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

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8 Running title: *Wolbachia* and antiviral protection in *D. suzukii*

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25 **Abstract**

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

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49 **Introduction**

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

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

73 biasing the host's sex ratio towards the production of females or, more commonly, by
74 impeding the reproduction of uninfected females through a sterility phenomenon called
75 Cytoplasmic Incompatibility (CI) (Werren *et al.*, 2008). Theory predicts that the spread of CI-
76 inducing *Wolbachia* in a population is under positive frequency-dependence and that their
77 maintenance depends on their transmission efficiency and on the intensity of CI (Turelli &
78 Hoffmann, 1995). *Wolbachia* can also successfully invade host populations by bringing direct
79 fitness benefits to infected individuals such as increasing fecundity (Dobson *et al.*, 2002;
80 Dobson *et al.*, 2004; Fry *et al.*, 2004; Weeks *et al.*, 2007; Unckless & Jaenike, 2012),
81 longevity (Gavotte *et al.*, 2010; Brelsfoard & Dobson, 2011; Alexandrov *et al.*, 2007;
82 Toivonen *et al.*, 2007) or provisioning nutrients (Brownlie & Johnson, 2009; Hosokawa *et al.*,
83 2010; Unckless & Jaenike, 2012). In addition, *Wolbachia* can protect its host against viruses
84 (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Osborne *et al.*, 2009; Bian *et al.*, 2010; Glaser *et*
85 *al.*, 2010; Blagrove *et al.*, 2012). Such benefits could explain the presence in natural
86 populations of *Wolbachia* strains that do not appear to rely on the reproductive manipulation
87 to spread. For example, the strain *wMel*, which induces a very low level of CI (Hoffmann *et*
88 *al.*, 1994; Hoffmann *et al.*, 1998), might be maintained in populations of *D. melanogaster*
89 because of positive effects such as the protection it confers against several RNA viruses
90 (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Similarly, *wAu*, which naturally infects *D.*
91 *simulans*, does not induce CI but confers strong protection against viruses (Osborne *et al.*,
92 2009; Martinez *et al.*, 2014). This antiviral protection, which has been observed only in
93 *Drosophila* and mosquitoes, has been shown to be highly variable according to the host
94 species and the *Wolbachia* strain (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Osborne *et al.*,
95 2009; Moreira *et al.*, 2009; Mousson *et al.*, 2010; Chrostek *et al.*, 2013; Chrostek *et al.*, 2014;
96 Martinez *et al.*, 2014).

97 Previous studies found that the prevalence of *wSuz* is highly variable in populations of *D.*
98 *suzukii* from North America (7 to 58%) and Europe (0 to 100%) (Hamm *et al.*, 2014; Cattel *et*
99 *al.*, 2016) and, until now, there is no indication that this strain can induce strong reproductive
100 manipulations in *D. suzukii* such as CI nor male killing (Hamm *et al.*, 2014; Cattel *et al.*,
101 2016). Moreover, in North American populations, it has been shown that *wSuz* is imperfectly
102 vertically transmitted by wild-caught *D. suzukii* females, which would cause the bacterium to
103 be lost from the population in the absence of any selection (Hamm *et al.*, 2014). All these
104 results suggest that *wSuz* may bring a fitness advantage to *D. suzukii* but yet no effect has
105 been found on fecundity, starvation tolerance or resistance to desiccation (Hamm *et al.*,
106 2014).

107 *wSuz* belongs to the supergroup A (Siozios *et al.*, 2013), which contains several
108 *Wolbachia* strains known to induce antiviral protection (Martinez *et al.*, 2014). In the present
109 study, we have thus tested whether *wSuz* can protect *D. suzukii* against viruses. Four host
110 lines were compared, two from France, a country which was recently invaded by *D. suzukii*,
111 and two from Japan, its native range (Cini *et al.*, 2012; Asplen *et al.*, 2015). Two RNA
112 viruses were tested, *Drosophila C virus* (DCV; highly pathogenic *Drosophila* virus) and the
113 Flock House virus (FHV; isolated from a beetle) (Scotti *et al.*, 1983; Huszar & Imler, 2008).
114 We found that *wSuz* is able to protect *D. suzukii* against these two viruses but that the
115 antiviral protection is very variable between the host lines. This beneficial effect could
116 explain its maintenance in natural populations.

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121 **Results**

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

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

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

138 The DCV titer was lower in *Wolbachia*-infected flies than in uninfected ones (Two-
139 way ANOVA, $F=15.22$, d.f.=1, $P<0.001$; Fig. 1B), and this effect of *Wolbachia* did not
140 depend on the line (Two-way ANOVA, *Wolbachia* x host interaction: $F=0.45$, d.f.=1,
141 $P=0.509$; Fig. 1B).

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145 *Wolbachia* effect on FHV infection

146 Given the difference in the degree to which *wSuz* increases the survival of *D. suzukii* after
147 DCV infection between lines we then investigated the effect of *wSuz* on FHV infection in
148 four genetic backgrounds: the effect of the French *Wolbachia* isolate, *wSuz-Fr*, in two French
149 backgrounds Fr-CP and Fr-BE, and the effect of the Japanese isolate, *wSuz-Jp*, in two
150 Japanese backgrounds Jp-OGH and Jp-YSG. A total of 800 flies were stabbed with FHV and
151 800 others with Ringer's solution (Fig. 2A). In the absence of viral infection neither
152 *Wolbachia* nor the host genetic background affected survival (Ringer control treatment, Cox's
153 mixed effect model, *Wolbachia* effect: $\chi^2=1.83$, d.f.=1, $P=0.180$; host effect: $\chi^2=1.43$, d.f.=3,
154 $P=0.7$; *Wolbachia* x host interaction: $\chi^2=1.22$, d.f.=3, $P=0.750$).

155 In FHV-infected flies, survival was significantly affected by the *Wolbachia* infection
156 ($\chi^2=31.88$, d.f.=4, $P<0.001$), the host genetic background ($\chi^2=39.55$, d.f.=6, $P<0,001$) and we
157 found a significant interaction between these two factors ($\chi^2=14.99$, d.f.=3, $P=0.002$). Because
158 we cannot exclude the possibility that the French and the Japanese lines are infected by a
159 different *Wolbachia* isolate (*wSuz-Fr* and *wSuz-Jp* respectively), we also tested the *Wolbachia*
160 and the host genetic background effects on infected flies' survival for the French and Japanese
161 lines separately. The French lines survival was significantly affected by the *Wolbachia*
162 infection ($\chi^2=17.75$, d.f.=2, $P<0.001$), the host genetic background ($\chi^2=34.14$, d.f.=2,
163 $P<0,001$) but there was no significant interaction between these two factors ($\chi^2=3.73$, d.f.=1,
164 $P=0.053$). In the Japanese lines, the survival rate was affected by the *Wolbachia* infection
165 ($\chi^2=14.18$, d.f.=2, $P<0.001$), the host genetic background ($\chi^2=10.54$, d.f.=2, $P=0.005$) and we
166 detected a significant interaction between these two factors ($\chi^2=8.41$, d.f.=1, $P=0.004$). By
167 comparison with the uninfected lines, the *wSuz* infection significantly increased the survival
168 of the Fr-BE and the Jp-YSG backgrounds (Tukey HSD, $P=0.012$ and $P<0.001$ respectively)

169 while it did not affect the survival of the Fr-CP and the Jp-OGH backgrounds (CP line,
170 $P=0.191$; OGH line, $P=0.849$) (Fig. 2A).

171 As for DCV, we also measured FHV titers and we found a significant effect of both
172 the *Wolbachia* infection status (Two-way ANOVA, $F=5.04$, d.f.=1, $P=0.03$) and the host
173 genetic background (Two-way ANOVA, $F=98.88$, d.f.=1 $P<0.001$) on the RNA copy number
174 (Fig. 2B), with a significant interaction between these two factors (Two-way ANOVA,
175 $F=11.54$, d.f.=1, $P<0.001$). As for the survival data analysis, we tested the influence of the
176 presence of *Wolbachia* and the host genetic background for the French and the Japanese lines
177 separately. For the French lines the RNA copy number was affected by *Wolbachia* infection
178 (Two-way ANOVA, $F=4.32$, d.f.=1, $P=0.045$), the host genetic background (Two-way
179 ANOVA, $F=189.82$, d.f.=1, $P<0.001$) with a significant interaction between these two factors
180 (Two-way ANOVA, $F=21.01$, d.f.=1, $P<0.001$). For the Japanese lines, we also found a
181 significant interaction between the *Wolbachia* infection and the host genetic background
182 (Two-way ANOVA, $F=13.18$, d.f.=1 $P<0.001$), a significant effect of the host genetic
183 background (Two-way ANOVA, $F=88.80$, d.f.=1, $P<0.001$) but we did not detect a significant
184 effect of the *Wolbachia* infection (Two-way ANOVA, $F=1.05$, d.f.=1, $P=0.311$). More
185 precisely, in the presence of *wSuz*, the RNA copy number significantly decreased (around
186 50% of reduction; Fig. 2B) in the Fr-BE and Jp-YSG backgrounds infected with *wSuz*-Fr and
187 *wSuz*-Jp isolates respectively (Tukey HSD, $P<0.001$ and $P=0.039$ respectively), the two lines
188 that exhibited a significant effect of *Wolbachia* on survival after FHV infection, and not in the
189 two other lines (Tukey HSD test, Fr-CP line, $P=0.665$; Jp-OGH line, $P=0.478$).

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193 *Wolbachia* density

194 *Wolbachia* density is known to be a major determinant of antiviral protection, with higher
195 densities being associated to higher levels of protection (Chrostek *et al.*, 2014; Martinez *et al.*,
196 2014). We therefore measured *wSuz* density in the four lines and found significant differences
197 (One-way ANOVA, $F=10.07$, $d.f.=3$, $P<0.001$; Fig. 3): the two Japanese's backgrounds (Jp-
198 OGH and Jp-YSG) showed a higher density than the two French backgrounds (Fr-CP and Fr-
199 BE), but there was no significant differences between the two French lines (both infected by
200 *wSuz*-Fr; Tukey HSD, $P=0.991$) and between the two Japanese lines (that both harbor the
201 Japan *Wolbachia* isolate ; Tukey HSD, $P=0.062$).

202

203 **Discussion**

204 We have found that *wSuz* can protect its host against RNA viruses. In certain lines individuals
205 infected with *wSuz* had higher survival and lower viral titers after infection with DCV and
206 FHV. It is known since 2008 that *Wolbachia* can protect *Drosophila* against RNA viruses
207 (Hedges *et al.*, 2008; Teixeira *et al.*, 2008), but this is the first time that it is described in *D.*
208 *suzukii*. In a recent study another direct fitness benefit of *Wolbachia* has been observed in an
209 Italian population of *D. suzukii* since infected females have a higher fecundity than uninfected
210 ones (Mazzetto *et al.*, 2015). These phenotypes can potentially explain the maintenance of
211 *Wolbachia* strains in natural populations without reproductive manipulation (Fenton *et al.*,
212 2011), as it has been found in American and European populations of *D. suzukii* (Hamm *et*
213 *al.*, 2014; Cattel *et al.*, 2016).

214 The variability of the *wSuz* prevalence could be the consequence of heterogeneity in
215 virus-induced selection similarly to what was observed in the Pea Aphid *Acyrtosiphon*
216 *pisum*. This species is protected against parasitoids by the symbiont *Hamiltonella defensa*,

217 which has variable prevalence among populations and is thought to be maintained by
218 negative-frequency dependent selection depending on the parasitism pressure in the field
219 (Oliver *et al.*, 2008). We found that *Wolbachia* mediated significant protection in *D. suzukii*
220 (Fr-CP for DCV, Fr-BE and Jp-YSG for FHV) was associated with reduced viral titer.
221 However, for DCV, the presence of *Wolbachia* correlates with a lower viral titer even when
222 no effect on the flies' survival was detected (Jp-OGH line). Several studies showed that
223 antiviral protection is generally explained by a phenomenon of resistance that reduces the
224 accumulation of virus but, in some cases, no differences in viral titers were observed despite
225 the protective effect (Teixeira *et al.*, 2008; Osborne *et al.*, 2009). In the latter case, it is
226 possible that *Wolbachia* does not affect the replication of the virus but rather makes the host
227 more tolerant to viral infection.

228 Experimental studies have shown that *Wolbachia*-mediated antiviral protection is a
229 common phenomenon in *Drosophila* and mosquitoes (Bian *et al.*, 2010; Hedges *et al.*, 2008;
230 Moreira *et al.*, 2009; Osborne *et al.*, 2009; Teixeira *et al.*, 2008; Chrostek *et al.*, 2013;
231 Chrostek *et al.*, 2014; Martinez *et al.*, 2014) but is strongly dependent on the *Wolbachia* strain
232 (Hedges *et al.*, 2008; Osborne *et al.*, 2009; Chrostek *et al.*, 2013; Chrostek *et al.*, 2014;
233 Martinez *et al.*, 2014). For instance, Martinez *et al.*, 2014 showed that among 19 *Wolbachia*
234 strains (originating from 16 *Drosophila* species) transferred into the same *D. simulans*
235 genotype, only half of them induced protection against DCV and FHV. The effect of host
236 genetics on protection is less well understood. However, the protective phenotype is affected
237 by the host species. For example, the strain *wInn* protects its natural host *D. innubila* against
238 FHV (Unckless & Jaenike, 2012) but has no effect in *D. simulans* (Martinez *et al.*, 2014).
239 Here, we found that the level of antiviral protection varied among the lines we used. This
240 difference was most dramatic in the DCV experiment, where we found large increases in the

241 survival of the French line but not the Japanese line. This difference could be caused by
242 genetic differences between the *Wolbachia* isolates, the flies or both. In the FHV experiment
243 we were able to compare the same *Wolbachia* isolates in two host genetic backgrounds. We
244 found a host background effect for both the Japanese and the French lines suggesting that host
245 factors may affect the expression of the *Wolbachia*-mediated protection. However, we would
246 caution that this needs further confirmation as we only have a single replicate line of each
247 *Wolbachia* isolate in each genetic background, so we cannot rule out other possible
248 differences (e.g. gut microbiota, or uncontrolled differences in the genetic background).
249 *Wolbachia* density is known to influence the level of protection (Osborne *et al.*, 2009;
250 Osborne *et al.*, 2012; Chrostek *et al.*, 2013; Chrostek *et al.*, 2014; Martinez *et al.*, 2014).
251 However, we didn't find any clear association between the level of protection and the density
252 of *Wolbachia*. The variation in antiviral protection could also be influenced by tissue tropism
253 of *Wolbachia* since Osborne *et al.*, 2012 highlighted that tissue tropism can partly explain
254 variations in the level of protection. Therefore it is possible that, in the *D. suzukii* lines used in
255 our study, the tissue tropism of *Wolbachia* was different despite showing very similar density
256 at the whole fly level.

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

265 **Experimental procedures**

266 *D. suzukii* lines and rearing

267 In this study, four lines of *D. suzukii* were used, two originating from France and two from
268 Japan. The French lines were collected in Compiègne (named Fr-CP) and in Bellegarde
269 (named Fr-BE) in 2011 and 2012 respectively and reared in large populations. The Japanese
270 lines have been obtained from the Ehime-fly stock center in 2011: they were sampled in
271 Yamagata (named Jp-YSG) (I#E-15016 YSG-11) and Tokyo (named Jp-OGH) (#E-
272 15014OGH06-03) in 2006. These lines have been chosen because two are free of *Wolbachia*
273 (Fr-BE and Jp-OGH) and the two others (Fr-CP and Jp-YSG) are 100% infected with
274 *Wolbachia* (see below for diagnostic PCR test). The flies were reared on a cornmeal diet
275 (agar: 1%, dextrose: 8.75%, maize: 8.75%, yeast: 2%, nipagin: 3%) and maintained in an
276 incubator at constant temperature (22°C) and humidity (70%) with a 12-hours light/dark
277 cycle. An MLST analysis performed on 6 genes (*ftsZ*, *fbpA*, *hcpA*, *coxA*, *gatB* and *wsp*)
278 revealed the *Wolbachia* isolates from Fr-CP and Jp-YSG lines to be the same sequence type
279 with 100% identity between the sequences. The sequences obtained in the present study are
280 recorded in Genbank as KS308222-7.

281

282 *Control of host genetic background and infection status*

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

286 Antibiotic treatments were performed for 3 generations in Fr-CP and Jp-YSG lines.

287 At each generation larvae were fed on medium with 0.25 mg.mL⁻¹ tetracycline. After 3
288 generations, 10 isofemale lines were established from treated females and the presence of

289 *Wolbachia* was checked by PCR as described below in mothers and then for 3 generations
290 more. Only one isofemale line was retained for each nuclear background (Fr-CP and Jp-YSG)
291 and maintained for 12 generations before the experiments. The absence of *Wolbachia* in these
292 lines was confirmed by real-time quantitative PCR (see below). Using this approach, we
293 obtained infected and cured lines with the same genetic background, Fr-CP or Jp-YSG.

294 To obtain infected and uninfected individuals with the same Fr-BE or Jp-OGH
295 genetic backgrounds, back-crosses were done for 8 generations. Two males from the
296 uninfected line (Fr-BE or Jp-OGH) were mated with single virgin females from the infected
297 lines from the same country, *i.e.* Fr-CP and Jp-YSG respectively. Backcrossing was
298 performed for a total of 8 generations which lead to an introgression of around 99.6% of the
299 nuclear background assuming no selection on the nuclear genome. However, compared with
300 the use of antibiotics treatments, lines obtained with this method have different mitochondrial
301 backgrounds. These two lines were maintained for 15 generations before the experiments. The
302 *Wolbachia* infection status of each line was verified by PCR just before the viral infection
303 experiment.

304

305 *Viral isolates*

306 Two viruses, *Drosophila C virus* (DCV) and *Flock House virus* (FHV), were used in this
307 study. DCV is a highly pathogenic *Drosophila* virus, which belongs to the family
308 Dicistroviridae (Huszar & Imler, 2008); FHV, which belongs to the Nodaviridae family, is not
309 a natural pathogen of *Drosophila* species and was initially isolated from a beetle (Scotti *et al.*,
310 1983). Viruses were produced and titrated as described by Martinez *et al.*, 2014. DCV was
311 produced and titrated in Schneider's Line 2 cells (SL-2) and FHV was titrated in Schneider
312 *Drosophila* Line 2 cells (DL2) (<https://dgrc.bio.indiana.edu/cells/Catalog>). For each infection

313 assay, one viral aliquot was defrosted just before the infection and diluted in Ringer's solution
314 (Sullivan *et al.*, 2000) to reach a viral concentration of 5×10^8 .mL⁻¹ TCID50 for DCV and
315 3.6×10^{10} .mL⁻¹ TCID50 for FHV.

316

317 *Survival assay*

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

328 For each line 3 days-old females were collected. After being anaesthetized with CO₂,
329 they were inoculated with DCV, FHV or Ringer's solution by stabbing flies. Groups of 20
330 stabbed flies were immediately placed into a vial of fly cornmeal medium and stored at 22°C.
331 Flies were transferred into fresh vials of food every 3 days and the number of dead flies was
332 recorded every day. The survival assay was replicated 5 times on independent cohorts of flies
333 across multiple days, corresponding to a total of 100 flies for each *Wolbachia* infection status
334 and virus infection treatment.

335

336

337 *Diagnostic polymerase chain reaction (PCR)*

338 The *Wolbachia* infection status of individuals was verified by PCR for each line just before
339 performing the experiments. DNA was extracted on pools of 10 individuals (one pool per
340 line) homogenized in 200 μ L of 5% w/v Chelex resin in water (Biorad) with 4 μ L of proteinase
341 K (20mg.mL⁻¹) and kept at 56°C for 3h. After 15min at 95°C, samples were centrifuged at
342 16000g for 4min and stored at -20°C. Presence of *Wolbachia* was checked by amplifying the
343 *Wolbachia* Surface Protein (*wsp*) gene using the primers *wsp81F* and *wsp691R* (Braig *et al.*,
344 1998, Table S1). PCR reactions were performed in 25 μ L volumes containing 100 μ M dNTP,
345 200nM primers, 0.5IU DreamTaq® DNA polymerase (Eurobio) and 1 μ L of DNA template.
346 Cycling conditions were 94°C (2min), 94°C (30sec), 52°C (30sec), 72°C (45sec), 72°C
347 (10min) for 35 cycles. PCR products were visualized in 1% agarose gels.

348

349 *Real-time quantitative PCR (qPCR)*

350 The *Wolbachia* density, DCV and FHV RNA copy number were measured by real-time
351 quantitative PCR (qPCR) on the Light Cycler™ system using primers listed in Table S1. To
352 estimate *Wolbachia* density, 10 pools of ten 3 days-old virus-free females for each line were
353 prepared and the DNA extracted using the Genra Pure gene Tissue Kit (Qiagen). The
354 *Wolbachia* density was measured by quantifying the copy number of the *Wolbachia* gene *ftsZ*
355 relative to the host gene *Rpl32* using Sso Advanced Universal Probes Supermix (BioRad;
356 2min at 95°C followed by 40 cycles of 10sec at 95°C and 20sec at 60°C). The 10 μ L of
357 multiplex reaction mix contained 400nM of *Rpl32* primers and 200nM of *ftsZ* primers, 5 μ L of
358 SsoADVUniver Probes Supermix, 200nM of each probe and 2 μ L of DNA sample. The
359 *Wolbachia* density was estimated by dividing the copy number of the *ftsZ* gene by the copy
360 number of the *Rpl32* host gene. The antiviral protection was also examined by measuring the

361 RNA copy number after infection by both viruses. 3 days-old females were stabbed with
362 DCV and FHV and frozen respectively 5 and 2 days after infection. After homogenization in
363 TRIzol Reagent (Ambion), RNA was extracted from 10 pools of 10 flies for each
364 experimental treatment using the RNA Easy Mini® kit following the manufacturer's
365 instructions (Qiagen). Reverse-transcription was done using SuperScript® III First-Strand
366 Synthesis System (Invitrogen) including a 30 min DNase digestion step at 37°C. The copy
367 number of the viral RNA was compared to the control gene *Rpl32*. The qPCR reactions for
368 DCV, FHV and *Rpl32* were done separately with the same conditions (30sec at 95°C
369 followed by 40 cycles of 10sec at 95°C and 20sec at 60°C). The 10µL reaction mix contained
370 200nM of each primer, 5µL of SsoADV Univer SYBR Green Supermix, and 1µL of DNA
371 sample. The RNA copy number and the *Wolbachia* density were estimated by calculating the
372 ratio: $\frac{E(\text{virus}/\text{Wolbachia})^{\Delta Ct}}{E(\text{host})^{\Delta Ct}}$ with $\Delta Ct = Ct_{\text{flygene}} - Ct_{\text{virus}/\text{Wolbachia}}$ where E corresponds to the
373 efficiency of the PCR reaction calculated from a dilution series for each set of primers ($E =$
374 $2^{\frac{1}{\text{linear regression slope}}}$) and Ct to the cycle threshold (Pfaffl, 2001).

375

376 *Statistical analysis*

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

382 Survival data for DCV, FHV and their respective controls (Ringer) were analyzed
383 separately. For each virus, two models were fitted to test a potential effect of the *Wolbachia*

384 infection and the genetic background on survival for the control treatment (Ringer) without
385 virus or after infection with a virus. The first model allowed testing whether *wSuz* infection
386 modifies survival independently of viral infection and indirectly confirm that the survival of
387 virus-infected flies cannot be explained by an inherent effect of *Wolbachia* on survival. The
388 effects of *Wolbachia*, host genetic background and their interaction were considered as fixed
389 effects and the replicate vials as a random effect. When a significant interaction was detected,
390 differences between *Wolbachia*-free and *Wolbachia*-infected flies within each host genetic
391 background were analyzed using pairwise comparisons (Tukey's Honest Significance test) (R
392 package multcomp).

393 Viral titers and *Wolbachia* density were analyzed on log₂-transformed data. For viral
394 titers, a two-way ANOVA allowed testing for the effect of *Wolbachia*, the host genetic
395 background and their interaction. A one-way ANOVA was done to test for the influence of
396 the host genetic background on *Wolbachia* density. Pairwise comparisons (Tukey's Honest
397 Significance test) were also done if a global effect of *Wolbachia* was detected.

398

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