP and P $= 0.99$ for Jp-OGH). As Fr-CP and Jp-OGH lines differ in both the host and bacterial genotypes, either of these may be causing the difference.

The DCV titre was lower in *Wolbachia*-infected flies than in uninfected ones [two-way analysis of variance (ANOVA), F $= 15.22$, df $= 1$, $P < 0.001$; Fig. 1B], and this effect of *Wolbachia* did not depend on the line (two-way ANOVA, *Wolbachia* $\times$ host interaction: F $= 0.45$, df $= 1$, $P = 0.509$; Fig. 1B).

Wolbachia *effect on FHV infection*

Given the difference in the degree to which *w*Suz increases the survival of *D. suzukii* after DCV infection amongst lines we then investigated the effect of *w*Suz

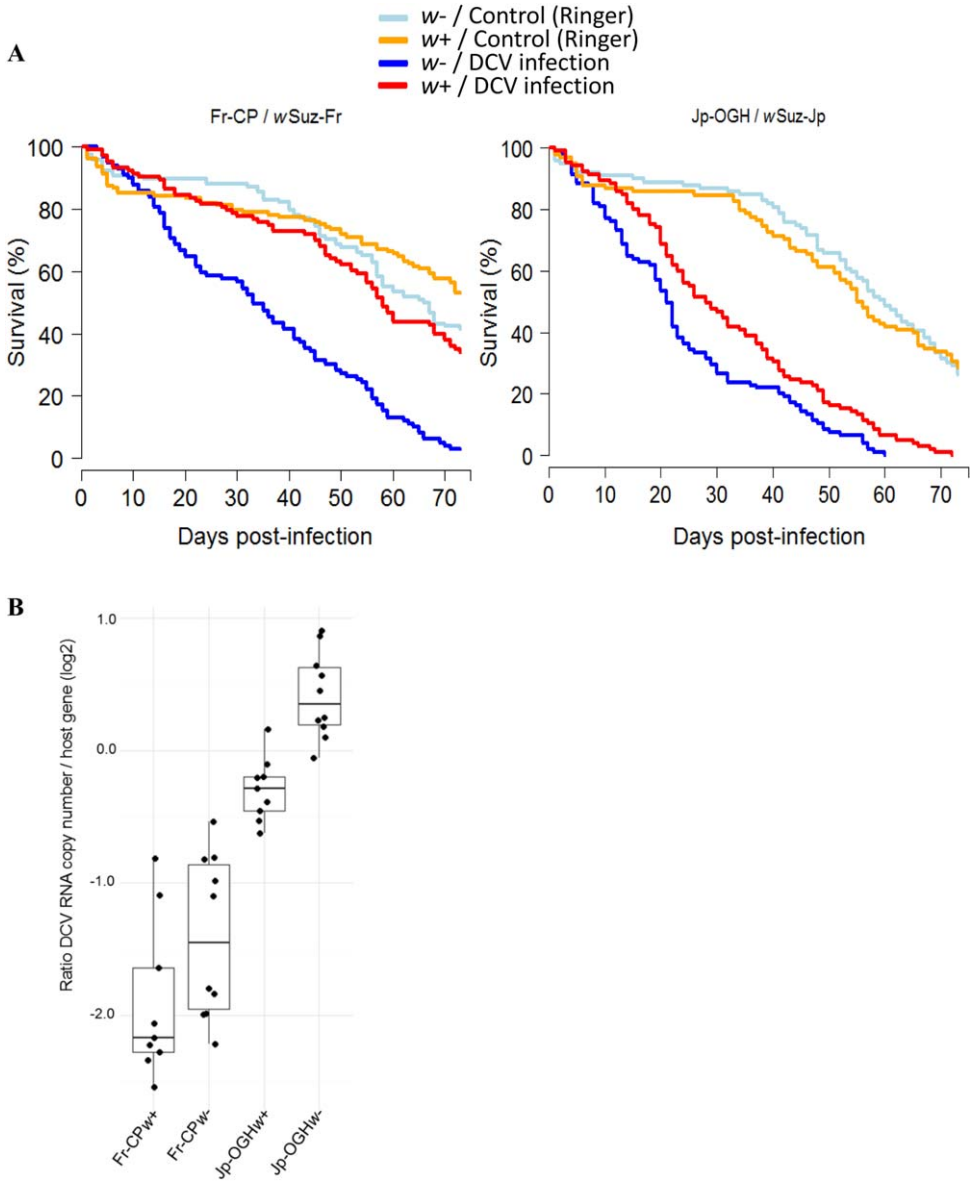
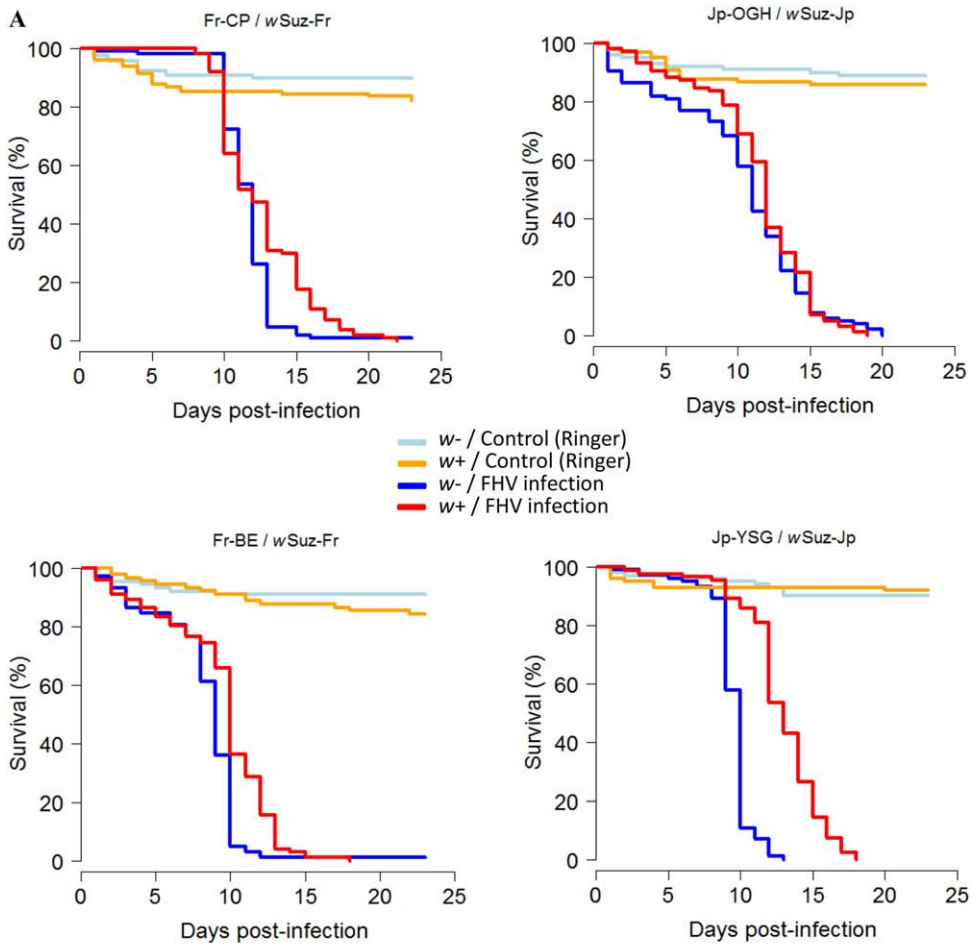


Figure 1. Effect of *Wolbachia* on fly survival and RNA copy number upon *Drosophila* C virus (DCV) infection with two *Wolbachia* isolates in different genetic backgrounds. (A) Survival of flies infected with DCV (dark blue and red lines) or Ringer's solution (light blue and orange lines). Dark blue and light blue lines indicate *Wolbachia*-free flies. Orange and red lines indicate *Wolbachia*-infected flies. (B) DCV RNA copy number infection 2 days post-infection in flies with two *Wolbachia* isolates in different genetic backgrounds. RNA copy number is expressed by the copy number of viral RNA relative to the host gene *Rpl32*.

on FHV infection in four genetic backgrounds: the effect of the French *Wolbachia* isolate, *w*Suz-Fr, in two French backgrounds Fr-CP and Fr-Bellegarde (BE), and the effect of the Japanese isolate, *w*Suz-Jp, in two Japanese backgrounds Jp-OGH and Jp-Yamagata (YSG). A total of 800 flies were stabbed with FHV and 800 others with Ringer's solution (Fig. 2A). In the absence of viral infection neither *Wolbachia* nor the host genetic background affected survival (Ringer control treatment, Cox's mixed effect model, *Wolbachia* effect: $\chi^2 = 1.83$, df = 1, $P = 0.180$; host effect: $\chi^2 = 1.43$, df = 3, $P = 0.7$; *Wolbachia* × host interaction: $\chi^2 = 1.22$, df = 3, $P = 0.750$).

In FHV-infected flies, survival was significantly affected by the *Wolbachia* infection ($\chi^2 = 31.88$, df = 4, $P < 0.001$) and by the host genetic background ($\chi^2 = 39.55$, df = 6, $P < 0{,}001$), and there was a significant interaction between these two factors ($\chi^2 = 14.99$, df = 3, $P = 0.002$). Because we could not exclude the possibility that the French and the Japanese lines are infected by a different *Wolbachia* isolate (*w*Suz-Fr and *w*Suz-Jp, respectively), we also tested the *Wolbachia* and the host genetic background effects on infected flies' survival for the French and Japanese lines separately. The French lines' survival was significantly affected by the *Wolbachia* infection ($\chi^2 = 17.75$, df = 2, $P < 0.001$) and by the host genetic background ($\chi^2 = 34.14$, df = 2, $P < 0{,}001$) but there was no significant interaction between these two factors ($\chi^2 = 3.73$, df = 1, $P = 0.053$). In the Japanese lines, the survival rate was affected by the *Wolbachia* infection ($\chi^2 = 14.18$, df = 2, $P < 0.001$) and by the host genetic



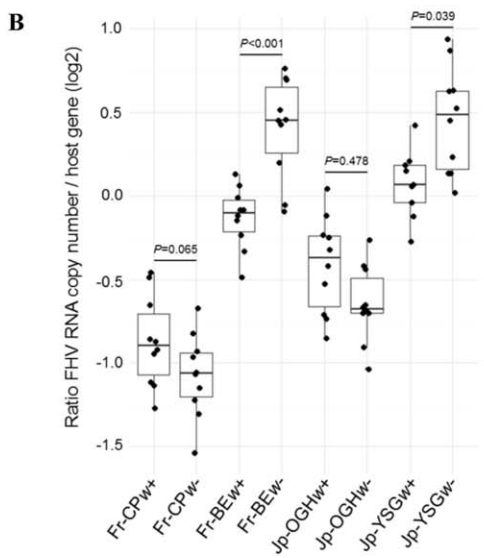


Figure 2. Effect of *Wolbachia* and host genetic background on fly survival and RNA copy number upon Flock House virus (FHV) infection. (A) Survival of flies infected with FHV (dark blue and red lines) or Ringer's solution (light blue and orange lines). Dark blue and light blue lines indicate *Wolbachia*-free flies. Orange and red lines indicate *Wolbachia*-infected flies. (B) Effect of *Wolbachia* and line on FHV RNA copy number 5 days post-infection. RNA copy number is expressed by the copy number of viral RNA relative to the host gene *Rpl32*. Tukey's honestly significant difference tests were performed for pairwise comparisons.

background ($\chi^2 = 10.54$, df = 2, $P = 0.005$), and we detected a significant interaction between these two factors ($\chi^2 = 8.41$, df = 1, $P = 0.004$). By comparison with the uninfected lines, the *w*Suz infection significantly increased the survival of the Fr-BE and the Jp-YSG backgrounds [Tukey's honestly significant difference (HSD), $P = 0.012$ and $P < 0.001$, respectively] whereas it did not affect the survival of the Fr-CP and the Jp-OGH

backgrounds (CP line, $P = 0.191$; OGH line, $P = 0.849$) (Fig. 2A).

As for DCV, we also measured FHV titres and we found a significant effect of both the *Wolbachia* infection status (two-way ANOVA, $F = 5.04$, $df = 1$, $P = 0.03$) and the host genetic background (two-way ANOVA, $F = 98.88$, $df = 1$ $P < 0.001$) on the RNA copy number (Fig. 2B), with a significant interaction between these two factors (two-way ANOVA, $F = 11.54$, $df = 1$, $P < 0.001$). As for the survival data analysis, we tested the influence of the presence of *Wolbachia* and the host genetic background for the French and the Japanese lines separately. For the French lines the RNA copy number was affected by *Wolbachia* infection (two-way ANOVA, $F = 4.32$, $df = 1$, $P = 0.045$) and by the host genetic background (two-way ANOVA, $F = 189.82$, $df = 1$, $P < 0.001$), with a significant interaction between these two factors (two-way ANOVA, $F = 21.01$, $df = 1$, $P < 0.001$). For the Japanese lines, we also found a significant interaction between the *Wolbachia* infection and the host genetic background (two-way ANOVA, $F = 13.18$, $df = 1$ $P < 0.001$) and a significant effect of the host genetic background (two-way ANOVA, $F = 88.80$, $df = 1$, $P < 0.001$), but we did not detect a significant effect of the *Wolbachia* infection (two-way ANOVA, $F = 1.05$, $df = 1$, $P = 0.311$). More precisely, in the presence of *w*Suz, the RNA copy number significantly decreased (around 50% reduction; Fig. 2B) in the Fr-BE and Jp-YSG backgrounds infected with *w*Suz-Fr and *w*Suz-Jp isolates, respectively (Tukey's HSD, $P < 0.001$ and $P = 0.039$, respectively), the two lines that exhibited a significant effect of *Wolbachia* on survival after FHV infection, and not in the two other lines (Tukey's HSD test, Fr-CP line, $P = 0.665$; Jp-OGH line, $P = 0.478$).

Wolbachia *density*

Wolbachia density is known to be a major determinant of antiviral protection, with higher densities being associated with higher levels of protection (Chrostek *et al.*, 2014; Martinez *et al.*, 2014). We therefore measured *w*Suz density in the four lines and found significant differences (one-way ANOVA, $F = 10.07$, $df = 3$, $P < 0.001$; Fig. 3): the two Japanese lines (Jp-OGH and Jp-YSG) showed a higher density than the two French backgrounds (Fr-CP and Fr-BE), but there was no significant difference between the two French lines (both infected by *w*Suz-Fr; Tukey's HSD, $P = 0.991$) or between the two Japanese lines (both harbour the Japanese *Wolbachia* isolate; Tukey's HSD, $P = 0.062$).

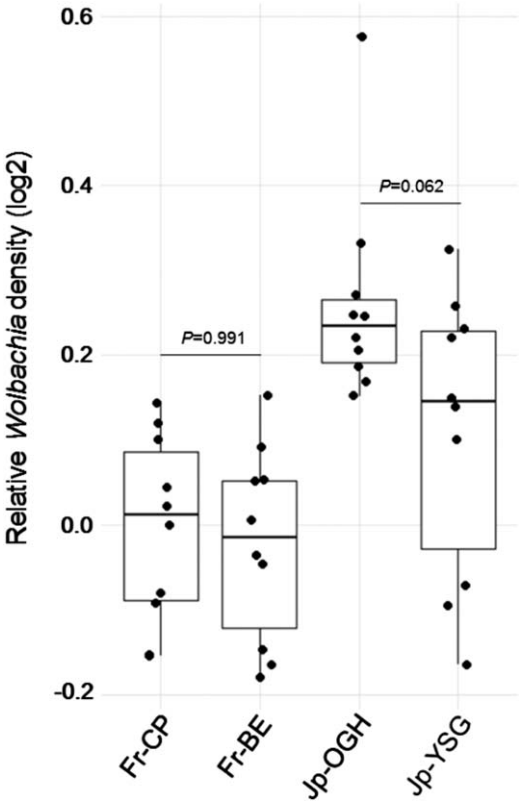


Figure 3. Relative *Wolbachia* density in different *Drosophila suzukii* genetic backgrounds. The *Wolbachia* quantity was normalized to that of the *Rpl32* host gene. Tukey's honestly significant difference tests were performed for pairwise comparisons.

Discussion

We have found that *w*Suz can protect its host against RNA viruses. In certain lines individuals infected with *w*Suz had higher survival and lower viral titres after infection with DCV and FHV. It has been known since 2008 that *Wolbachia* can protect *Drosophila* against RNA viruses (Hedges *et al.*, 2008; Teixeira *et al.*, 2008), but this is the first time that it has been described in *D. suzukii*. In a recent study another direct fitness benefit of *Wolbachia* was observed in an Italian population of *D. suzukii*: infected females had higher fecundity than uninfected ones (Mazzetto *et al.*, 2015). These phenotypes can potentially explain the maintenance of *Wolbachia* strains in natural populations without reproductive manipulation (Fenton *et al.*, 2011), as has been found in North American and European populations of *D. suzukii* (Hamm *et al.*, 2014; Cattel *et al.*, 2016).

The variability in *w*Suz prevalence could be a consequence of heterogeneity in virus-induced selection similar to that observed in the pea aphid, *Acyrthosiphon pisum*. This species is protected against parasitoids by the symbiont *Hamiltonella defensa*, which has variable prevalence amongst populations and is thought to be maintained by negative-frequency dependent selection depending on the extent of parasitism pressure in the field (Oliver *et al.*, 2008). We found that *Wolbachia* mediated significant protection in *D. suzukii* (Fr-CP for DCV,

Fr-BE and Jp-YSG for FHV) and that this protection was associated with reduced viral titre. However, for DCV, the presence of *Wolbachia* correlated with a lower viral titre even when no effect on the flies' survival was detected (Jp-OGH line). It has been shown that antiviral protection is generally explained by a phenomenon of resistance that reduces the accumulation of virus but, in some cases, no differences in viral titres were observed despite the protective effect (Teixeira *et al*., 2008; Osborne *et al*., 2009). In the latter case, it is possible that *Wolbachia* does not affect the replication of the virus but rather makes the host more tolerant to viral infection.

Experimental studies have shown that *Wolbachia*-mediated antiviral protection is a common phenomenon in *Drosophila* and mosquitoes (Hedges *et al*., 2008; Teixeira *et al*., 2008; Moreira *et al*., 2009; Osborne *et al*., 2009; Bian *et al*., 2010; Chrostek *et al*., 2013; Chrostek *et al*., 2014; Martinez *et al*., 2014) but is strongly dependent on the *Wolbachia* strain (Hedges *et al*., 2008; Osborne *et al*., 2009; Chrostek *et al*., 2013; Chrostek *et al*., 2014; Martinez *et al*., 2014). For instance, Martinez *et al*. (2014) showed that amongst 19 *Wolbachia* strains (originating from 16 *Drosophila* species) transferred into the same *D. simulans* genotype, only half of them induced protection against DCV and FHV. The effect of host genetics on protection is less well understood. However, the protective phenotype is affected by the host species. For example, the strain *w*Inn protects its natural host *Drosophila innubila* against FHV (Unckless & Jaenike, 2012) but has no effect in *D. simulans* (Martinez *et al*., 2014). Here, we found that the level of antiviral protection varied amongst the lines we used. This difference was most dramatic in the DCV experiment, in which we found large increases in the survival of the French line but not the Japanese line. This difference could be caused by genetic differences between the *Wolbachia* isolates, the flies or both. In the FHV experiment we were able to compare the same *Wolbachia* isolates in two host genetic backgrounds. We found a host background effect for both the Japanese and the French lines, suggesting that host factors may affect the expression of the *Wolbachia*-mediated protection. However, we would caution that this needs further confirmation as we only had a single replicate line of each *Wolbachia* isolate in each genetic background, so we cannot rule out other possible differences (eg gut microbiota, or uncontrolled differences in the genetic background). *Wolbachia* density is known to influence the level of protection (Osborne *et al*., 2009; 2012; Chrostek *et al*., 2013; 2014; Martinez *et al*., 2014). However, we did not find any clear association between the level of protection and the density of *Wolbachia*. The variation in antiviral protection could also be influenced by tissue tropism of *Wolbachia* as Osborne *et al*. (2012) highlighted that this can partly explain variations in the level of protection. Therefore it is possible that, in the *D. suzukii* lines used in our study, the tissue tropism of *Wolbachia* was different, despite showing very similar density at the whole fly level.

The importance of antiviral protection in natural populations of *D. suzukii* is unknown. It has been estimated that *Wolbachia* would need to generate a fitness benefit of 20% to be maintained in populations (Hamm *et al*., 2014). To achieve this RNA viruses would need to be causing significant harm to the flies in nature and *Wolbachia* would need to be mitigating much of this harm. The effects of the presence of *Wolbachia* on viral titre and survival that we observed were mostly smaller than in many previous studies (Hedges *et al*., 2008; Teixeira *et al*., 2008; Chrostek *et al*., 2013; Chrostek *et al*., 2014; Martinez *et al*., 2014). However, it is not possible to extrapolate this to effects in nature without further work.

Experimental procedures

D. suzukii *lines and rearing*

In this study, four lines of *D. suzukii* were used, two originating from France and two from Japan. The French lines were collected in Compiegne (named Fr-CP) and in Bellegarde (named Fr-BE) in 2011 and 2012, respectively, and reared in large populations. The Japanese lines were obtained from the Ehime-fly Stock Center in 2011: they were sampled in Yamagata (named Jp-YSG; l#E-15016 YSG-11) and Tokyo (named Jp-OGH; #E-15014OGH06-03) in 2006. These lines were chosen because two are free of *Wolbachia* (Fr-BE and Jp-OGH) and the two others (Fr-CP and Jp-YSG) are 100% infected with *Wolbachia* (see below for diagnostic PCR test). The flies were reared on a cornmeal diet (agar: 1%, dextrose: 8.75%, maize: 8.75%, yeast: 2%, nipagin: 3%) and maintained in an incubator at constant temperature (22°C) and humidity (70%) with a 12-h light/dark cycle. An MLST analysis performed on six genes [*ftsZ*, *fbpA*, *hcpA*, *coxA*, *gatB* and *Wolbachia* surface protein (*wsp*)] revealed the *Wolbachia* isolates from Fr-CP and Jp-YSG lines to be the same sequence type, with 100% identity between the sequences. The sequences obtained in the present study are recorded in GenBank as KS308222–7.

Control of host genetic background and infection status

We used two different methods to obtain *Wolbachia*-infected and *Wolbachia*-free lines with similar genetic backgrounds: antibiotic treatments of the infected lines and introgression of *Wolbachia* into uninfected lines by back-crossing.

Antibiotic treatments were performed for three generations in Fr-CP and Jp-YSG lines. Larvae in each generation were fed on medium with 0.25 mg/ml tetracycline. After three generations, 10 isofemale lines were established from treated females and the presence of *Wolbachia* was checked by PCR as described below in mothers and then for three generations more. Only one isofemale line was retained for each nuclear

background (Fr-CP and Jp-YSG) and maintained for 12 generations before the experiments. The absence of *Wolbachia* in these lines was confirmed by real-time quantitative PCR (qPCR; see below). Using this approach, we obtained infected and cured lines with the same genetic background, Fr-CP or Jp-YSG.

To obtain infected and uninfected individuals with the same Fr-BE or Jp-OGH genetic backgrounds, back-crosses were performed for eight generations. Two males from the uninfected line (Fr-BE or Jp-OGH) were mated with single virgin females from the infected lines from the same country, ie Fr-CP and Jp-YSG, respectively. Backcrossing was performed for a total of eight generations, which led to an introgression of around 99.6% of the nuclear background assuming no selection on the nuclear genome. However, compared with the use of antibiotic treatments, lines obtained with this method have different mitochondrial backgrounds. These two lines were maintained for 15 generations before the experiments. The *Wolbachia* infection status of each line was verified by PCR just before the viral infection experiment.

Viral isolates

Two viruses, DCV and FHV, were used in this study. DCV is a highly pathogenic *Drosophila* virus, which belongs to the family Dicistroviridae (Huszar & Imler, 2008); FHV, which belongs to the Nodaviridae family, is not a natural pathogen of *Drosophila* species and was initially isolated from a beetle (Scotti *et al.*, 1983). Viruses were produced and titrated as described by Martinez *et al.* (2014). DCV was produced and titrated in Schneider's Line 2 cells and FHV was titrated in Schneider Drosophila Line 2 cells (https://dgrc.bio.indiana.edu/cells/Catalog). For each infection assay, one viral aliquot was defrosted just before the infection and diluted in Ringer's solution (Sullivan *et al.*, 2000) to reach a viral concentration of 5×10^8/ml 50% Tissue Culture Infective Dose (TCID50) for DCV and 3.6×10^{10}/ml TCID50 for FHV.

Survival assay

In order to test for a potential protective effect of *w*Suz, we measured the survival of flies after infection with DCV, FHV or mock infection with Ringer's solution. To infect flies, a 0.1-mm diameter anodized steel needle (26002-15, Fine Science Tools, San Francisco, CA, USA) was bent, 0.25 mm from the end, dipped in viral solution and the bent part of the needle pricked into the pleural suture on the thorax of the flies (Longdon *et al.*, 2013). For DCV, we followed the survival of *Wolbachia*-free or *Wolbachia*-infected flies of the Fr-CP and Jp-OGH lines only. As, in that first experiment, we observed variation depending on the geographical origin of the flies, we performed the second experiment with FHV using all four genetic backgrounds (Fr-CP, Fr-BE, Jp-OGH and Jp-YSG). Survival of Ringer's controls was followed in parallel for these two experiments.

For each line 3-day-old females were collected. After being anaesthetized with CO_2, they were inoculated with DCV, FHV or Ringer's solution by stabbing them as described above. Groups of 20 stabbed flies were immediately placed into a vial of fly cornmeal medium and stored at 22°C. Flies were transferred into fresh vials of food every 3 days and the number of dead flies was recorded every day. The survival assay was replicated five times on independent cohorts of flies across multiple days, corresponding to a total of 100 flies for each *Wolbachia* infection status and virus infection treatment.

Diagnostic PCR

The *Wolbachia* infection status of individuals was verified by PCR for each line just before performing the experiments. DNA was extracted on pools of 10 individuals (one pool per line) homogenized in 200 μl 5% w/v Chelex resin in water (Bio-Rad, Hercules, CA, USA) with 4 μl proteinase K (20 mg/ml) and kept at 56°C for 3 h. After 15 min at 95°C, samples were centrifuged at 16 000 *g* for 4 min and stored at −20°C. Presence of *Wolbachia* was checked by amplifying the *wsp* gene using the primers wsp81F and wsp691R (Braig *et al.*, 1998; Table S1). PCR reactions were performed in 25 μl volumes containing 100 μM Désoxyribonucléotides (dNTP), 200 nM primers, 0.5 International Unit DreamTaq® DNA polymerase (Eurobio, Paris, France) and 1 μl DNA template. Cycling conditions were 94°C (2 min), 94°C (30 s), 52°C (30 s), 72°C (45 s), 72°C (10 min) for 35 cycles. PCR products were visualized in 1% agarose gels.

qPCR

The *Wolbachia* density, DCV and FHV RNA copy number were measured by qPCR on a Light Cycler™ system (Roche Life Science, USA) using the primers listed in Table S1. To estimate *Wolbachia* density, 10 pools of 10 3-day-old virus-free females for each line were prepared and the DNA extracted using a Gentra Pure gene Tissue Kit (Qiagen, Valencia, CA, USA). The *Wolbachia* density was measured by quantifying the copy number of the *Wolbachia* gene *ftsZ* relative to the host gene *Rpl32* using Sso Advanced Universal Probes Supermix (Bio-Rad; 2 min at 95°C followed by 40 cycles of 10 s at 95°C and 20 s at 60°C). The 10 μl of multiplex reaction mix contained 400 nM *Rpl32* primers, 200 nM *ftsZ* primers, 5μl Sso Advanced Universal Probes Supermix, 200 nM of each probe and 2μl of DNA sample. The *Wolbachia* density was estimated by dividing the copy number of the *ftsZ* gene by the copy number of the *Rpl32* host gene. The antiviral protection was also examined by measuring the RNA copy number after infection by both viruses. Three-day-old females were stabbed with DCV and FHV and frozen 5 and 2 days after infection, respectively. After homogenization in TRIzol Reagent (Ambion, Thermo Fisher Scientific, MA USA), RNA was extracted from 10 pools of 10 flies for each experimental treatment using an RNA Easy Mini® kit following the manufacturer's instructions (Qiagen). Reverse-transcription was carried out using a SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) including a 30 min DNase digestion step at 37°C. The copy number of the viral RNA was compared to that of the control gene *Rpl32*. The qPCR reactions for DCV, FHV and *Rpl32* were carried out separately under the same conditions (30 s at 95°C followed by 40 cycles of 10 s at 95°C and 20 s at 60°C). The 10 μl reaction mix contained 200 nM of each primer, 5 μl Sso Advanced Universal Probes Supermix SYBR Green Supermix and 1 μl DNA sample. The RNA copy number and the *Wolbachia* density

were estimated by calculating the ratio: $\frac{E(\text{virus}/Wolbachia)^{\Delta Ct}}{E(\text{host})^{\Delta Ct}}$ with $\Delta Ct = Ct_{flygene} - Ct_{virus/Wolbachia}$ where E is the efficiency of the PCR reaction calculated from a dilution series for each set of primers $\left[E = 2\left(\frac{1}{linear\ regression\ slope}\right)\right]$ and Ct is the cycle threshold (Pfaffl, 2001).

Statistical analysis

Survival data were analysed with a Cox's proportional hazards mixed-effect model using the coxme package in R (R Core Team, 2013). The Cox's model estimates hazard ratios with the probability of a *Wolbachia*-infected fly dying at a given time-point divided by the probability of a *Wolbachia*-free fly dying. Flies that were alive at the end of the experiment were treated as censored data.

Survival data for DCV, FHV and their respective controls (Ringer) were analysed separately. For each virus, two models were fitted to test a potential effect of the *Wolbachia* infection and the genetic background on survival for the control treatment (Ringer) without virus or after infection with a virus. The first model allowed us to test whether *w*Suz infection modifies survival independently of viral infection and indirectly confirm that the survival of virus-infected flies cannot be explained by an inherent effect of *Wolbachia* on survival. The effects of *Wolbachia*, host genetic background and their interaction were considered as fixed effects and the replicate vials as a random effect. When a significant interaction was detected, differences between *Wolbachia*-free and *Wolbachia*-infected flies within each host genetic background were analysed using pairwise comparisons (Tukey's HSD test) (R package multcomp).

Viral titres and *Wolbachia* density were analysed on $\log_2$-transformed data. For viral titres, a two-way ANOVA allowed us to test for the effect of *Wolbachia*, the host genetic background and their interaction. A one-way ANOVA was conducted to test for the influence of the host genetic background on *Wolbachia* density. Pairwise comparisons (Tukey's HSD test) were also carried out if a global effect of *Wolbachia* was detected.

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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:

Table S1. Primers and probes used in this study.