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1 **Back and forth *Wolbachia* transfers reveal efficient strains to control**  
2 **spotted wing drosophila populations**

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32

33 **Abstract**

34 1. Since its recent invasion of the European and American continents, the spotted wing  
35 *Drosophila*, *Drosophila suzukii*, has become a burden of the fruit industry. Armed with a  
36 highly sclerotized ovipositor, females can lay eggs in a wider variety of ripening and healthy  
37 fruits than other *Drosophila* species. Economic losses due to *Drosophila suzukii* reach  
38 millions of dollars annually and methods to control natural populations in the field mainly  
39 rely on the use of chemical pesticides.

40 2. We tested if *Wolbachia* bacteria represents a potential ally to control this pest. These  
41 symbionts are naturally present in many insects and often induce a form of conditional  
42 sterility called Cytoplasmic Incompatibility (CI): the offspring of infected males die, unless  
43 the eggs are rescued by the compatible infection, inherited from the mother that protects the  
44 embryo. A long-recognised, a strategy called the Incompatible Insect Technique (IIT) makes  
45 use of the CI phenotype to control insect populations through the mass release of infected  
46 males. To implement this technique in *D. suzukii*, we used back and forth *Wolbachia* transfers  
47 between *D. suzukii* and *D. simulans* to identify *Wolbachia* strains that can sterilize *D. suzukii*  
48 females despite the presence of *wSuz*, a natural *Wolbachia* infection in this species.

49 3. We identified two *Wolbachia* strains as potential candidates for developing IIT in *D.*  
50 *suzukii*. Both induce a very high level of CI in this pest which is not attenuated by the  
51 presence of *wSuz* in females. Moreover, the newly transferred *Wolbachia* do not affect the  
52 fitness or the mating competitiveness of the sterilizing males.

53 4. *Synthesis and applications*. Although several critical steps still need to be tested and  
54 developed outside the laboratory to achieve the control of *Drosophila suzukii* using  
55 Incompatible Insect Technique. By an experimental approach in large population cage, we  
56 showed that releases of transinfected males limits population size. Thus, we provide in this

57 study the proof of concept that this technique can be a very promising approach to control *D.*  
58 *suzukii* populations.

59

## 60 **Introduction**

61 *Drosophila suzukii*, the spotted wing *Drosophila*, has become a major burden for fruit growers  
62 since its recent invasion of the European and American continents (Goodhue *et al.* 2011;  
63 Calabria *et al.* 2012; Cini, Ioriatti & Anfora 2012; Cini *et al.* 2014; Asplen *et al.* 2015).  
64 Although the vast majority of *Drosophila* species are not fruit pests, *D. suzukii* is able to lay  
65 eggs on a wide variety of healthy ripening fruits, thanks to a sclerotized ovipositor (Mitsui,  
66 Takahashi & Kimura 2006). Internal larval feeding represents a direct damage that can also  
67 facilitate secondary infections by pathogens as fungi, yeasts or bacteria (Cini, Ioriatti &  
68 Anfora 2012; Hamby *et al.* 2012; Ioriatti *et al.* 2015). Most damages are reported on red  
69 fruits, with an approximate \$500 million annual loss in the US (Goodhue *et al.* 2011). In  
70 France and in Northern Italy, up to 100% destruction was reported on cranberries,  
71 strawberries and sweet cherries (Cini, Ioriatti & Anfora 2012). Control of *D. suzukii*  
72 populations in the field largely relies on chemical pesticides, a practice with serious  
73 drawbacks because of its use close to harvest and the consequent risk of high amount of  
74 residues left on fruits. In brief, there is an urgent need for developing effective, specific, and  
75 environmentally-friendly methods to fight against *D. suzukii*.

76 In this context, suppression of pest populations through the mass release of sterilizing  
77 males seems relevant, as it is highly specific, not polluting, and does not require the  
78 introduction of a new species into the environment (Bourtzis *et al.* 2014; Lees *et al.* 2015). In  
79 the Sterile Insect Technique (SIT), males are sterilized by irradiation and released for mating  
80 with wild females (Knipling 1955). This approach has already shown its effectiveness against  
81 agricultural insect pests like the New World screw-worm fly (Lindquist, Abusowa & Hall

82 1992). Genetic population suppression approaches, such as Oxitec's RIDL technology could  
83 also be considered in principle (Black, Alphey & James 2011), but this technique faces  
84 serious regulatory issues in European Union. Finally, one can make use of the bacterial  
85 endosymbiont *Wolbachia pipientis* to produce sterilizing males (Boller *et al.* 1976; Riegler &  
86 Stauffer 2002), which is the option explored here.

87 The genus name "*Wolbachia*" designates a highly diverse clade of maternally  
88 transmitted intracellular symbionts of arthropods and nematodes, belonging to the  $\alpha$ -  
89 proteobacteria (Werren 1997). It is well known for its ability to manipulate host reproduction  
90 through different strategies that maximize its spread and maintenance in host populations.  
91 Among these, the most common appears to be Cytoplasmic Incompatibility (CI), a sperm-egg  
92 incompatibility occurring in crosses between infected males and uninfected females or  
93 between males and females infected by incompatible *Wolbachia* strains, leading to the death  
94 of embryos (Werren 1997). CI can be understood as resulting from a modification/rescue  
95 system (*mod/resc*) where the *mod* function modifies the sperm during spermatogenesis and  
96 the *resc* function, expressed in the egg, rescues the embryo through an interaction with the  
97 modified sperm. Recent findings indicate that a pair of genes located in a *Wolbachia* prophage  
98 operon, including a deubiquitylating enzyme, are very likely to encode the *mod* and *resc*  
99 functions (Beckmann, Ronau & Hochstrasser 2017; LePage *et al.* 2017). Depending on  
100 *Wolbachia* and host factors, some strains can both induce and rescue CI, while others express  
101 only the *resc* function, meaning that they do not induce CI but can rescue the embryos against  
102 the *mod* function expressed by other *Wolbachia* strains (Poinsot *et al.* 1998). CI has led to the  
103 proposal and development of the Incompatible Insect Technique (IIT), analogous to the SIT,  
104 where sterilization of the targeted populations is achieved by the release of *Wolbachia*-  
105 infected males incompatible with the resident females (Boller *et al.* 1976; Riegler & Stauffer  
106 2002). The first successful application of IIT was achieved in Burma where the target

107 population of *Culex pipiens*, vector of the filariasis, was almost eliminated (Laven 1967).  
108 Promising results were also obtained more recently in mosquito species under semi-field  
109 (Chambers *et al.* 2011; Moretti & Calvitti 2013; Atyame *et al.* 2015) and field (O'Connor *et*  
110 *al.* 2012) conditions. While initially limited to species where natural *Wolbachia* infections  
111 allowed the expression of CI, this method came to the fore again within the last decade,  
112 thanks to the possibility to transfer *Wolbachia* strains between species (Hughes & Rasgon  
113 2014). This approach has been successfully tested in the agricultural pest *Ceratitis capitata*,  
114 naturally uninfected, where the transfer of two *Wolbachia* strains from *Rhagoletis cerasi* led  
115 to the expression of a high level of CI (Zabalou *et al.* 2004b).

116 Here we aim at developing this promising approach in *D. sukukii*, where we face an  
117 additional challenge linked to the presence of *wSuz*, a natural *Wolbachia* strain, found in most  
118 *D. sukukii* populations but at variable frequencies (Hamm *et al.* 2014; Cattel *et al.* 2016a).  
119 Data indicated that *wSuz* is the only natural infection in *D. sukukii* where it does not induce  
120 strong CI (Hamm *et al.* 2014; Cattel *et al.* 2016a). These results indicate that the  
121 implementation of IIT in *D. sukukii* requires the introduction of a foreign *Wolbachia* strain  
122 that would induce a strong CI in *D. sukukii* and, crucially, a CI that would not be rescued by  
123 *wSuz*.

124 To identify such a strain, we first introduced *wSuz* in *Drosophila simulans* where  
125 many *Wolbachia* infections are maintained. This allowed us to assess the rescue capacities of  
126 *wSuz* against multiple CI-inducing strains after a single trans-infection experiment. We thus  
127 selected three promising strains that were then introduced in *D. sukukii* leading to the  
128 identification of two strains that induce a nearly complete CI in this background, regardless of  
129 the presence of *wSuz* in females. In addition, the transinfected males showed a similar  
130 competitiveness compared to naturally infected or uninfected males and are able to induce a  
131 high level of CI during all their life. Finally, we demonstrated that in large population cages,

132 the IIT can be very efficient to limit the increase of *D. sukukii* populations size. We thus  
133 obtained *D. sukukii* lines combining the properties required for an effective implementation of  
134 IIT.

135

## 136 **Materials and methods**

137

### 138 MICRO-INJECTIONS AND MATERNAL TRANSMISSION MEASUREMENT

139 Micro-injections were performed between *D. sukukii* and *D. simulans*, in both directions,  
140 using a micro-capillary needle to transfer the cytoplasm of infected embryos into uninfected  
141 ones, at the Fly Facility of the Department of Genetics of the University of Cambridge,  
142 following Poinot *et al.* (1998). Adult females emerging from the injected embryos (G0  
143 females) were crossed with uninfected males of the same genetic background (line STCP in  
144 *D. simulans* and Fr-BE-Ø in *D. sukukii*) and allowed to lay eggs during 5 days. We checked  
145 the presence of *Wolbachia* by PCR (see Table S1 for the protocol) in all G0 females and kept  
146 the offspring of the infected ones. This process was repeated until a perfect maternal  
147 transmission of *Wolbachia* was observed. All injected lines were maintained in the lab for at  
148 least 8 generations before beginning experiments.

149

### 150 *WOLBACHIA* STRAINS, *DROSOPHILA* LINES AND REARING PROCEDURES

151 Crossing experiments in *D. simulans* involved 10 *Wolbachia* strains, all belonging to the  
152 supergroup A (Martinez *et al.* 2015), 9 of which have been used in earlier studies (Poinot *et*  
153 *al.* 1998; Zabalou *et al.* 2008; Veneti *et al.* 2012; Martinez *et al.* 2014) (see Table S2).

154 In *D. sukukii*, we used one isofemale line, named Fr-BE-Ø, naturally free of  
155 *Wolbachia*, collected in 2012 in Bellegarde (France) and maintained since as a mass  
156 population. A line infected by *wSuz* (Fr-BE-*wSuz*) was obtained by back-crosses (see Cattel

157 *et al.* 2016b). The Fr-BE-Ø and Fr-BE-*wSuz* lines were maintained for respectively ~50 and  
158 40 generations before the crossing experiments. The Fr-BE-Ø line was also the recipient for  
159 injections of *Wolbachia* strains from *D. simulans*.

160 *D. simulans* and *D. sukukii* lines were reared on a cornmeal diet (agar: 1%, dextrose:  
161 8.75%, maize: 8.75%, yeast: 2%, nipagin: 3%) and maintained in an incubator at constant  
162 temperature (22°C) and humidity (50%) with a 12-hours light/dark cycle.

163

#### 164 CROSSING EXPERIMENTS

165 To analyze the *resc* function of *wSuz* in *D. simulans* we first performed mass crosses using  
166 infected females carrying *wSuz* with males infected by each of the *Wolbachia* strains  
167 available (see Table S2). Freshly emerged adults were sexed and placed separately into  
168 cornmeal diet tubes to ensure the virginity of flies. Ten virgin males (3 to 5-days old) and ten  
169 virgin females (5 to 6-days old) were allowed to mate in food vials for 24h. Females were  
170 then allowed to oviposit for 48h on grape-juice agar in a petri dish. The total number of  
171 hatched and unhatched eggs was recorded 48h after removal of the females. Six such mass  
172 crosses were performed for each *Wolbachia* strain tested.

173 Based on the results of mass crosses, three candidate strains were identified (*wRi*,  
174 *wTei* and *wHa*). To quantify more precisely the CI relationships between *wSuz* and these  
175 candidate *Wolbachia* strains, individual crosses were performed in *D. simulans* following the  
176 same protocol except that we used only 3-days old virgin males and 5-days old virgin females  
177 and that the egg hatch rates were estimated individually.

178 In *D. sukukii*, in order to make sure that mating had taken place, mating was either  
179 observed, confirmed by the hatching of at least one egg, or by the presence of sperm in the  
180 spermathecae. We also confirmed *D. sukukii* female's virginity before mating by placing them  
181 individually in a petri dish for egg-laying during 48h. Only 13 of the 70 females tested laid



182 eggs, but none of them hatched, confirming their virginity. For all individual crosses, at least  
183 20 repetitions were obtained, excluding females that laid fewer than 10 eggs. To account for  
184 variation in background embryonic mortality (not related to CI), we used a corrected index of  
185 CI ( $CI_{corr}$ ) (Poinsot *et al.* 1998) calculated as follows :  $CI_{corr} = [(CI_{obs} - CCM)/(100 - CCM)] * 100$ ,  
186 where  $CI_{obs}$  is the percentage of unhatched eggs observed in a given incompatible cross,  
187 and CCM is the mean mortality observed in the control crosses.

188

## 189 MEASURE OF LIFE HISTORY TRAITS

190 *Wolbachia* infection can negatively affect the fitness of its host depending on the *Wolbachia*  
191 strain, the host genotype and the environmental conditions. Because such costs could  
192 undermine the effectiveness of the IIT, we measured different life history traits on the lines  
193 transinfected by *wHa* and *wTei* and compared them to the naturally infected and uninfected  
194 lines (Fr-BE-Ø and Fr-BE-*wSuz*). The larval development conditions were standardized in  
195 these experiments by depositing 50 eggs of each line in 2mL of cornmeal diet placed in a tube  
196 with agar and sugar (10%). We prepared at least 12 such tubes per line and used the freshly  
197 emerged adults for the different measures.

198 We first measured the survival of the pre-adult stages, that is, from egg to adult. In  
199 each tube where 50 eggs were deposited, we counted the number of adults that emerged. We  
200 then measured the adult survival for both sexes. For each line, 10 freshly emerged adults were  
201 placed into a tube containing sweetened 10% agar (10 replicates per sex) and the mortality  
202 was recorded every day. Finally, to measure fecundity and hatch rate, we placed 10 virgin  
203 males and 10 females (1-day old) in a cornmeal diet tube during 48h for mating. Females  
204 were then allowed to oviposit for 48h on grape-juice agar in a petri dish. The total number of  
205 eggs and their hatch rate were recorded 48h after removal of the females. At least 18  
206 repetitions were performed for each line.

207 THE EFFET OF MALE AGE ON CI INTENSITY

208 To quantify the effect of ageing on CI levels in *D. sukukii* transinfected lines, we used the  
209 same protocol as described above for the mass crosses experiments in *D. simulans*. The effect  
210 of male age on CI intensity was tested for three ages, 3-4, 7-8 or 11-12 days (6 replicates per  
211 age), by crossing males infected by *wHa* or *wTei* with females naturally infected or not by  
212 *wSuz*. Control crosses were performed (between uninfected males and females, and between  
213 males and females carrying *wSuz*) in order to assess the effect of male age on hatch rate  
214 regardless of CI. Here again a corrected CI index ( $CI_{corr}$ ) was used in our analysis.

215

216 MATING COMPETITIVENESS OF TRANSINFECTED MALES

217 The mating competitiveness was tested for males infected by *wHa* by mixing 40 virgin  
218 females with different ratios of these sterilizing males with males carrying *wSuz* or uninfected  
219 males in cages of 30×30×30 cm. Five ratios were tested (4 replicates per ratio): 1:1  
220 ( $20\sigma:20\sigma wHa:40\varnothing$ ), 1:5 ( $7\sigma:35\sigma wHa:40\varnothing$ ), 1:10 ( $4\sigma:40\sigma wHa:40\varnothing$ ) and two  
221 control ratios, 1:0 ( $40\sigma wHa:40\varnothing$ ) and 0:1 ( $40\sigma:40\varnothing$ ). Similarly, the same ratios were  
222 used with males and females carrying *wSuz* instead of being uninfected. As for previous  
223 experiments, we used 5-6 days-old females and 3-4 days-old males. Females were first placed  
224 in the cages followed by the simultaneous release of all males, and mating was allowed for  
225 48h, with food and water supply (two recipients containing 50 ml of cornmeal diet, and two  
226 sweetened water sources with 10% sugar). Thereafter, females were allowed to oviposit for  
227 48h on grape-juice agar in petri dishes which were then replaced with new ones for another  
228 48h. The total number of hatched and unhatched eggs was recorded 48h after removal the  
229 petri dishes from the cage. We computed the competitiveness index (C) (Fried 1971) to  
230 compare the performance of sterilizing and compatible males, which is defined as follows:  
231  $C=(N/S)*[(H_c-H_i)/(H_i-H_s)]$ , where N is the number of “compatible” males, S is the number of

232 incompatible males,  $H_c$  is the hatch rate in the compatible crosses,  $H_i$  the hatch rate observed  
233 in the different ratios tested and  $H_s$  is the hatch rate in clutches from females exclusively  
234 crossed with incompatible males. Similarly, expected hatch rate values in male competition  
235 experiments were calculated as follows:  $[(N * H_c) + (S * H_i)] / (S + N)$ .

236

## 237 PROOF OF CONCEPT OF THE IIT EFFECTIVENESS

238 The IIT can be used to decrease the population size of the targeted species but also to limit the  
239 introduction and the population growth, and this is the point we tested here using males  
240 infected by  $wHa$ . Two cages of 3x3x2 meters were placed separately in climatic chambers  
241 with similar conditions of temperature (22°C), humidity (50%), and light (light/dark cycle of  
242 12-hours). In each cage, we placed 6 bottles of 1L of a cornmeal diet with a red fruits mixture  
243 (red fruits: 50%, agar: 2.6%, yeast: 12%, maize flour: 18%, sugar: 17%, nipagin: 0.4%) and  
244 10 bottles of 10cL of sweetened water (10%). In the control cage, 20 4-6 days old mated  
245 females and 20 males (half uninfected and half infected by  $wSuz$ ) were introduced at the  
246 beginning of the experiment and then again every 7 days. In the second cage, called the “IIT  
247 cage”, the same protocol was followed except that in addition to the 40 individuals released  
248 every 7 days, we introduced simultaneously 260 sterilizing males, corresponding to a ratio of  
249 13:1 ( $260 \sigma wHa : 20 \sigma (10 \sigma \emptyset + 10 \sigma wSuz)$ ). This experiment lasted 62 days, that is, about 9  
250 weeks, so that 360 individuals were released in the control cage and 2700 in the IIT cage  
251 (among which 2340 were sterilizing males). The aim of the experiment was to follow the  
252 evolution of the population size over time. Six nesting sites (grape-juice agar in a petri dish of  
253 9 cm) were placed in each cage and renewed every 48h. For each nesting site, the number of  
254 eggs laid was used as a proxy of the population size and the hatch rate was determined. At the  
255 end of the experiment, all living flies were captured and counted.

256

257 STATISTICAL ANALYSIS

258 We used generalized linear mixed models (GLMM) (binomial family) to analyse all hatch  
259 rates data.

260 - For the mass and individual crosses in *D. simulans* and in *D. sukukii*, the *Wolbachia* strain  
261 was included as a fixed factor and the replicates as a random factor.

262 - For the mating competitiveness analysis, the factors “ratio” and “female’s status” were  
263 included as fixed explanatory factors and the replicates as a random factor. Exact binomial  
264 tests were then used to compare the observed and expected hatch rates in the mating  
265 competitiveness experiment. Survival data were also analyzed by GLMM (gamma  
266 distribution and inverse link). The *Wolbachia* infection was included as a fixed explanatory  
267 factor and the replicates vials as a random factor.

268 - Fecundity data was analyzed using a GLMM (poisson family); the *Wolbachia* strain was  
269 included as a fixed effect and the replicates as a random factor.

270 - The survival rate from the egg to adult stage was analyzed with a linear mixed-effects model  
271 (Gaussian distribution) where the *Wolbachia* infection was included as fixed explanatory factor  
272 and the replicates as a random factor.

273 - In the last experiment, the number of eggs laid and the hatch rate were analysed using a  
274 GLM with a poisson and binomial families respectively.

275 Analysis were performed in R version 3.3.0 (R Core Team 2016), using the package *lme4* for  
276 all mixed models (Bates *et al.* 2014).

277

278 **Results**

279 *w*Suz INJECTION AND CROSSING EXPERIMENTS IN *D. SIMULANS*

280 The cytoplasm of *D. sukukii* embryos infected by *w*Suz was injected into 234 *D. simulans*  
281 *Wolbachia*-free embryos. Seven isofemale lines proved to be infected by *Wolbachia* in G0.

282 Patterns of maternal transmission of *wSuz* are presented in Fig. S1. One isofemale line  
283 showing a 100% transmission from G2 to G3 (20 individuals tested) was selected for CI tests.

284 CI experiments were designed to select potential *Wolbachia* strains for the sterilization  
285 of *D. sukuzii* populations, that is, strains that would induce CI even when females carry *wSuz*.  
286 To this end, we performed crosses in *D. simulans* between males infected by candidate strains  
287 and females carrying *wSuz*. In control crosses, *i.e.* crosses between males and females both  
288 infected by *wSuz*, the hatch rate was 97.5%. Among the 10 *Wolbachia* strains tested, known  
289 to induce CI in this genetic background (STCP, Martinez *et al.* 2015), three did not appear to  
290 induce CI against *wSuz*-infected females. Indeed, hatch rates were not significantly reduced in  
291 crosses involving the *wStv*, *wPro* and *wMelCS* strains compared to the control crosses (Fig.  
292 1). This indicates that *wSuz* does express a functional *rescue* in *D. simulans* against these  
293 strains, and discards them as potential candidates. On the contrary, reduced hatch rates were  
294 observed in crosses involving males carrying the other strains. Following Poinot *et al.* (1998)  
295 we computed a CI index ( $CI_{corr}$ ) taking into account the basal embryonic mortality, to indicate  
296 only the proportion of embryos killed by CI. The highest  $CI_{corr}$  levels were observed with *wRi*  
297 (57,99%  $\pm$ 13,73), *wHa* (82,59%  $\pm$ 11,60) and *wTei* (84,74%  $\pm$ 11,78) (Fig. 1).

298 Focusing on these three promising strains, we performed individual crosses between  
299 males carrying these strains and females either uninfected (used as control) or infected with  
300 *wSuz* to characterize more precisely the rescue capabilities of *wSuz*. We thus showed that the  
301 presence of *wSuz* in females partially rescues the CI induced by *wRi* (with a 29% decrease of  
302  $CI_{corr}$ ) ( $z=3.53$ ,  $P<0.001$ ) and *wTei* (with a 23% decrease in  $CI_{corr}$ ) ( $z=3.57$ ,  $P<0.001$ ) but not  
303 *wHa* ( $z=0.09$ ,  $P=0.97$ ) (Fig. 2). At that stage, *wHa* thus appeared to be the most promising  
304 strain, but we injected all the three strains into *D. sukuzii* in case host effects would change  
305 the rescue capabilities of *wSuz* in its natural host.

306

## 307 CI EXPRESSION OF *WOLBACHIA* CANDIDATES IN *D. SUZUKII*

308 The cytoplasm of *D. simulans* embryos infected by *w*Ri, *w*Ha or *w*Tei was injected into  
309 uninfected *D. suzukii* embryos. As for *D. simulans*, isofemale lines were created from infected  
310 females for 2 more other generations until perfect transmission of *Wolbachia* was observed  
311 (from G2 to G3; see details in Table S3). For each *Wolbachia* strain, one isofemale line was  
312 then selected for crossing experiments.

313 We performed CI crosses to assess (i) if *w*Ri, *w*Ha and *w*Tei can induce CI in *D.*  
314 *suzukii* (transinfected males crossed with uninfected females) and (ii) if *w*Suz in its natural  
315 host is able to rescue these effects (transinfected males crossed with females infected by  
316 *w*Suz). We found that *w*Ha and *w*Tei induce strong CI when infected males are crossed with  
317 uninfected females in *D. suzukii* (95.57% and 96.46% CI<sub>corr</sub>, respectively), in contrast to *w*Ri  
318 for which the percentage of unhatched eggs was nearly as low as in the control compatible  
319 crosses (18.21% CI<sub>corr</sub>) (Fig. 3). In addition, the low CI induced by *w*Ri was fully rescued by  
320 the presence of *w*Suz in females, while the strong CI induced by *w*Ha and *w*Tei was not.  
321 When males infected by *w*Ha were crossed with females carrying *w*Suz, only 34 eggs hatched  
322 in total out of the 960 eggs laid (3.54%). Moreover, no egg hatched in 13 of the 30 individual  
323 crosses. For the *w*Tei strain, 33 eggs hatched out of 842 (3.92%) with 0% hatch rates in 25 of  
324 the 33 individual crosses. In two crosses, higher hatch rates (94.7% and 54.6 %) were seen,  
325 suggesting the induction of CI may occasionally fail or that occasionally some males can be  
326 uninfected, although an infection rate of 100% has always been observed in the line used here.

327

## 328 LIFE HISTORY TRAITS OF THE TRANSINFECTED LINES

329 Adult longevity data revealed a significant effect of the infection status on this trait in both  
330 males and females ( $\chi^2= 121.95$ , d.f=3,  $P<0.001$ ;  $\chi^2= 38.98$ , d.f=3,  $P<0.001$ ). However,  
331 this effect does not indicate any physiological cost of the *w*Ha or the *w*Tei *Wolbachia* strain.

332 On the contrary, males and females infected by *w*Tei had a greater longevity than the other  
333 lines (Fig. S2A and B). In females, there was no significant difference between the three other  
334 lines (uninfected, *w*Suz and *w*Ha) (Fig. S2A) while in males, individuals infected by *w*Suz  
335 showed a higher longevity than uninfected ones (Fig. S2B).

336 The infection status also affects the fecundity with a significantly larger number of  
337 eggs laid in 48h in the *w*Tei ( $26.39 \pm 12.03$ ) and *w*Suz lines ( $26.22 \pm 8.17$ ) (between which no  
338 significant difference was found; GLMM:  $z = -0.32$ ,  $P = 0.752$ ). No significant difference was  
339 detected between the uninfected ( $21.15 \pm 10.82$ ) and the *w*Ha ( $22.29 \pm 9.14$ ) lines (GLMM:  $z = -$   
340  $0.74$ ,  $P = 0.456$ ) (Fig. S3A). We also found an effect of *Wolbachia* infection on hatch rates,  
341 irrespective of CI (Fig. S3B): the *w*Tei and uninfected lines show higher basal hatch rates  
342 ( $91.33\% \pm 15.59$  and  $94.59\% \pm 9.14$  respectively) than the *w*Ha and *w*Suz lines ( $83.31\%$   
343  $\pm 25.50$  and  $83.83\% \pm 25.36$ ).

344 The infection status also appears to impact survival rates at the pre-adult stage, that is,  
345 from egg to adult. Uninfected individuals showed a significantly higher survival rate than the  
346 other lines ( $\emptyset$ :  $75.33\% \pm 6.34$ ; *w*Suz:  $64.38\% \pm 12.63$ ; *w*Tei:  $64.00\% \pm 8.49$ ; *w*Ha:  $62.17\%$   
347  $\pm 3.86$ ), while there was no significant difference between the three infected lines (Fig. S4).

348

#### 349 MALE AGE AND CI INTENSITY

350 In the control crosses, although hatch rates varied slightly between experiments performed  
351 with young or old males, there was no trend indicating an increase or decrease of basal hatch  
352 rates with male age (Fig. 4).

353 In contrast, we observed an overall decrease in CI intensity with male age (Fig. 4A  
354 and B). In crosses between males infected by *w*Ha and uninfected females, the  $CI_{\text{corr}}$  dropped  
355 from  $97.65\%$  ( $\pm 2.81$ ) in 3-4-days-old males to  $79.72\%$  ( $\pm 11.97$ ) in 11-12-days-old males. In

356 crosses with *wSuz*-infected females, the *wHa*  $CI_{corr}$  dropped from 97.30% ( $\pm 2.50$ ) to 78.22%  
357 ( $\pm 9.36$ ) (Fig. 4A).

358 The decrease in CI intensity was larger in crosses involving *wTei*-infected males,  
359 dropping from 94.77% ( $\pm 5.00$ ) to 60.68% ( $\pm 16.35$ ) and from 93.22% ( $\pm 6.02$ ) to 60.71%  
360 ( $\pm 10.78$ ) in crosses with uninfected and *wSuz* females, respectively (Fig. 4B). Notably, these  
361 experiments also confirmed that females infected by *wSuz* cannot rescue the CI induced by  
362 males infected by *wHa* or *wTei*, regardless of male age.

363

#### 364 MATING COMPETITIVENESS OF STERILIZING MALES

365 In this experiment, we selected the *wHa* strain, one among the two candidates, because its CI  
366 effect was less affected by male age (full data is provided in Table S4). We first confirmed  
367 that *wHa*-infected males induced nearly 100% CI in *D. suzukii*, regardless of the presence of  
368 *wSuz* in females, with an average hatch rate of 0.40% ( $\pm 1.74$ ) and 0.44% ( $\pm 0.56$ ) in crosses  
369 with uninfected and *wSuz* females, respectively (Fig. 5A). Accordingly, we observed that  
370 hatch rates decrease when the proportion of sterilizing males increases (all ratios tested  
371 produce significantly different hatch rates, except the 1:5 and 1:10 ratios, GLMM:  $z=0.63$ ,  
372  $P=0.60$ ; Fig. 5A). No effect of females' infection status on hatch rates was detected (GLMM:  
373  $z=-0.50$ ,  $P=0.62$ ). The hatch rates observed in the ratios 1:0 and 0:1 (without transinfected  
374 males or only transinfected males, respectively) allowed us to calculate the hatch rate  
375 expected under the assumption of a similar mating competitiveness between sterilizing and  
376 compatible males. For each ratio tested, the observed and expected hatch rates were very  
377 close, with slight significant deviations. In three cases, the C index (Fried 1971) was less than  
378 1 (meaning that the transinfected males are less competitive than the compatible males) but  
379 the reverse was observed in 3 cases (Fig. 5B). Overall, these results indicate that uninfected  
380 and *wSuz* males have very similar mating capacity.



## 381 PROOF OF CONCEPT OF THE IIT EFFECTIVENESS

382 We finally aimed at assessing if repeated releases of sterilizing males could mitigate the  
383 increase of a *D. sukukii* population. We performed experiments in two cages of 3x3x2 meters.  
384 In these cages, a small *D. sukukii* population was introduced (20 mated females and 20 males,  
385 half uninfected and half infected by *wSuz*) and then again every 7 days. In the control cage,  
386 where no *wHa*-infected males were introduced, the number of eggs laid per time unit (48h)  
387 increased substantially during the first 38 days, which corresponds to about two generations  
388 (Fig. 6A). The increase continued until the end of the experiment, reaching 1408 eggs laid in  
389 48h. In the IIT cage, where *wHa*-infected males were regularly introduced, the number of  
390 eggs laid per 48h remained low and stable. It was between 0 and 49 per time unit until the  
391 53th days, and slightly increased to reach 308 eggs per time unit at the end of the experiment.  
392 The multiple releases of sterilizing males thus allowed to keep the population size 5 times  
393 smaller than the control cage and this effect is significant (GLM:  $z=-60.74$ ,  $P<0.001$ ).  
394 Accordingly, the hatch rate observed in the IIT cage was significantly different to the one  
395 obtained in the control cage (GLM:  $z=-20.14$ ,  $P<0.001$ ) (Fig. 6B). Among the 380 eggs laid in  
396 the IIT cage in the last 48h, only 120 were viable (39%) compared with the 1150 hatched eggs  
397 among 1408 in the control cage (82%). At the end of the experiment, that is, after 62 days, all  
398 live individuals were caught. We thus counted 2184 individuals in the control cage (55%  
399 females), and 664 individuals in the IIT cage (41% females). At the end of the experiment,  
400 there were thus 4.3 times less females in the IIT cage than in the control one.

401

## 402 Discussion

403 This study aimed at identifying strains of *Wolbachia* that could be candidates for controlling  
404 *D. sukukii* populations through IIT. We achieved this goal in several steps. We first  
405 transferred *wSuz*, the natural infection of *D. sukukii*, into *D. simulans* to test its ability to

406 rescue the CI induced by the many *Wolbachia* strains maintained in *D. simulans*. We thereby  
407 selected three *Wolbachia* candidates based on incompatibility with *wSuz*, injected them into  
408 *D. sukukii*, and validated two as highly promising for the development of IIT.

409 In the course of these experiments, we confirmed previously described CI patterns,  
410 namely a strong host effect affecting compatibility relationships in a strain-specific manner  
411 (Reynolds & Hoffmann 2002; Weeks, Tracy Reynolds & Hoffmann 2002). We will first  
412 discuss these elements before highlighting critical future developments for the effective  
413 implementation of IIT in *D. sukukii*. We found strong variation in CI intensity (*mod* function)  
414 depending on host factors: *wRi* induces only moderate CI in *D. sukukii* although it is well  
415 known to induce strong CI in its natural host *D. simulans* (Hoffmann, Turelli & Simmons  
416 1986; this study), but also after transfer in *D. melanogaster* (Poinsot *et al.* 1998). On the  
417 contrary, *wTei* does not induce CI in its natural host, *D. teissieri* (Zabalou *et al.* 2004a), but  
418 induces a high level of CI in both *D. sukukii* (this study) and *D. simulans* (Martinez *et al.*  
419 2015; this study). The *wSuz* infection exhibits the exact same pattern in its natural host and in  
420 *D. simulans*: it induces very low albeit significant CI (see Fig. S5A, B; 63.7% hatch rate and  
421 64.2% respectively). It is clear from these data that a given host should not be generally  
422 considered as permissive or refractory to *Wolbachia* infection and CI, given these strong host  
423 genotype-by-*Wolbachia* strain interactions. Moreover, the analysis of the CI relationship  
424 between *wSuz* and other *Wolbachia* strains in *D. simulans* and in *D. sukukii* reveals that not  
425 only the induction of CI (*mod* function) is host dependent, but also the ability to rescue CI  
426 (*resc* function).

427 Beyond these CI-relevant results, the main point of this study was to identify  
428 *Wolbachia* strains that could be used as biological control of *D. sukukii* populations through  
429 IIT. By performing back and forth *Wolbachia* transfers between *D. sukukii* and *D. simulans*,  
430 we identified two candidates, *wHa* and *wTei*. These two strains induce a very high level of CI

431 in *D. sukukii*, which is not attenuated by the presence of *wSuz* in females. A number of  
432 additional results further confirm that these transinfected lines can be envisaged to implement  
433 the control of *D. sukukii* populations. First, *wHa* and *wTei* show a perfect maternal  
434 transmission. Second, transinfected males induce a high level of CI throughout their life,  
435 despite a reduction with ageing. Finally, transinfected males do not suffer from reduced  
436 mating competitiveness or other fitness costs. Accordingly, we showed that the repeated  
437 release of sterilizing males can limit the explosion of a *D. sukukii* large cage population.  
438 Overall, this study provides, at a laboratory scale, a proof of concept that the IIT approach can  
439 be powerful to control *D. sukukii* populations.

440 An efficient IIT program must rely on efficient methods to avoid the release of fertile  
441 females, which could result in population replacement rather than population suppression  
442 (Bourtzis *et al.* 2014). In case of accidental release, the newly introduced infection would  
443 easily spread across *D. sukukii* populations, since the resident infection induces only very low  
444 CI. Notably, the fact that the *wHa* and *wTei* strains are mutually incompatible (Zabalou *et al.*  
445 2008) means that one strain might still be used for population control in case of accidental  
446 release and invasion of the first strain. To circumvent the difficult sexing step, IIT could be  
447 coupled with moderate irradiation (Brelsfoard, St Clair & Dobson 2009; Bourtzis *et al.* 2014;  
448 Zhang *et al.* 2015) that would be sufficient to sterilize females without affecting the life  
449 history traits and competitiveness of males which would be fully sterile thanks to the  
450 *Wolbachia* (Calvitti *et al.* 2012; Bourtzis *et al.* 2014). Finally, IIT relies on the massive  
451 production of males which seems achievable for such a small and polyphagous insect, but  
452 requires additional developments.

453 The existence of *D. sukukii* lines transinfected by CI inducing *Wolbachia* strains  
454 represents a critical step toward the implementation of IIT. Our results indicate that these lines  
455 carry a number of crucial properties that make them usable in practice to limit population size

456 in large population cages. While additional developments are still needed, we are now much  
457 closer to make IIT a credible alternative to pesticides to control *D. suzukii* populations.

458

#### 459 **Author's contributions**

460 JC, SC, FV, PG and LM conceived the project and designed its methodology; JC, TA, KN  
461 and DL collected the data; JC analyzed the data; JC, SC, FV, PG and LM led the writing of  
462 the manuscript. All authors contributed critically to the drafts and gave final approval for  
463 publication.

464

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472

#### 473 **Data accessibility**

474 Data available from the Dryad Digital Repository. DOI: 10.5061/dryad.4c7kq (Cattel *et al.*  
475 2018).

476

#### 477 **Supporting Information**

478 **Table S1.** Primers used in this study

479 **Table S2.** Details of all *D. simulans* lines used in this study

480 **Table S3.** Data on microinjections and maternal transmission of *Wolbachia* strains in *D.*  
481 *suzukii*

482 **Table S4.** Data on mating competitiveness of *w*Ha-transinfected males

483 **Figure S1.** Details of transmission rate of *w*Suz in isofemale line of *D. simulans*

484 **Figure S2.** Effect of *Wolbachia* infection on *D. suzukii* survival

485 **Figure S3.** Effect of *Wolbachia* infection on the fecundity and the hatch rate in *D. suzukii*

486 **Figure S4.** Effect of *Wolbachia* infection on the survival rate from the egg to adult stage

487 **Figure S5.** CI induced by *w*Suz in *D. suzukii* and *D. simulans*

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635 **Figures**

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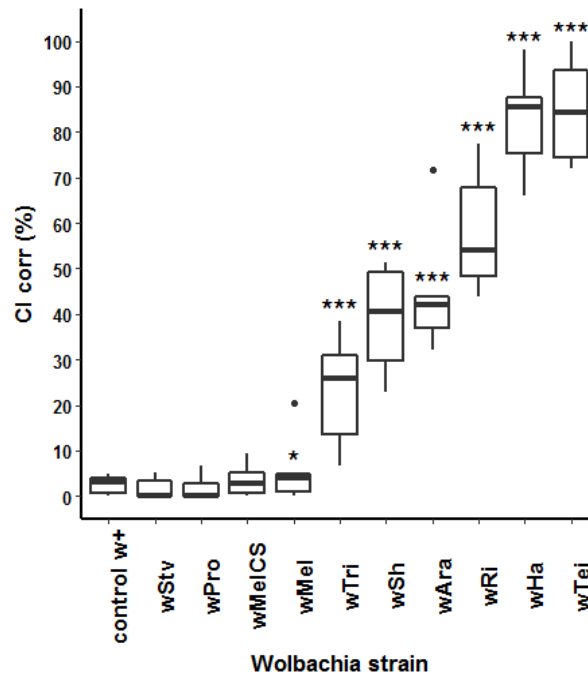
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648 **Fig .1.** CI levels estimated in mass crosses in *D. simulans* between infected males (*Wolbachia*  
649 strains named on the horizontal axis) and *wSuz*-infected females. The  $CI_{corr}$  index removes the  
650 basal embryonic mortality (estimated in control crosses); it is thus a measure of the CI-related  
651 mortality. GLMM (binomial family) was performed for comparisons with the control crosses.  
652 \*\*\*:  $P < 0.001$ ; \*:  $P < 0.05$ .

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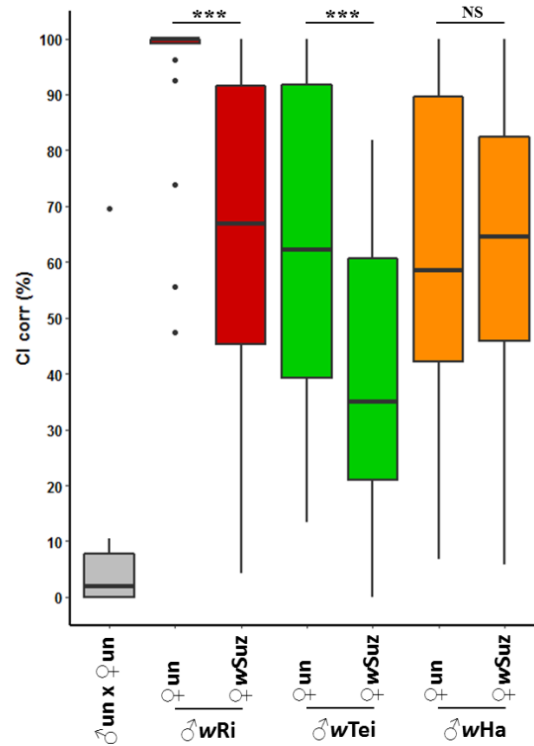
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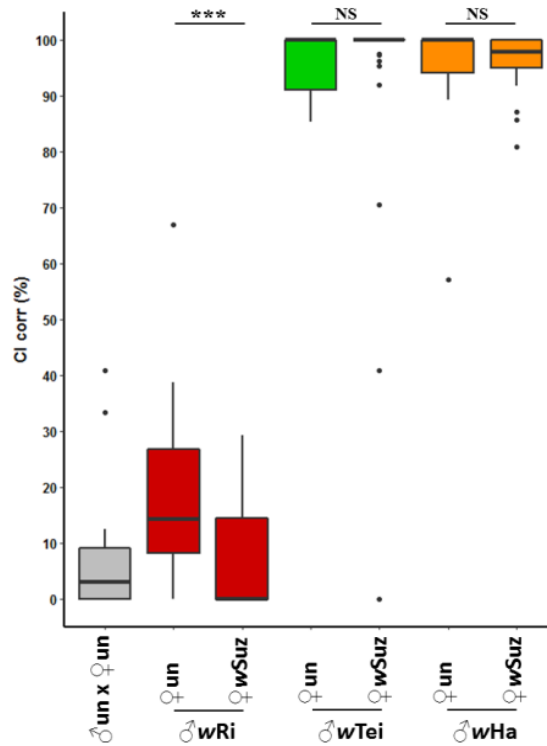
668 **Fig .2.** Assessment of the *resc* capabilities of *wSuz* in *D. simulans*. un: uninfected, *wSuz*:  
669 infected by *wSuz*, *wRi*: infected by *wRi*, *wTei*: infected by *wTei*, *wHa*: infected by *wHa*. The  
670  $CI_{corr}$  index removes the basal embryonic mortality (estimated in control crosses); it is thus a  
671 measure of the CI-related mortality. 20 repetitions were performed for each type of cross.  
672 \*\*\*:  $P < 0.001$ .

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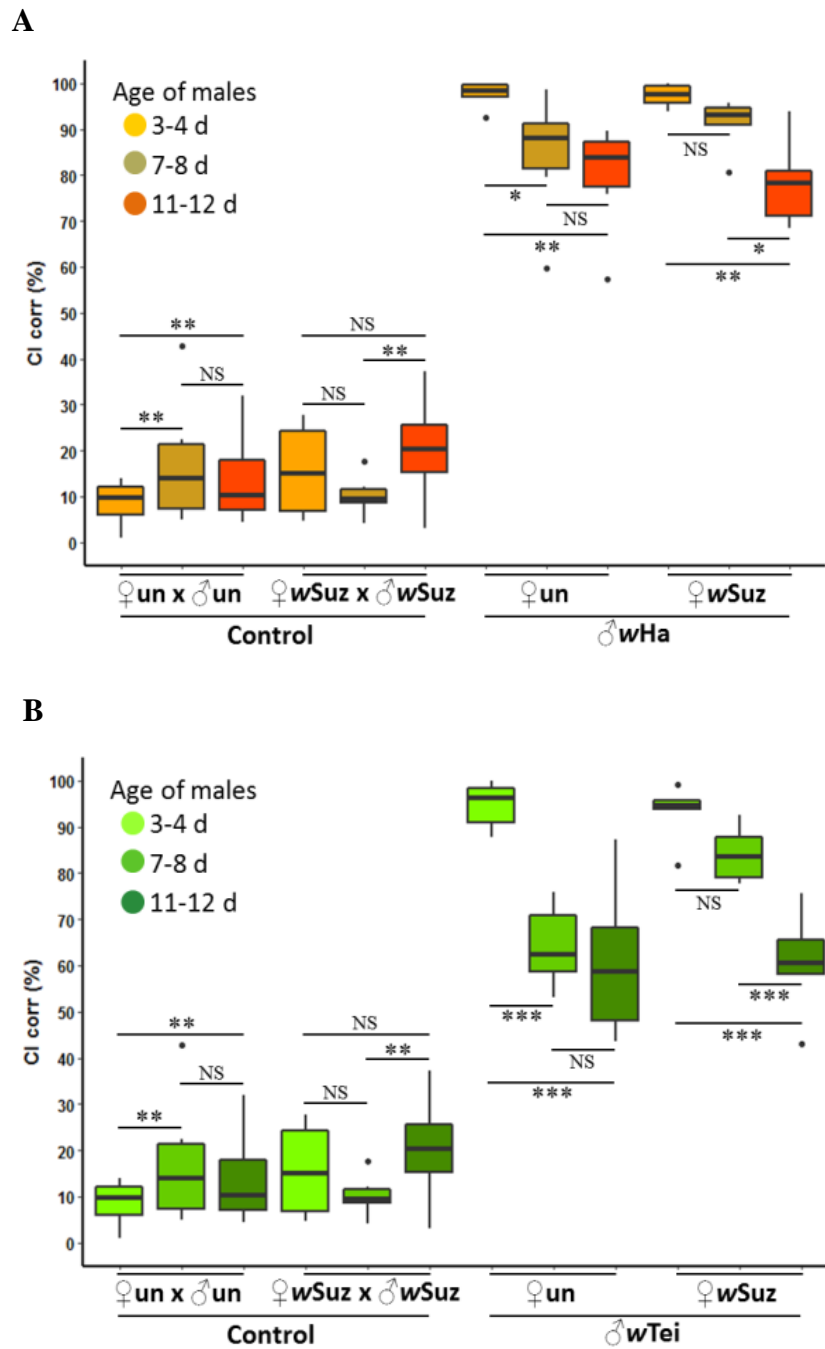
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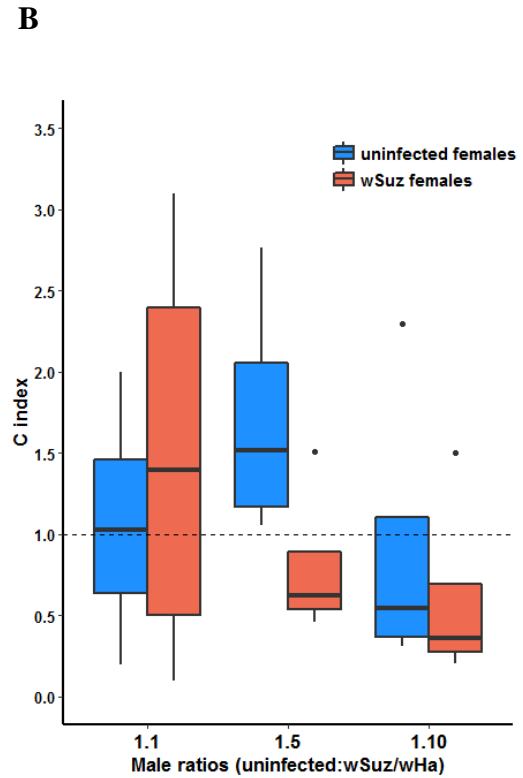
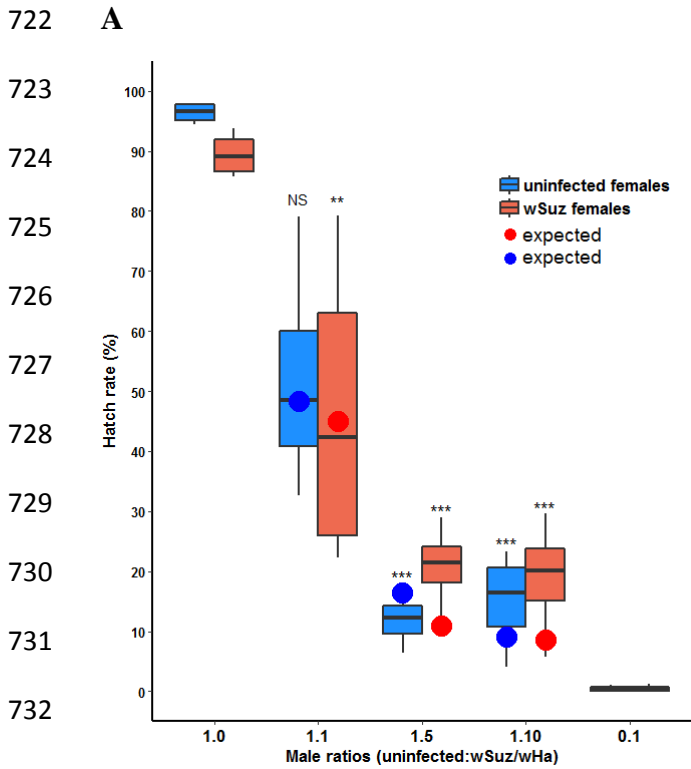
687 **Fig .3.** Measure of the *mod* function of *wRi*, *wHa* and *wTei* and the *resc* function of *wSuz* in  
 688 *D. suzukii*. un: uninfected, *wSuz*: infected by *wSuz*, *wRi*: infected by *wRi*, *wTei*: infected by  
 689 *wTei*, *wHa*: infected by *wHa*. The  $CI_{corr}$  index removes the basal embryonic mortality  
 690 (estimated in control crosses); it is thus a measure of the CI-related mortality. \*\*\*:  $P < 0.001$ .

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**Fig. 4.** Effect of male age on CI intensity in *D. sukuzii*. For each transinfected line (*wHa* or *wTei*), three different ages were tested, 3-4, 7-8 and 11-12 days. A: sterilizing males infected by *wHa*. B: sterilizing males infected by *wTei*. un: uninfected, *wSuz*: infected by *wSuz*, *wHa*: infected by *wHa*, *wTei*: infected by *wTei*. The  $CI_{corr}$  index removes the basal embryonic mortality (estimated in control crosses); it is thus a measure of the CI-related mortality. \*\*\*: $P < 0.001$ , \*\*: $P < 0.01$ , \*: $P < 0.05$ .



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735 **Fig. 5.** Mating competitiveness of the *wHa*-transinfected males. A: observed and expected  
 736 hatch rates; as expected, hatch rates decrease as the proportion of sterilizing males is  
 737 increased. Exact binomial tests were performed to compare the observed and expected hatch  
 738 values. \*\*\*:  $P < 0.001$ ; \*\*:  $P < 0.01$ . B: competitiveness index (C). Both expected hatch rates  
 739 and C index were computed following Fried (1971) (see Material and Methods for details).

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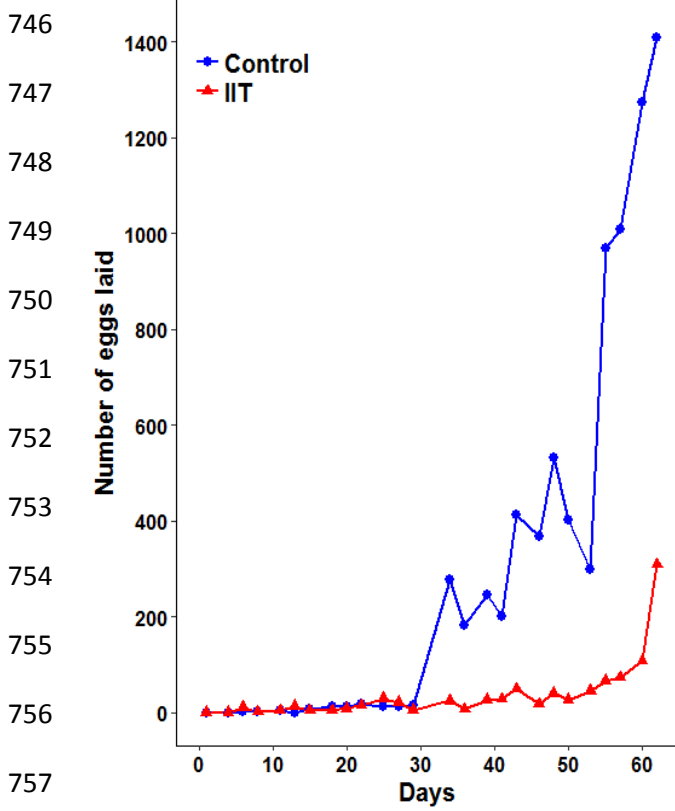
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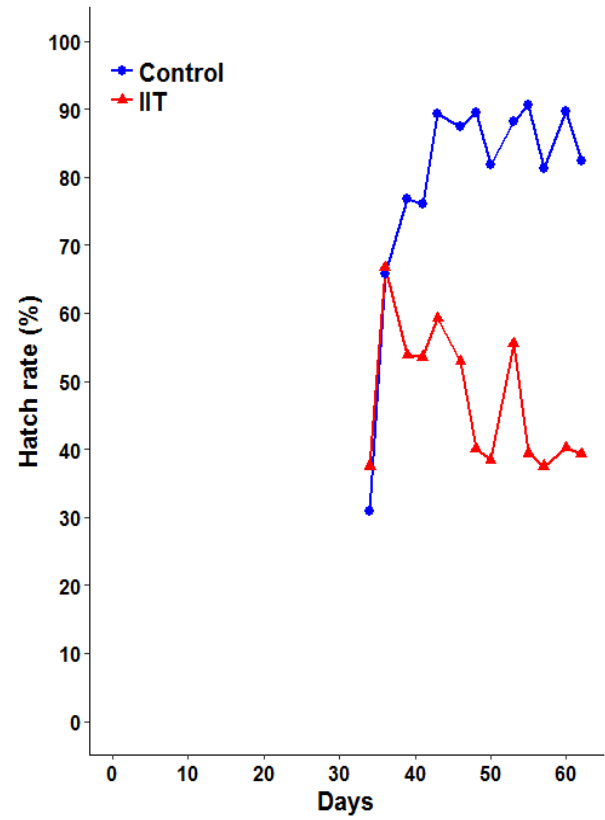
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745 **A**



**B**



758 **Fig. 6.** Evaluation of the IIT effectiveness to limit *D. sukuzii* population growth in a large  
759 climatic chamber. A: number of eggs laid per 48h at regular intervals. B: hatch rates at regular  
760 intervals.

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