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Julien Cattel, Katerina Nikolouli, Thibault Andrieux, Julien Martinez, Francis Jiggins, et al.. Back and forth Wolbachia transfers reveal efficient strains to control spotted wing drosophila populations. *Journal of Applied Ecology*, 2018, 55 (5), pp.2408-2418. 10.1111/1365-2664.13101 . hal-01916800

HAL Id: hal-01916800

<https://hal.science/hal-01916800>

Submitted on 7 Jan 2019

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

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25 **Key-words:** Cytoplasmic Incompatibility, Incompatible Insect Technique, Pest control,
26 spotted wing drosophila, *Wolbachia*, fruit industry, pesticide, bacteria

27 Running title: IIT design against *D. sukukii*
28 Number of words: 7549
29 Number of tables and figures: 0 table, 6 figures
30 Supporting information: 4 tables, 5 figures
31 Number of references: 43
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 α -
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. suzukii* 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♂:20♂*wHa*:40♀), 1:5 (7♂:35♂*wHa*:40♀), 1:10 (4♂:40♂*wHa*:40♀) and two
221 control ratios, 1:0 (40♂*wHa*:40♀) and 0:1 (40♂:40♀). 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_{corr}, 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_{corr}) (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 ± 12.03) and *w*Suz lines (26.22 ± 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 ± 10.82) and the *w*Ha (22.29 ± 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 ± 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 ± 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_{corr} dropped
355 from 97.65% (± 2.81) in 3-4-days-old males to 79.72% (± 11.97) in 11-12-days-old males. In

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

358 The decrease in CI intensity was larger in crosses involving *wTei*-infected males,
359 dropping from 94.77% (± 5.00) to 60.68% (± 16.35) and from 93.22% (± 6.02) to 60.71%
360 (± 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% (± 1.74) and 0.44% (± 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

465 **Acknowledgments**

466 This work was funded by the CNRS (IFR41-UMR5558) and supported by the ONEMA (APR
467 Biodiversité-Ecophyto), the ANR (ANR-15-CE21-0017-01) and the FWF (I 2604-B25).
468 Julien Cattel was the recipient of a PhD studentship from the Rhône-Alpes region (“ARC
469 Program” Grant). We also warmly thank Yuk Sang Chan at Cambridge University, for his
470 invaluable competence on *Wolbachia* transinfections, and Aymeric Bellemain and Justine
471 Planche for their technical contribution.

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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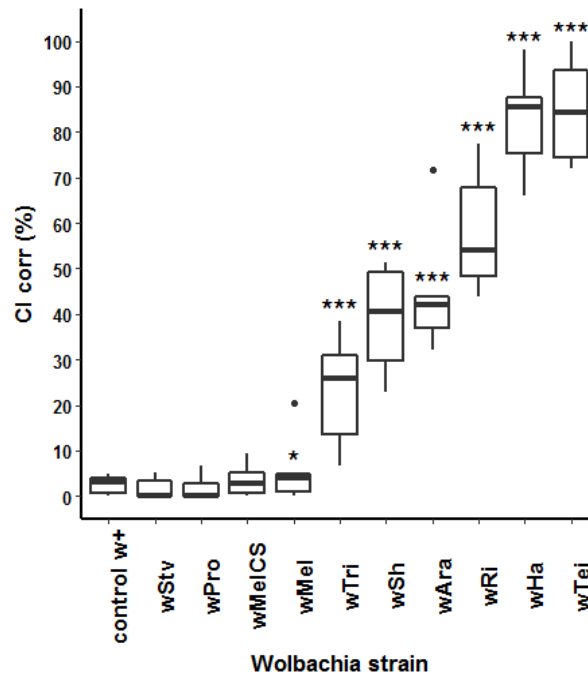
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648 **Fig .1.** CI levels estimated in mass crosses in *D. simulans* between infected males (*Wolbachia*
649 strains named on the horizontal axis) and *wSuz*-infected females. The CI_{corr} index removes the
650 basal embryonic mortality (estimated 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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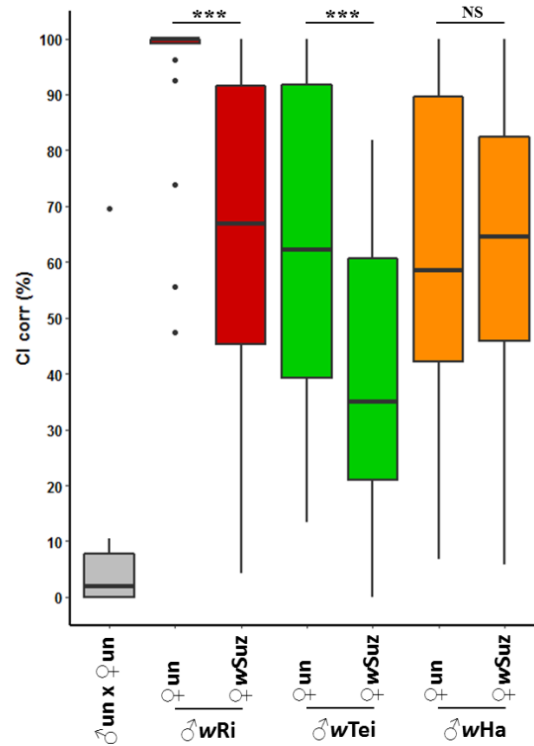
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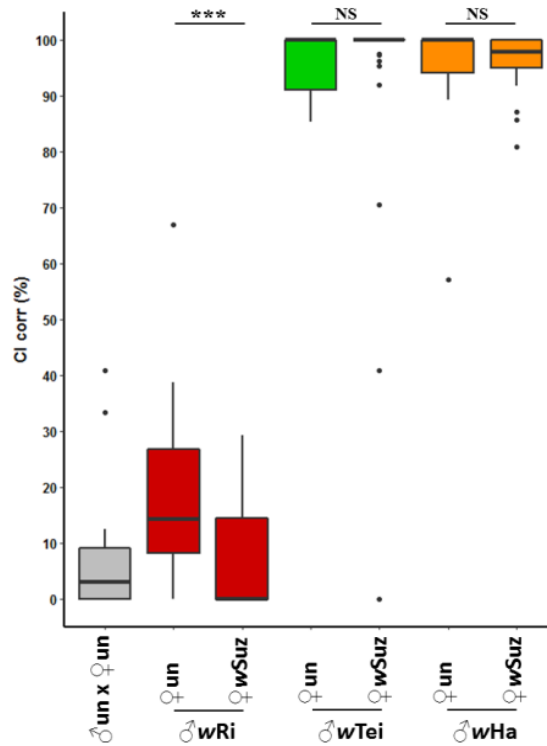
668 **Fig .2.** Assessment of the *resc* capabilities of *wSuz* in *D. simulans*. un: uninfected, *wSuz*:
669 infected by *wSuz*, *wRi*: infected by *wRi*, *wTei*: infected by *wTei*, *wHa*: infected by *wHa*. The
670 CI_{corr} index removes the basal embryonic mortality (estimated in control crosses); 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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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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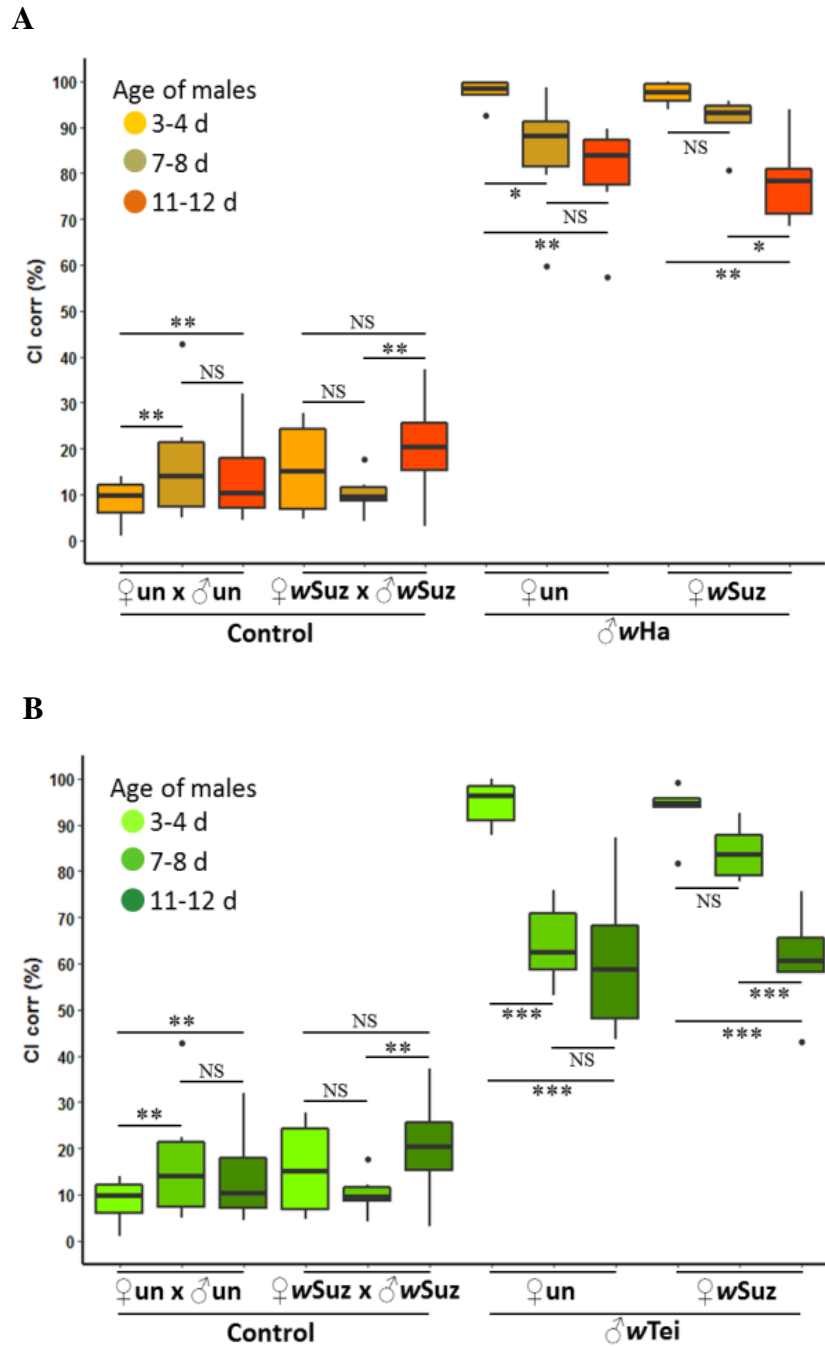
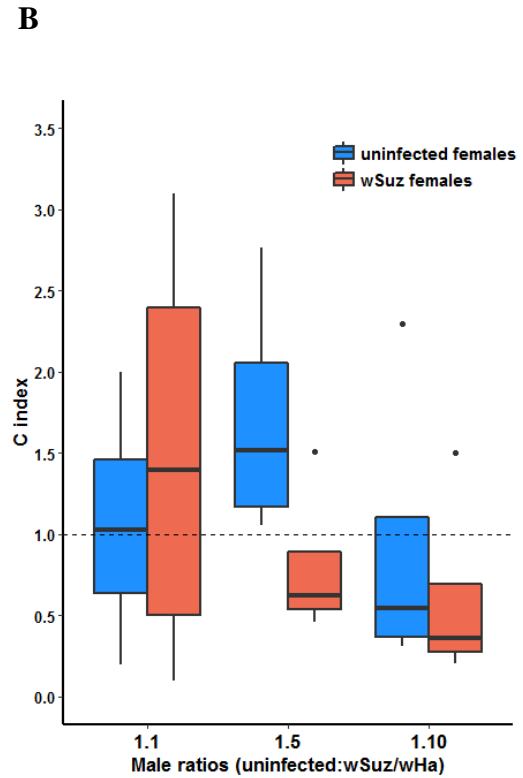
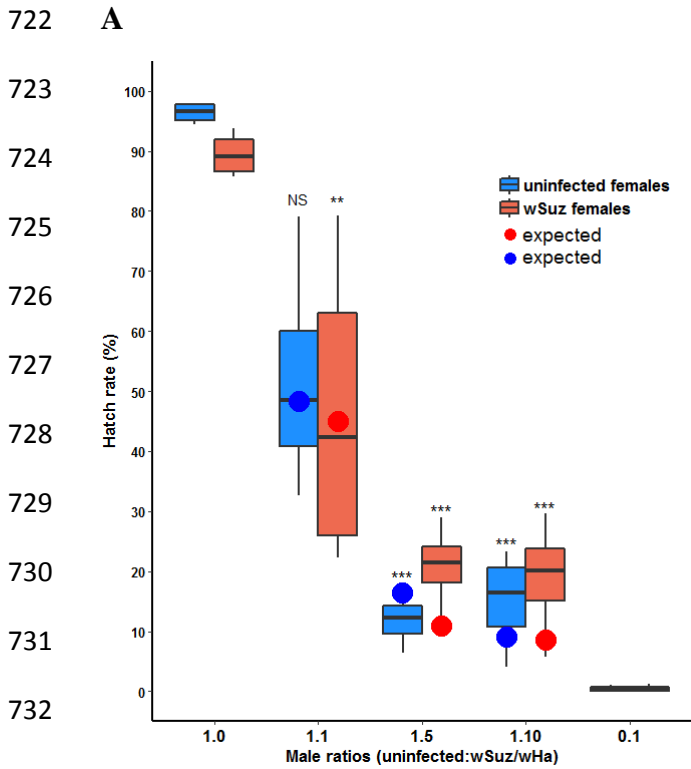


Fig. 4. Effect of male age on CI intensity in *D. sukuzii*. For each transinfected line (*wHa* or *wTei*), three different ages were tested, 3-4, 7-8 and 11-12 days. A: sterilizing males infected by *wHa*. B: sterilizing males infected by *wTei*. un: uninfected, *wSuz*: infected by *wSuz*, *wHa*: infected 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. ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$.



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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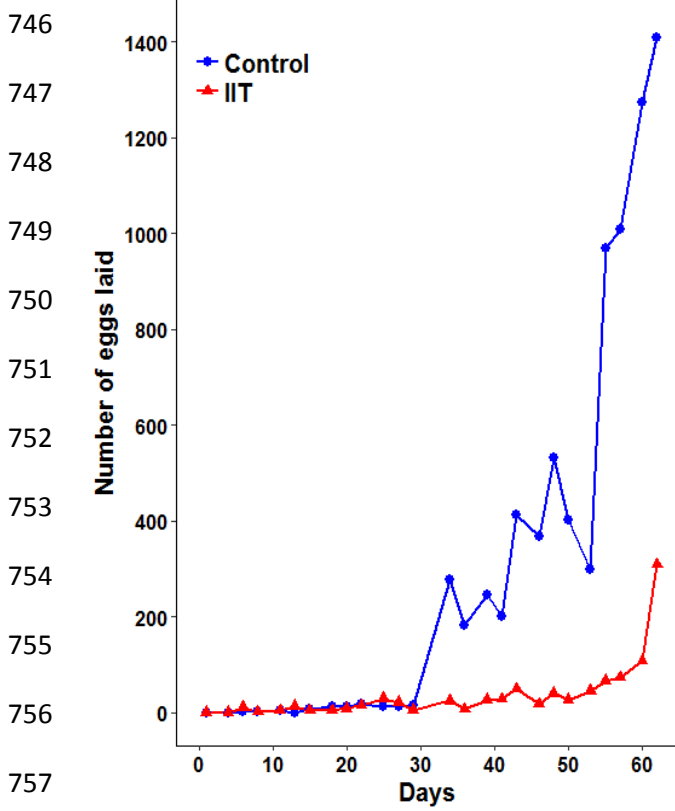
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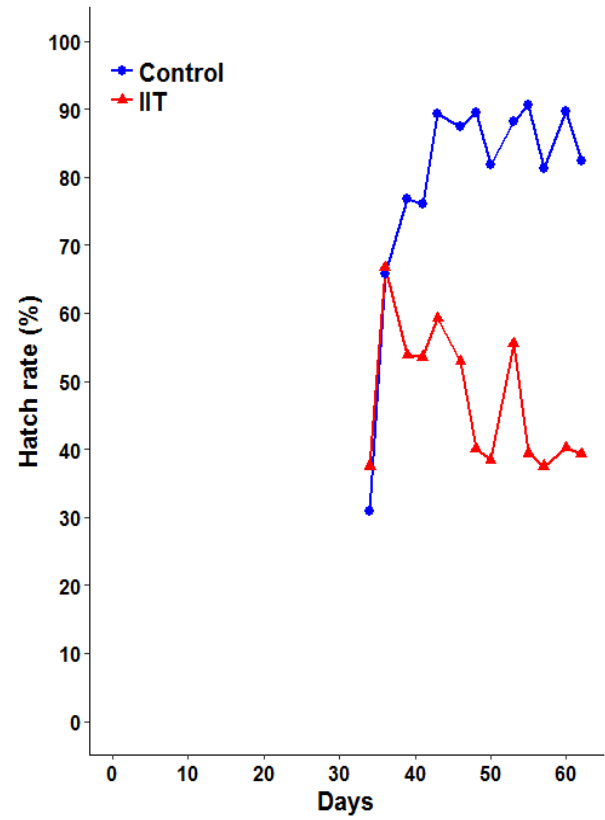
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B



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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