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Symbiont-driven male mating success in the neotropical *Drosophila paulistorum* superspecies

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► **To cite this version:**

Daniela I Schneider, Lee Ehrman, Tobias Engl, Martin Kaltenpoth, Aurélie Hua-Van, et al.. Symbiont-driven male mating success in the neotropical *Drosophila paulistorum* superspecies. *Behavior Genetics*, 2019, 49, pp.83-98. 10.1007/s10519-018-9937-8 . hal-02155257

HAL Id: hal-02155257

<https://hal.science/hal-02155257>

Submitted on 13 Jun 2019

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1 **Title:** Symbiont-driven male mating success in the neotropical *Drosophila paulistorum*
2 superspecies

3

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18 **Running head:** Symbiont-driven male mating success

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27

28

29 **Abstract**

30

31 Microbial symbionts are ubiquitous associates of living organisms but their role in
32 mediating reproductive isolation (RI) remains controversial. We addressed this
33 knowledge gap by employing the *Drosophila paulistorum*-*Wolbachia* model system.
34 Semispecies in the *D. paulistorum* species complex exhibit strong RI between each
35 other and knockdown of obligate mutualistic *Wolbachia* bacteria in female *D.*
36 *paulistorum* flies trigger loss of assortative mating behavior against males carrying
37 incompatible *Wolbachia* strains. Here we set out to determine whether *de novo* RI can
38 be introduced by *Wolbachia*-knockdown in *D. paulistorum* males. We show that
39 *Wolbachia*-knockdown *D. paulistorum* males (i) are rejected as mates by wild type
40 females, (ii) express altered sexual pheromone profiles, and (iii) are devoid of the
41 endosymbiont in pheromone producing cells. Our findings suggest that changes in
42 *Wolbachia* titer and tissue tropism can induce *de novo* premating isolation by directly
43 or indirectly modulating sexual behavior of their native *D. paulistorum* hosts.

44

45 **Key words**

46

47 *Wolbachia*, *Drosophila*, reproductive isolation, premating isolation, pheromones

48

49 **Introduction**

50

51 Potential impacts of microbial symbionts as drivers of host speciation have been
52 discussed frequently (Bordenstein et al. 2001; Jaenike et al. 2006; Telschow et al.
53 2007; Brucker and Bordenstein 2012; Telschow et al. 2014; Gebiola et al. 2016), but
54 their broader evolutionary significance fostering speciation remains controversial
55 (Brucker and Bordenstein 2013, 2014; Chandler and Turelli 2014; Turelli et al. 2014;
56 Najarro et al. 2015; Leftwich et al. 2017), and standard models of speciation commonly

57 disregard such impacts (Coyne and Orr 2004; Brucker and Bordenstein 2012; Miller
58 and Schneider 2012). Several theoretical models have nominated the maternally
59 transmitted reproductive tract endosymbiont *Wolbachia* as a candidate worthy of
60 consideration (Telschow et al. 2007; Telschow et al. 2014). The α -proteobacterium
61 *Wolbachia* is arguably the most prevalent intracellular invertebrate infection on the
62 planet, infecting as many as 40% of all terrestrial arthropods (Zug and Hammerstein
63 2012). *Wolbachia* have attracted attention as participants in arthropod symbioses
64 because they successfully manipulate host biology in manifold ways, ranging from
65 reproductive parasitism like cytoplasmic incompatibility (CI), male killing, feminization
66 and parthenogenesis to nutritional supplementation, fecundity and protection from
67 pathogens and parasites (Werren et al. 2008; Teixeira et al. 2008; Brownlie et al. 2009;
68 Fast et al. 2011; Moriyama et al. 2015). Although *Wolbachia* are mainly found as
69 facultative endosymbionts in most insect hosts (Martinez et al. 2015 and references
70 therein), they also can evolve fixed obligate associations such as in the parasitoid wasp
71 *Asobara* (Dedeine et al. 2001), bedbugs (Hosokawa et al. 2010), or neotropical fruit
72 flies belonging to the *Drosophila paulistorum* species complex (Miller et al. 2010).
73 In the latter case, this superspecies is under incipient speciation in the wild and
74 consists of six semispecies (Dobzhansky and Spassky 1959), named Amazonian
75 (AM), Andean Brazilian (AB), Centroamerican (CA) Interior (IN), Orinocan (OR) and
76 Transitional (TR) expressing strong RI at the pre- and postzygotic level in inter-
77 semispecies crosses (reviewed in Ehrman & Powell 1982). As recently found they all
78 carry different loads of obligate mutualistic but distinctive *Wolbachia* strains (Miller et
79 al. 2010), which can cause strong cytoplasmic incompatibilities (embryonic mortality)
80 plus complete hybrid male sterilities in reciprocal crosses between different
81 semispecies in the laboratory. Because *Wolbachia* are obligate endosymbionts in this
82 system, no uninfected flies can be generated (Ehrman 1968; Kernaghan and Ehrman
83 1970; Miller et al. 2010) to test for classic bidirectional CI as feasible in facultative

84 symbioses such as in *Culex pipiens* mosquitos (reviewed in Werren et al. 2008). The
85 association between the obligate *Wolbachia* symbiont (earlier designated
86 *Mycoplasma-like-organisms*, MLOs; reviewed in Ehrman and Powell 1982) and the
87 induction of postmating isolation, however, is based on earlier and recent findings that
88 hybrid lethality and male sterility are partly reversible upon mild antibiotic or heat
89 treatments of the parents before mating (Ehrman 1968; Kernaghan and Ehrman 1970;
90 Miller & Schneider, unpublished).

91 In two earlier publications, Dobzhansky and collaborators (Dobzhansky and Pavlovsky
92 1966, 1971) observed the spontaneous evolution of *de novo* postmating isolation as
93 some lines, which were originally fully compatible with the Orinocan reference strain
94 O11, were no longer compatible with this semispecies or any other semispecies of *D.*
95 *paulistorum*. This resulted in occurrence of high embryonic F₁ mortality and complete
96 male hybrid sterility, presumably because of drift and isolation in these lines in the
97 laboratory. However, the role of endosymbionts on the formation of premating
98 mechanisms has been less studied in the past (Dobzhansky and Pavlovsky 1966,
99 1971; Miller et al. 2010). Importantly, in facultative symbioses, where *Wolbachia* has
100 not reached fixation in their natural hosts as in *D. melanogaster* or *D. yakuba* group
101 species, the authors did not detect any *Wolbachia* effect on premating isolation
102 (Sharon et al. 2010; Arbuthnott et al. 2016; Cooper et al. 2017).

103 This current study is based on the recent observation that *D. paulistorum* semispecies
104 show strong premating isolation through female mate choice for intra-semispecific
105 (homogamic) males (Fig. 1A, left panel). Such behavior is lost upon *Wolbachia*-
106 knockdown in females, i.e., significant titer reduction but not clearance, resulting in
107 random mating between *per se* incompatible, heterogamic mates (Miller et al. 2010;
108 Fig. 1A, right panel). Knockdown instead of clearance is performed because *Wolbachia*
109 is a fixed obligate mutualist in *D. paulistorum*, providing vital but still undetermined
110 functions for its native hosts (Miller et al. 2010). More recently, we could demonstrate
111 selective neurotropism (the affinity to nervous tissue) of native *D. paulistorum*

112 *Wolbachia* to defined female and male brain regions, known as functionally important
113 for mating behavior and memory (Strunov et al. 2017). In addition to governing
114 assortative mating behavior of *D. paulistorum* females, we speculate that perturbations
115 of the *Wolbachia-Drosophila* homeostasis in males might induce assortative mating
116 behavior between *per se* compatible, homogamic mates, at least under experimental
117 conditions (Fig. 1B). Finally we speculate that under certain conditions like obligate
118 mutualism and tropism of the endosymbiont to host organs associated with sexual
119 behavior, *Wolbachia* can act as an influential and dynamic factor for modulating sexual
120 behavior, which, because of their sensitivity to exogenous factors such as stress and
121 antibiotics (Reynolds and Hoffmann 2002; Mouton et al. 2006; Weeks et al. 2007), plus
122 their high innate mutability (Chrostek et al. 2013; Schneider et al. 2013; Newton and
123 Sheehan 2014), can possibly initiate the process of sexual isolation, at least under
124 experimental laboratory conditions. Their potential impact on speciation in nature,
125 however, remains elusive.

126 The key components of premating sexual isolation mechanisms are visual, acoustic,
127 and chemical courtship signals. In many insects, sexual pheromones play an important
128 role as olfactory signals influencing mate choice (Blomquist and Bagnères 2010;
129 Chung and Carroll 2015; Dembeck et al. 2015). In *D. paulistorum*, males from the six
130 semispecies exhibit characteristic sexual pheromone profiles (same compounds but
131 different quantities), including four male-specific long-chained ester compounds, which
132 are recognized by females (Kim et al. 2004; Chao et al. 2010). In contrast to other
133 *Drosophila* species such as *D. melanogaster*, no female-specific compounds are
134 present in this system. Hence, the authors of previous studies (Kim et al. 2004; Chao
135 et al. 2010) concluded that, based on chemical profiles, *D. paulistorum* females accept
136 homogamic (compatible), but reject heterogamic (incompatible) males as mates (Chao
137 et al. 2010). Forced mating between heterogamic mates carrying incompatible
138 *Wolbachia* strains is highly detrimental for both host and symbionts by triggering high
139 levels of embryonic lethality and complete male sterility among F₁ hybrids (Ehrman

140 1968; Kernaghan and Ehrman 1970; Miller et al. 2010). Hence strong selection should
141 act on both intimate partners to evolve or maintain such mechanisms for mate
142 recognition.

143 Here we experimentally investigated whether bacterial symbionts are capable of
144 initiating artificial *de novo* reproductive isolation (RI) by targeting host-derived olfactory
145 cues. Therefore, we have (i) transiently knocked down obligate *Wolbachia* in *D.*
146 *paulistorum* males, (ii) assayed their mating success in homogamic choice assays with
147 wild type females, (iii) monitored their sexual pheromone profiles, and (iv) investigated
148 the presence of *Wolbachia* in putative pheromone-producing cells.

149

150 **Methods**

151

152 **Fly strains and generation of *Wolbachia*-knockdown (kd), axenic (et), and** 153 **penicillin/streptomycin-treated (ps) flies**

154

155 Reference strains for two semispecies from the *Drosophila paulistorum* superspecies
156 were chosen for this study (Amazonian, A28 and Orinocan, O11; originally described
157 in Burla et al. 1949; Dobzhansky and Pavlovsky 1966). The *Wolbachia* infection status
158 (*wPau*) of these *D. paulistorum* strains was previously described in Miller et al. 2010.

159 In brief, A28 carries very low densities of the *Wolbachia wPauAM* strain, and O11 is
160 infected with the high-titer *wPauOR* strain (Miller et al. 2010). *Wolbachia* positive
161 controls for Polymerase chain reaction (PCR) assays were *D. willistoni* P98 and JS6.3,
162 both lab strains carrying native high-titer *Wolbachia (wWil)*; Miller and Riegler 2006).

163 The *D. melanogaster* H2-strain (*wMel*; Miller and Riegler 2006) was used as an
164 additional *Wolbachia* positive control. *Wolbachia* negative controls for PCR were *D.*

165 *simulans* NouméaTC (Poinsot et al. 2000), *D. melanogaster w¹¹¹⁸* (BDSC, USA), and

166 *D. willistoni* Willi3 (14030-0811.2 DSSC, USA). Flies were reared on Formula 4-24

167 *Drosophila* instant food (Carolina, USA) at 24-25°C on a 12 hour light-dark cycle.

168 *Wolbachia*-knockdown lines A28^{kd} and O11^{kd} were generated by mass-treating wild
169 type flies with 0.2% (w/v) rifampicin (Duchefa, Netherlands) added to Formula 4-24
170 *Drosophila* instant food (Carolina, USA) over three sequential generations (T₁-T₃). The
171 low dosage of the antibiotic is sublethal and allows for reduction of mutualistic
172 *Wolbachia* in *D. paulistorum* (Miller et al. 2010). Offspring from T₃ parents were then
173 transferred onto antibiotic-free food for more than 10 generations under a mass-rearing
174 regime designated as knockdown pools (A28^{kd}, O11^{kd}; Supporting Information Fig. S1).
175 In parallel, F₂^{kd} was used to initiate 8-10 isofemale lines per semispecies (A28^{kd-l},
176 O11^{kd-l}; Supporting Information Fig. S1). Biological assays were performed at different
177 generations between F₄^{kd} and F₁₃^{kd} and their respective *Wolbachia* load was monitored
178 by qPCR (see below). Gut flora restoration (gfr) lines were generated by feeding the
179 native gut microbiome to knockdown flies as follows: A28^{wt} and O11^{wt} virgin females
180 were kept on instant food for 2-3 days to collect feces. Flies were then removed and
181 the corresponding knockdown (kd) flies were transferred onto the feces-containing
182 food vials. After egg deposition adults were removed and emerging flies were
183 consequently used as gfr lines. Axenic ('gut microbe-free flies') were generated by
184 consequently washing freshly collected eggs from A28^{wt} and O11^{wt} with 70% ethanol
185 for five minutes to surface-sterilize them. This treatment prevented larvae ingesting
186 microbes from the outer layer of the eggshell when hatching. Collected eggs were then
187 transferred into food vials and hatching F₁ and F₂ adults (A28^{et}, O11^{et}) were
188 consequently used for mate choice assays. Penicillin/streptomycin-treated (ps) flies
189 were generated by supplementing Formula 24 *Drosophila* instant food (Carolina, USA)
190 with a 1:100 dilution of a 100x pen/strep stock solution (10000 units/ml penicillin, 10000
191 µg/ml streptomycin). A28^{wt} and O11^{wt} were kept on this food for at least one week to
192 lay eggs and the hatching F₁ (A28^{ps}, O11^{ps}) was consequently used for mate choice
193 assays against the wt counterparts. Strains are reported in Table S1.

194

195 **Quantification of wPau *Wolbachia***

196

197 DNA was extracted from a pool of ten three-day old flies using Gentra Puregene
198 chemistry (Qiagen, Germany). Concentrations of DNA were measured on a Nanodrop
199 2000 spectrophotometer. Consequently, diagnostic *Wolbachia*-PCR (quantitative real
200 time PCR) was performed using the *Wolbachia* outer surface protein gene *wsp*
201 (Yamada et al. 2007), and the *Wolbachia*-specific 16S rRNA gene (16SW_RTf 5'-
202 CCTGATCCAGCCATGCCGCAT-3', 5'-16SW_RTf CGGCTGCTGGCACGGAGTTA-
203 3'). The *wsp* primer set generates a 69 bp amplicon, and the 16S rRNA set produces
204 a 77 bp fragment. *Wolbachia* titers obtained with MxPro QPCR v4.10 Software (Agilent
205 Technologies, USA) were normalized against *Drosophila* ribosomal protein *RPS17*
206 (RPSmel_f 5'-CACTCCCAGGTGCGTGGTAT-3', RPSwil_r 5'-
207 GGAAACGGCGGGCACGTA-3'). A temperature profile of 95°C for 3 sec, 60°C for 20
208 sec, and 72°C for 6 sec was used for 45 cycles. All samples were run in duplicates on
209 a Stratagene MxPro4000 cycler. Quantitative PCR with *wsp* was run to confirm results
210 obtained with the 16S rRNA primer set. Only results for the latter one are presented in
211 the manuscript.

212

213 **Measuring sexual isolation *via* multiple mate choice assays**

214

215 Mate choice assays between wild type, knockdown and control assays involved double
216 blind direct multiple-mating observations carried out mornings at room temperature in
217 daylight. Virgin flies were aged two to three days (females isolated from males), and
218 half were marked *via* distal wing clips before running the choice assay. These marks
219 were rotated (wing to wing and knockdown to wild type). Such minute abrasions have
220 tested neutral regarding behavioral influences in this superspecies (Dobzhansky and
221 Pavlovsky 1966; Leonard and Ehrman 1983). For each mate choice assay five replicas
222 and 120 mating events (240 individual flies) were scored (12A ♀♀ + 12B ♀♀ + 12A
223 ♂♂ + 12B ♂♂ differentiated by alternating wing clips; Supporting information Fig. S2A,

224 B). Flies were placed (females first) in mating observation chambers (10 cm in
225 diameter) without anesthetization and the following parameters were scored until all
226 flies copulated in approximately 30-40 minutes: the time each mating took place (from
227 start of observations; each copulation approximately lasted 15-17 minutes); its
228 sequence among other *copulae* which occurred; where in the chamber the mating pair
229 was located; the kind of female involved; and the kind of male involved. Recording the
230 location of copula, even upside down, prevented scoring a copula more than once.
231 Sexual isolation index (SII) was computed according to the following formula
232 (Malogolowkin-Cohen et al. 1965).

233 $SII = (nho - nhe) / N$, where *nho* is the number of homogamic matings, *nhe* is the number
234 of heterogamic matings, and *N* the total number of matings. SIIs range from -1.00
235 (preference for unlikes, heterogamy) through 0 (random mating) to +1.00 (preference
236 for likes, homogamy). This experimental design is not devoid of bias. Indeed, when the
237 number of partners is finite and remating is limited, the choice of the last individuals is
238 conditioned by the choice of the previous ones. This could lead to erroneous signals
239 for assortative mating, e.g. when both males and females from the same population
240 tend to mate earlier than those from the other population (Ehrman and Parsons 1981).
241 However, unbiased rates of assortative mating could be estimated by accounting for
242 the order of mating pairs, using a statistical model described extensively in Supporting
243 Methods. This method provides the maximum-likelihood estimates of the assortative
244 mating coefficient (called eSII, or *h* in the model and Supporting Methods) and
245 accounts for the possibility of remating in males. Several remating rates were tested
246 and did not influence much the estimation of SII (see Figure S3), then in practice a
247 remating rate of 0.5 was chosen for the general analysis (Tables S2-S4). To avoid
248 convergence issues, the female remating rate was set to 0.001. Two additional
249 parameters (biases in mating order for males and females) were also estimated by the
250 model (Table S5). However, their biological interpretation is not that straightforward as
251 they may correspond to either a general preference for one population of one sex, or

252 a different mating speed between both populations. The statistical departure of the SII
253 from 0 was estimated by a likelihood ratio test. Simulations showed that this procedure
254 efficiently corrects for such mating order biases, and was even slightly more powerful
255 than homogeneity (Fisher) tests when mating order was unbiased.

256 The model was implemented in R version 3.1.1 (R Core Team 2016), the code is
257 available in Supporting Methods, along with the raw data. A comparison of the
258 uncorrected SII and the model-estimated SII is provided in Supporting Information Fig.
259 S3 and Tables S2-S4). Hereafter, we will refer only to the estimated SII that will be
260 simply called eSII.

261

262 **Analysis of cuticular hydrocarbons (CHCs) *via* gas chromatography-ion trap** 263 **mass spectrometry (GC-MS)**

264

265 Male virgin flies were collected, isolated from females, and aged to three days. Flies
266 were pooled in glass vials and submerged in 1 ml of HPLC-grade hexane (Carl Roth,
267 Germany). Replicates consisted of 10 males per sample. We did nine replicates for
268 A28^{wt} and 10 replicates for each A28^{kd}, O11^{wt} and O11^{kd}. Two µg of octadecane (C18)
269 per sample was added as the internal standard for absolute quantification of CHCs.
270 Extraction of CHCs was performed for 10 minutes at room temperature under constant
271 agitation, after which the flies were removed. Extracts were evaporated to about 20-
272 30 µl of hexane under a constant stream of argon, and then transferred to a 150 µl
273 GC-µ-vial (CZT, Germany). 1 µl aliquots were injected into a Varian 450GC gas
274 chromatograph coupled to a Varian 240MS mass spectrometer (Agilent Technologies,
275 Germany). A DB5-MS capillary column (30m x 0.25 mm diameter, film thickness: 0.25
276 µm, Agilent Technologies, Germany) was used and the GC was programmed from
277 150°C to 300°C at 15°C/min with a 27 min final isothermal hold. Helium, with a constant
278 flow rate of 1 ml/min, was used as carrier gas. Recording of mass spectra was
279 performed using electron ionization (EI-MS) in external ionization mode and data

280 acquisition plus quantifications were done with MS Workstation v6.9.3 Software
281 (Agilent Technologies, Germany). Consequently, peaks were identified by their mass
282 spectra in comparison to previously published hydrocarbon profile analyses of *D.*
283 *paulistorum* (Kim et al. 2004; Chao et al. 2010) and *D. melanogaster* (Ueyama et al.
284 2005). Hydrocarbon quantities were calculated from peak areas and then centered log-
285 ratio-transformed according to Aitchinson 1986. To test for differences in chemical
286 profiles between wild type and knockdown individuals, principal component analyses
287 (PCAs) based on eigenanalysis of covariances were performed to reduce numbers of
288 variables. Consequently, resulting PCs were used for discriminant analyses (DAs), to
289 test for among-group differences.

290

291 **Fluorescence *in situ* hybridization (FISH) on *D. paulistorum* oenocytes**

292

293 Ten to 15 female and male abdomen per semispecies were dissected in RNase-free
294 1x phosphate buffered saline (PBS). After removing inner organs, cuticles were fixed
295 in 3.7% formaldehyde in RNase-free PBS for 20 minutes at room temperature and
296 consequently washed with PBTX (1x PBS, 0.3% Triton-X 100). After permeabilization
297 with 70% ethanol over night at 4°C, samples were hybridized overnight in 10%
298 formamide, saline sodium citrate (SSC) and 0.5 nmol of CAL Fluor Red 590-labeled
299 customized *Wolbachia* 16-23S rRNA probe (Biosearch Technologies, USA; Schneider
300 et al., *in press*). Samples were then washed in 10% formamide and SSC, stained with
301 DAPI-SSC (1µg/ml) and mounted in Roti[®]-Mount FluorCare (Carl Roth, Germany) on
302 sterilized microscope slides. Cuticles were analyzed on an Olympus confocal
303 microscope. Beam paths were adjusted to excitation/emission peaks of used
304 fluorophores: 569/591 nm for CAL Fluor Red 590 (*Wolbachia*), and 350/450 nm for
305 4',6-Diamidin-2-phenylindol (DAPI). Images were processed with Fiji software
306 (<http://fiji.sc>). Staining was repeated twice and a minimum of 15 flies was assayed in
307 each experiment.

308

309 **Results**

310

311 **Knockdown of obligate *Wolbachia* in *Drosophila paulistorum* semispecies is of** 312 **transient nature**

313

314 To test whether manipulation of native *Wolbachia* titers and associated disruption of
315 the host-symbiont homeostasis can trigger *de novo* reproductive isolation (RI) in *D.*
316 *paulistorum*, we generated *Wolbachia*-knockdown (kd) males of two different
317 semispecies (Amazonian: A28, and Orinocan: O11) by mild rifampicin treatment for
318 three consecutive generations (T1-T3) and in the following generations on regular
319 media without antibiotics (F_1^{kd} - F_{14}^{kd}) for restoration. For details see Supporting
320 Information Table S1, Fig. S1. Global *Wolbachia* titers of these males ($A28^{kd}$, $O11^{kd}$)
321 were tested against wild type (wt) controls ($A28^{wt}$, $O11^{wt}$) in quantitative real time PCR
322 (qPCR) assays targeting the *Wolbachia* 16S ribosomal RNA gene. Assays obtained
323 from F_7^{kd} (knockdown generation 7) revealed massive titer reduction to about 5 and
324 14% of the average native wt *Wolbachia* titers (Fig. 2A,B).

325 To further assess whether *Wolbachia* titers return to native levels in later generations
326 post kd, we tested symbiont load by F_{14} and earlier generations. Whereas in O11 the
327 global symbiont titer was close to wt-levels at F_{14} (90%; Fig 2B), A28 flies have
328 gradually reached only 23% of their wt-titer at this time point (Fig. 2A).

329

330 **Mate choice assays reveal *de novo* assortative mating phenotype of wild type** 331 **(wt) females towards *Wolbachia*-knockdown males**

332

333 Among sympatric *D. paulistorum* semispecies, no hybrids are formed inter-
334 semispecifically in nature (Dobzhansky and Spassky 1959). When performing inter-
335 semispecific mate choice assays involving $A28^{wt}$ and $O11^{wt}$ semispecies, we observed

336 strong RI, revealed by a high estimated Sexual Isolation Index (eSII) (Ehrman 1965;
337 Ehrman and Powell 1982; Supporting Information: Table S2, assays 1,2). In contrast,
338 intra-semispecific control assays showed no assortative mating in standard
339 interbreeding 'pool' lines (Fig. 3 A,B wt x wt (purple) and Supporting Information Table
340 S2, assays 3,4). Results of statistical testing are summarized in Table S2.

341 In order to test our hypothesis that manipulation of the native *Wolbachia* titer and the
342 associated disruption of the host-symbiont homeostasis in *D. paulistorum* might trigger
343 *de novo* premating isolation intra-semispecifically, we assayed mating behavior of
344 *Wolbachia*-kd pool lines towards wt flies of the same semispecies (Fig. 3A,B, orange,
345 and Table S3, assays 1-6). Assays run with knockdown flies at generation F₄ or F₅
346 post-treatment, consistently showed high eSII, i.e. mating between likes rather than
347 unlikes. This points towards *de novo* mate discrimination between wt and kd flies from
348 the same semispecies that significantly differ in their symbiont load (kd F₇ in Fig 2A,B;).
349 However, at generation F₁₃ homogamy was less pronounced among all of them, and
350 eSII did not differ significantly from what is expected under random mating. Importantly,
351 although O11 flies showed rapid increase of *Wolbachia* levels by kd F₁₄ (Fig. 2B), only
352 a slower, gradual titer increase was observed in A28 (Fig. 2A). Hence the restoration
353 of the random mating phenotype in O11 might directly correlate with the global
354 restoration of the symbiont level at faster pace, whereas the reversion to random
355 mating of A28 flies might depend on a critical titer threshold of *Wolbachia* and/or their
356 tropism to sensitive host tissues such as oenocytes (see discussion).

357 To rule out that drift effects were responsible for observed *de novo* sexual isolation,
358 we additionally assayed wt and kd lines that were reared as ten isofemale lines per
359 semispecies (A28^{wt-i}, O11^{wt-i} and A28^{kd-i}, O11^{kd-i}, respectively). First, two out of ten
360 randomly picked control isofemale lines (wt-i) representing each semispecies were
361 tested against each other (intra) in F₁₀ in order to verify random mating between them
362 (Fig. 3C,D wt-i x wt-i, purple, and Supporting Information Table S2, assays 5-8). The
363 observed low eSII's indicated the absence of any drift-dependent sexual isolation. To

364 test for emerging sexual isolation between kd-isofemale lines upon drift (as a response
365 to *Wolbachia*-kd and consequent restoration) we performed intra-semispecific choice
366 assays using independent F₈ isofemale knockdown lines (kd-i). Surprisingly in this
367 case the assays revealed significant isolation between them (*p* values <10⁻⁴; Fig. 3C,D,
368 pink, and Supporting Information Table S3, assays 7,8).

369 Taken together, control assays between independent wt isofemale lines rule out drift
370 as a potential factor for triggering *de novo* premating sexual isolation observed
371 between wt and kd lines, whereas independent knockdown lines have possibly drifted
372 apart from each other upon isolation having potentially different levels and/or individual
373 tissue tropisms of *Wolbachia* during restoration at F₈. Overall, the emergence of high
374 eSIs in intrastrain assays suggests that even slight perturbations of the native
375 *Wolbachia* - *D. paulistorum* homeostasis and/or tropism are sufficient to induce *de*
376 *novo* RI in this system, even between individual knockdown isofemale lines. This latter
377 and quite intriguing observation, however, will need further investigations, which are
378 beyond the scope of our current analyses (see discussion).

379

380 **No evidence for influence of gut microbiota on *de novo* assortative mating** 381 **phenotype**

382

383 Earlier and recent studies, however, have addressed the potential influence of diet and
384 consequently the gut microbiome on host sexual isolation not only in *D. pseudoobscura*
385 (Dodd et al. 1989), but also in highly inbred lines of *D. melanogaster* (Sharon et al.
386 2010; Ringo et al. 2011). Here, sexual isolation can be triggered by manipulating the
387 fly's gut microbiome, based on a certain dietary regime. However, two recent studies
388 have questioned the general role for gut bacteria or diet composition in driving
389 reproductive isolation in *D. melanogaster* (Najarro et al. 2015; Leftwich et al. 2017). In
390 the light of these still controversial findings obtained from inbred *D. melanogaster*
391 strains, we have tested *D. paulistorum* gut flora restored (gfr) lines, where kd lines

392 were fed with wt feces, in parallel with our unfed kd lines (see Methods section for
393 details). Assays with these lines and wt flies revealed almost complete SII suggesting
394 that gut flora restoration does not affect the *de novo* assortative mating phenotype (p
395 values $< 10^{-4}$, Fig. 3E, and Supporting Information Table S4, assays 1,2). To further
396 exclude an effect of the gut microbiome associated with the egg smear, we assayed
397 axenic flies, i.e., eggs washed with ethanol (et, Fig. 3F, and Supporting Information
398 Table S4, assays 3,4), and penicillin/streptomycin-treated flies (ps, Fig. 3G, purple,
399 and Supporting Information Table S4, assays 5,6). Mating behavior with both sets were
400 compatible with random mating (absence of assortative mating). Hence, in contrast to
401 rifampicin, which acts on *Wolbachia* levels, neither sterilizing eggs, nor treatments with
402 penicillin/streptomycin had any effect on mate choice behavior in this system.
403 Furthermore, to test whether penicillin/streptomycin affects mate behavior of females
404 we performed inter-semispecific control assay between wildtype A28 and
405 penicillin/streptomycin-treated O11 flies that still revealed high sexual isolation (ps, Fig.
406 3G, yellow, Supporting Information Table S4, assay 7, p value $< 10^{-4}$), confirming that
407 penicillin/streptomycin-sensitive gut microbes in females have no effect on mating
408 behavior in this system.

409 Importantly, we are aware that none of our control assays on its own can exclude the
410 spurious presence of some hidden non-*Wolbachia* microbe affecting mate behavior.
411 However, the combination of three independent control assays, i.e., gut-flora
412 restoration, egg-surface sterilization and penicillin/streptomycin-treatment, strongly
413 supports our hypothesis that endosymbiotic *Wolbachia*, which are resilient to
414 penicillin/streptomycin and egg surface sterilizing treatments (Audsley et al. 2017;
415 Leclerq et al. 2017, Ye et al. 2017), are interfering with mate discrimination of *D.*
416 *pauistorum* in both sexes and not any other bacteria.

417 Concordantly, only rifampicin treatment massively reduces *Wolbachia* load and
418 induces *de novo* assortative mating behavior of wildtype females against knockdown
419 males from the same semispecies (this study) that also selectively triggers loss of

420 assortative mating behavior in knockdown females against males from a different
421 semispecies (Fig. 3G, yellow; Miller et al. 2010).

422

423 **Sexual pheromone profiles are altered in *D. paulistorum* knockdown males**

424

425 Based on the observed *de novo* assortative mating phenotype, we tested whether the
426 pheromone composition of *Wolbachia*-kd males was altered in comparison with wt
427 males. Males from all six *D. paulistorum* semispecies exhibit characteristic pheromone
428 profiles (all share the same 15 major compounds but in varying quantities), including
429 four male-specific long-chain esters, which are recognized by females as a
430 semispecific blend because of the differences in their relative concentrations
431 (Supporting Information Figure S4; Kim et al. 2004; Chao et al. 2010). In contrast to
432 other *Drosophila* species such as *D. melanogaster*, no female-specific compounds are
433 known in *D. paulistorum* (Chao et al. 2010) and mate decisions appear – to our current
434 knowledge - to be exclusively made by females (Ehrman 1965; Ehrman and Parsons
435 1981). Here we extended hydrocarbon profile analyses to a total of 27 compounds
436 found in all tested semispecies by adding two new, unknown compounds (Supporting
437 Information Table S6, compounds 9, 13) and by resolving the original C33, C35, and
438 C37 peak clusters (C = chain length of the compound; 33, 35, and 37 carbon atoms).
439 In full agreement with earlier reports (Chao et al. 2010; Kim et al. 2004), discriminant
440 analyses of sex pheromone profiles from A28^{wt} and O11^{wt} males revealed major
441 differences among the semispecies (Supporting Information Figure S5A). In order to
442 test for differences in male pheromone profiles upon *Wolbachia*-kd, we compared
443 profiles between F₇ kd males and wt males for each semispecies. Based on the 27
444 quantified components (Supporting Information Table S6), seven principal
445 components were extracted capturing 87.7% of the total variance for A28^{kd} vs. A28^{wt}
446 and 86.2% for O11^{kd} vs. O11^{wt}. The discriminant analysis revealed statistically
447 significant differences between A28^{wt} and A28^{kd} males (Wilks' Lambda = 0.059,

448 $X^2=39.6$, $df=6$, $p < 0.0001$) and also between $O11^{wt}$ and $O11^{kd}$ (Wilks' 446 Lambda =
449 0.117, $X^2=34.3$, $df=6$, $p < 0.001$, Supporting Information Figure S5B).

450 To further analyze differences in pheromone profiles between wt and kd males, we
451 calculated the relative quantities of the four male-specific compounds in *D. paulistorum*
452 male profiles (11-docosenyl-acetate, DA, 19-triacontenyl-acetate, TA, di-unsaturated
453 acetate $C_{32}H_{60}O_2$, DU, and methyl-(Z)-tetradecanoate, MD). As shown in Figure 4, we
454 observed most drastic changes in quantities of these compounds in $A28^{kd}$ males,
455 where all four male-specific compounds were decreased between 9- and 23-fold when
456 compared to wt levels (Fig. 4A; $p < 0.0001$ for all four compounds). Although not as
457 drastic as $A28^{kd}$, in $O11^{kd}$ males at least one male-specific compound also showed
458 significant changes: compared to $O11^{wt}$ males, MD was reduced 5-fold (Fig. 4B; $p =$
459 0.0399).

460

461 ***Wolbachia* colonize oenocytes in *D. paulistorum***

462

463 In insects, pheromone components are mainly synthesized in specialized cells located
464 under the fly's cuticle, the oenocytes (reviewed in Blomquist and Bagnères 2010;
465 Chung and Carroll 2015; Dembeck et al. 2015). We hypothesized that these
466 specialized cells could serve a primary somatic target for *Wolbachia* to manipulate host
467 pheromone profiles in *D. paulistorum*. Since male pheromone profiles were altered
468 upon *Wolbachia* kd, we tested the infection status of male oenocytes in both *D.*
469 *paulistorum* wt and kd individuals. As shown in Fig. 5, oenocytes from $O11^{wt}$ females
470 (Fig. 5A,A') and males (Fig. 5B,B') harbor *Wolbachia*. We could also detect the
471 symbiont in oenocytes of $A28^{wt}$ males (not shown). Post *Wolbachia*-kd oenocytes,
472 however, are cleared from the symbiont (F_4 $O11^{kd}$; males in Fig. 5C; females in 5C').
473 Since our mate choice assays indicated reversion to wt mating behavior, i.e., loss of
474 *de novo* assortative mating, around $F_{13/14}$ post *Wolbachia*-kd, we tested whether this
475 phenotype correlates with a potential recolonization of *Wolbachia* to oenocytes around

476 this generation post kd. As shown in Fig. 5D, oenocytes of F₁₃ O11^{kd} males are, at
477 least partially, recolonized by the endosymbiont. Oenocytes of other *Drosophila* hosts
478 like the wMel-infected *D. melanogaster* strain Harwich-2, (males in Fig. 5E, females in
479 5E') in which *Wolbachia* have evolved facultative symbiotic interactions and do not
480 affect mate behavior, are devoid of the symbiont. This is in contrast to *D. paulistorum*,
481 where wPau *Wolbachia* are obligate mutualists affecting host behavior (Miller et al.
482 2010 and this study). Oenocytes derived from the *Wolbachia*-uninfected *D. willistoni*
483 strain Willi3, a sister species of *D. paulistorum*, were used as negative controls (Fig.
484 5F). These data suggest that the oenocyte-tropism of wPau is a phenotypic specificity
485 for the *Wolbachia*-*D. paulistorum* system.

486

487 **Discussion**

488

489 ***Wolbachia*-knockdown provokes a *de novo* assortative mating phenotype**

490 *D. paulistorum* semispecies are very sensitive to standard antibiotic treatments
491 (Ehrman 1968; Kernaghan and Ehrman 1970) since in this symbiosis, *Wolbachia* is a
492 fixed obligate mutualist providing vital, but still undetermined functions for its native
493 hosts (Miller et al. 2010). Similar *Wolbachia*-dependencies were found in the parasitoid
494 wasp *Asobara tabida* (Dedeine et al. 2001; Pannebakker et al. 2007), bedbugs of the
495 genus *Cimex* (Hosokawa et al. 2010; Nikoh et al. 2014; Moriyama et al. 2015), and in
496 the rice water weevil *Lissorhoptrus oryzophilus* (Chen et al. 2012). Furthermore, even
497 partial depletion of fixed mutualistic *Wolbachia* from filarial nematodes affects host
498 fitness and fecundity but also triggers overexpression of host nuclear and
499 mitochondrial genes in order to partially compensate the loss of some gene functions
500 provided by *Wolbachia* (Pfarr et al. 2008; Strübing et al. 2010).

501 Here we have assayed for behavioral consequences of symbiont knockdowns in males
502 of two different *D. paulistorum* semispecies, i.e., *Wolbachia* low-titer Amazonian (A28)
503 and high titer Orinocan (O11) strains. Our data strongly suggest that in both systems

504 partial knockdown of the endosymbiont in males significantly affects their
505 attractiveness for homogamic wt-females, which seems to correlate with global titer
506 levels. However, restoration to random mating in later generations post treatment (Fig.
507 3A,B) seems to correlate with the gradual quantitative reconstitution of global symbiont
508 titers that are fast in O11 (Fig. 2B), but not in A28 (Fig. 2A). In our assays, A28 flies
509 expressing random mating at F13 still show significantly lower global *Wolbachia* levels
510 than wt flies. We speculate that reversion to random mating of A28 flies observed at
511 later generations post-treatment might not depend on the global restoration of the
512 symbiont titer in this semispecies to wt, but possibly because of a critical titer threshold
513 and/or the symbiont's tropism to sensitive tissues and organs associated with mating
514 behavior. In order to test this hypothesis, however, detailed temporal and spatial *in situ*
515 quantifications of the symbiont in different host tissues during the restoration phase
516 are required, which were beyond the scope of our current study.

517 As shown by this and earlier studies, under laboratory conditions different *D.*
518 *pauistorum* semispecies express strong assortative mating behavior against each
519 other, which is sensitive to rifampicin and tetracycline (Miller et al. 2010), but
520 insensitive to penicillin and streptomycin that do not target the *Wolbachia*
521 endosymbiont (this study). It seems feasible although not tested that strict female mate
522 choice might prevent mating between incompatible members of different *D.*
523 *pauistorum* semispecies also in nature. If true, this behavior has most likely coevolved
524 in conjunction with the obligate endosymbiont to avoid detrimental reproductive
525 phenotypes like hybrid mortality and male sterility in crosses between inter-
526 semispecies (Ehrman 1968; Kim and Ehrman 1998). Here we have evaluated the
527 potential of *Wolbachia* to trigger assortative mating behavior in *D. pauistorum* towards
528 *per se* compatible mates under laboratory conditions. Our results suggest that kd-
529 males appear sexually less attractive for their wt-female counterparts in mate choice
530 assays upon manipulation of the native *wPau-Wolbachia* titer since they were
531 expressing high SII in all assays. Such high SII were previously found only between

532 members of different *D. paulistorum* semispecies (Ehrman 1965; Malogolowkin-Cohen
533 et al. 1965) or between *Drosophila* species that are sexually isolated from each other
534 (reviewed in Spieth and Ringo 1983; Martin and Hosken 2003; Anderson and Kim
535 2005; Castrezana and Markow 2008). Our data also indicate that *de novo* sexual
536 isolation in *D. paulistorum* under our experimental conditions is transient as eSIs
537 reverted to random mating around generation F₁₃ post kd (only 45-57% homogamy)
538 together with the observed re-colonization of male oenocytes. However, experimental
539 reduction of native *D. paulistorum-Wolbachia* titers was sufficient to alter males so that
540 they were subsequently rejected by wt-females in the two semispecies tested, at least
541 transiently. Interestingly we observed significant assortative mating between
542 independently established kd-isofemale lines of the same semispecies, a finding most
543 likely caused by drift. In such, heterogeneity of kd isolines could be a result of different
544 temporal and spatial dynamics of *Wolbachia* recolonization of behavioral important
545 tissues in the individual lines post knockdown. To test this drift hypothesis for kd lines,
546 however, detailed quantitative and qualitative analyses of global symbiont titer levels,
547 but also their *in situ* tropism and densities will be necessary from multiple staged flies
548 and tissues of the same generation. In our current study, extensive analyses like these
549 were not possible due to limitations of fly material at this time point.

550

551 **Host pheromonal signatures may be a target for *Wolbachia* to signal the**
552 **infection state and to impact mate choice**

553

554 In many insects, sexual pheromones serve as recognition cues for mate choice
555 between and within species and are hence important players in reproductive isolation
556 (Coyne et al. 1994; Savarit et al. 1998; Ferveur 2005). Moreover, such cues might
557 even be prone to manipulation by pathogenic bacteria, as recently shown in *D.*
558 *melanogaster* (Keesey et al. 2017).

559 In our study, the observed alteration of pheromone blends in kd-males may explain
560 why females prefer wt-mates carrying the intact native profile. This finding is
561 corroborated by studies showing that in lekking sandflies (*Lutzomyia*), female mate
562 choice is influenced by the amount of pheromones released by males before potential
563 mating (Jones and Hamilton 1998; Jones et al. 2000). Another study demonstrated the
564 influence of *Wolbachia* on host odor-linked mate preference in the terrestrial isopod
565 *Armadillidium vulgare* (Richard 2017). In A28^{kd} and O11^{kd} males, relative quantities of
566 pheromone compounds were affected differently, suggesting that the quantitative
567 change in at least one of the male-specific components, but possibly also in
568 conjunction with some other non-sex-specific CHCs, might be sufficient to trigger
569 rejection of kd-males by wt-females in choice experiments. Importantly, our findings
570 are in line with a recent study suggesting that *Wolbachia* can influence pupal
571 communication between females and males in *Drosophila melanogaster* by
572 modulating CI levels (Pontier and Schweissguth 2015, 2017; but also see Jacquet et
573 al. 2017). However, as shown earlier, facultative *Wolbachia* symbionts of *D.*
574 *melanogaster* adults do not affect mate choice behavior in this system at all, but
575 possibly other gut microbes (Sharon et al. 2010; Ringo et al. 2011; Arbuthnott et al.
576 2016). Importantly, a very recent study has severely questioned the potential impact
577 of the gut microbiome on reproductive isolation since the microbiome of *D.*
578 *melanogaster* is not fixed but reported as flexible and environmental determined
579 (Leftwick et al. 2017). In the mutualistic *D. paulistorum-Wolbachia* system, however,
580 where the endosymbiont is fixed by serving vital but still undetermined functions to
581 their native host (Miller et al. 2010) we suggest that these obligate *Wolbachia* have
582 also a direct or indirect impact on adult cuticular hydrocarbon (CHC) profiles, where
583 male-specific and sex-unspecific compounds change quantitatively upon *Wolbachia*-
584 kd in adult males.

585 The presence of *Wolbachia* in oenocytes of O11^{wt} males implies a direct or indirect
586 interaction of the symbiont with host pheromone production and mate choice behavior

587 as a consequence. The potential effects of the endosymbiont in oenocytes on female
588 pheromone blending, however, await elucidation. If the presence of *Wolbachia* in male
589 oenocytes is essential to express the semispecies-specific pheromone profile, loss of
590 the symbiont from male oenocytes (F₄ post kd) might explain why pheromone profiles
591 are altered and knockdown males are consequently rejected as mate partners from
592 homogamic wt-females. Based on our mate choice assays, the *de novo* assortative
593 mating phenotype behavior is transient and results in reversion to a wt-like situation
594 around F₁₃/F₁₄ post knockdown. Assuming a direct link between *Wolbachia* tropism in
595 the oenocytes and expression of the male pheromone profile, we expected a
596 recolonization of oenocytes in parallel with reversion to random mating phenotype. We
597 could confirm such recolonization, and thus potentially explain reversion to random
598 mating, in oenocytes of F₁₃ O11^{kd} males. Our finding is particularly interesting in the
599 light of the contrasting situation in *D. melanogaster*, where *Wolbachia* do not play a
600 role in mate choice (Sharon et al. 2010; Ringo et al. 2011; Arbuthnott et al. 2016). In
601 our experimental setup, we did not detect *Wolbachia* in *D. melanogaster* oenocytes,
602 which most likely explains why the symbiont does not affect mate choice and
603 pheromone expression in this model system (Sharon et al. 2010). Finally, in contrast
604 with the strict neurotropism of obligate *Wolbachia* to defined brain regions of *D.*
605 *pauistorum*, (Strunov et al. 2017) native *Wolbachia* are randomly dispersed at low
606 densities in *D. melanogaster* brains (Albertson et al. 2009). However, it remains to be
607 elucidated how *Wolbachia* manipulate male pheromone expression and female mate
608 choice in *D. pauistorum*.

609 To conclude, our combined data strongly imply that artificial reduction of the obligate
610 *Wolbachia* endosymbiont of *D. pauistorum* males significantly reduces mating
611 success with homogamic wildtype females belonging to the same semispecies.
612 Although these and earlier findings in *D. pauistorum* lead to interpretations based on
613 associations rather than causation and their functional and molecular bases are still
614 undetermined, we propose the following model for the potential impact of the *D.*

615 *paulistorum* endosymbiont on RI and host speciation in this system. In contrast to most
616 facultative *Wolbachia*-insect associations, where the symbiont is not fixed and does
617 not serve vital host functions, *D. paulistorum* semispecies have evolved vital
618 mutualistic associations with their endosymbiont (Miller et al. 2010) as well as strict
619 tissue tropisms to reproductive host organs, such as the primordial germline cells of
620 embryos and adult gonads (Miller et al. 2010), but also to defined larval and adult brain
621 regions associated with sexual behavior (Strunov et al. 2017) and pheromone
622 producing oenocytes (this study). As implicated from the results of this study even
623 partial depletions of the mutualist from their primary somatic host targets, such as
624 oenocytes, might directly or indirectly alter pheromone signatures of males, which are
625 no longer accepted as mates from stress-free wt females.

626 It appears likely that spontaneous symbiont knockdowns might also happen in the wild
627 by stochastic exposure of the host to natural antibiotics or heat stress. This *de novo*
628 assortative phenotype expressed by females against aberrant homogamic males can
629 significantly disrupt gene flow within populations via premating isolation, at least
630 transiently. As shown earlier, however, *Wolbachia*-kd males are accepted by kd
631 females, which randomly mate even with heterogamic males under lab conditions
632 (Miller et al. 2010). Under this scenario two sexually isolated reproductive groups
633 would emerge and coexist next to each other. Since population sizes of *Wolbachia*
634 have dropped significantly upon such external stresses, it seems quite likely that
635 genetic drift (Chrostek et al. 2013; Schneider et al. 2013; Newton et al. 2014) can
636 cause disruptive diversification of earlier-compatible *Wolbachia* variants that
637 consequently trigger high cytoplasmic incompatibilities plus complete male sterility at
638 postmating levels.

639 Hence it seems plausible that in Dobzhansky's earlier studies (Dobzhansky and
640 Pavlovsky 1966; 1971) where spontaneous emergence of strong post mating
641 incompatibilities between long term isolated sub lines of the same *D. paulistorum*
642 semispecies were observed, he had actually detected the final outcome of this

643 symbiotic stress and drift effect, observed in our current experimental study. Planned
644 studies, however, should elucidate the mechanistic basis of *Wolbachia*-influence on
645 male pheromone production and whether similar scenarios observed under lab
646 conditions also take place in nature.

647

648 **Acknowledgments**

649

650 We thank Bertha Inocencio Green and Traude Kehrer for fly work and Dr. Taina Chao
651 for help with analyses of *D. paulistorum*. We also thank the Max Planck Society for
652 supporting this study.

653

654 **Compliance with Ethical Standards**

655

656 **Funding:** This study was funded by two grants (P22634-B17, P28255-B22) from the
657 Austrian Science Fund to W.J.M., and by a grant (FA0701-9528) from the COST Action
658 to D.I.S. The Max Planck Society was also supporting this study (T.E., M.K.) and L.E.
659 was awarded an E+E grant (E2 Grant 331-15).

660

661 **Conflict of Interest:** The authors declare that they have no conflict of interest.

662 **Ethical Approval:** For this type of study formal consent is not required. This article
663 does not contain any studies with human participants or animals performed by any of
664 the authors.

665

666 **Informed Consent:** Not applicable.

667

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905
906

907 **Figure Legends**

908

909 **Figure 1**

910 Impact of *Wolbachia* on mate choice in *D. paulistorum*. (A) Mate choice in *D.*
911 *paulistorum*: *Wolbachia*-knockdown females lose assortative mating behavior against
912 heterogamic wild type males. Left: wild type females (blue) infected with *Wolbachia* A
913 (*wA*) express strong assortative mating behavior (red block) against males of a
914 different semispecies (red) carrying a different, incompatible *Wolbachia* (*wB*). Right:
915 *Wolbachia* kd females (symbolized with red flash) with reduced titers of *wA* lose
916 assortative mating behavior (grey arrows) and hence mate randomly with males from
917 same (blue) but also from different/incompatible (red) semispecies. Female

918 knockdown phenotype: loss of assortative mating behavior. Data taken from (Miller et
919 al. 2010). (B) Mate choice in *D. paulistorum*: *Wolbachia*-knockdown males are rejected
920 by homogamic wildtype females. Left: wild type males are accepted randomly (grey
921 arrows) for mating by females of the same semispecies (all blue), carrying the
922 same/compatible *Wolbachia* (both *wA*). Right: *Wolbachia* kd males (indicated by red
923 flash) with reduced titers of *wA* are rejected as mate partners (red block) from wild type
924 females of the same semispecies. Thickness of arrows indicates strength of mate
925 choice between partners: thick black arrow = high mate preference, red block =
926 assortative mating, and grey medium arrow = lack of mate choice, i.e., random mating.
927 Abbreviations: kd knockdown.

928

929 **Figure 2**

930 Quantitative analysis of *Wolbachia* titers in wild type (wt) and knockdown (kd) *D.*
931 *paulistorum* males. Bars represent *Wolbachia* titers measured *via* quantitative real time
932 PCR on DNA from whole body extracts of A28 (A), and O11 (B) using *Wolbachia* 16S
933 rRNA normalized against *Drosophila* ribosomal protein 17 (*RPS17*). Each purple bar
934 represents the mean *Wolbachia* titer in wt males (mean from 8-10 biological replicates
935 is plotted). Orange bars represent titers in F₇/F₁₄ kd males (post treatment); individual
936 biological replicates are shown as black dots. In addition, for A28^{kd}, F₁₀ titers were
937 tested. Kd-titers are relative to wt ones (set to 1). Each sample was run in technical
938 duplicates. Error bars represent the standard deviation of the biological replicates.
939 Asterisks indicate significance of differences between titer levels. *P* values were
940 calculated using *Fisher's* exact test. Abbreviations: wt wild type, F₇, F₁₀, F₁₄ fly
941 generations 7, 10 and 14.

942

943 **Figure 3**

944 Violin plots showing estimated sexual Isolation Index (eSII) in multiple choice assays
945 between *D. paulistorum* wild type (wt), *Wolbachia*-knockdown (kd), axenic (et), and

946 penicillin/streptomycin-treated (ps) flies. (A,B) Intra-semispecific mate choice assays
947 with pool lines. Wild type (wt) flies (control, in purple) or knockdown (kd) flies (in
948 orange) were assayed at generations F₄, F₅, F₁₃, against wt flies, for A28 and O11.
949 (C,D) Intra-semispecific mate choice assays with isofemale lines. wt vs. wt (purple),
950 and kd vs. kd (pink) mating choice assays were performed between different isofemale
951 lines, respectively at generation F₁₀ and F₈. (E) Intra-semispecific assays between wt
952 and gut-flora-restored flies (gfr) performed at generation F₁₀ for A28 and O11. (F) Intra-
953 semispecific assays between wt and ethanol-washed wt flies (et) performed at
954 generation F₂ for A28 and O11. (G) Intra-semispecific assays between wt and
955 penicillin/streptomycin-treated flies (ps), performed at generation F₃ for the A28 and
956 O11. The last assay (yellow) is an additional inter-semispecific assay between wt and
957 ps flies. For all assays, the model-estimated SII was used on either individual replicates
958 (dots), or considering the 5 replicates (horizontal bars). Asterisks denote assays for
959 which the likelihood ratio test (LRT) is highly significant (p values < 10⁻⁴), the null model
960 being random mating (SII = 0). All the p values are reported in table S2-S4).

961

962 **Figure 4**

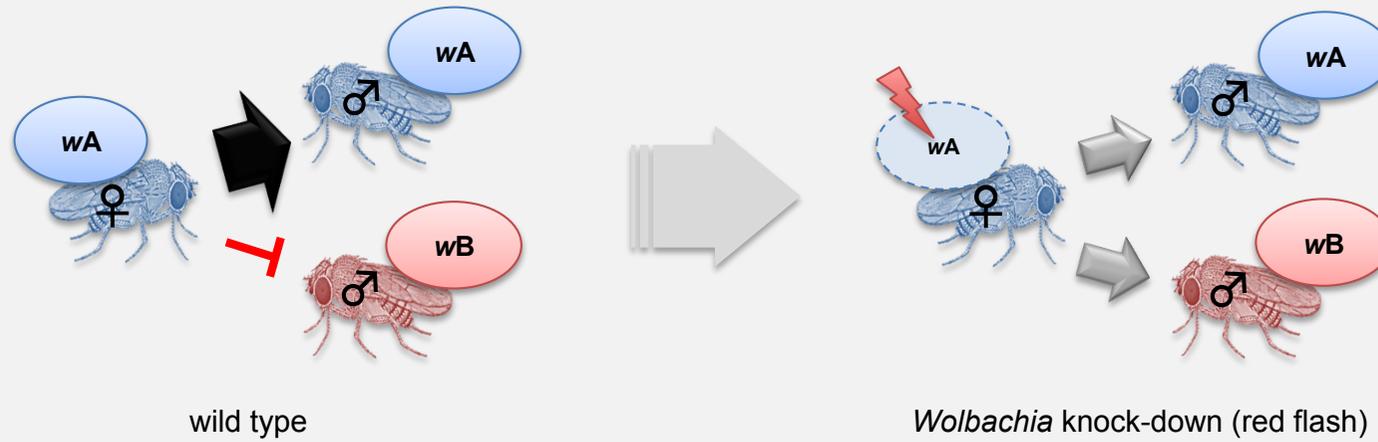
963 Quantitative changes in *D. paulistorum* male-specific pheromone compounds upon
964 *Wolbachia*-knockdown. Violin plots show percentage of the four male-specific ester
965 compounds of the total pheromone profiles (27 compounds) of A28 (A) and O11 (B).
966 Individual replicates for wild type controls (wt) are shown in purple, and in orange for
967 F₇ knockdown males (kd). Horizontal bars correspond to the mean. Tables below violin
968 plots list corresponding percent change of compounds plus n-fold increase (↑) or
969 decrease (↓) between wt and kd. Asterisks indicate statistical significance based on
970 two-tailed *P* values from *Student's t* tests. Abbreviations: DA 11-docosenyl-acetate, TA
971 19-triacontenyl-acetate, MD methyl-(Z)-tetradecanoate, DU di-unsaturated acetate
972 C₃₂H₆₀O₂.

973

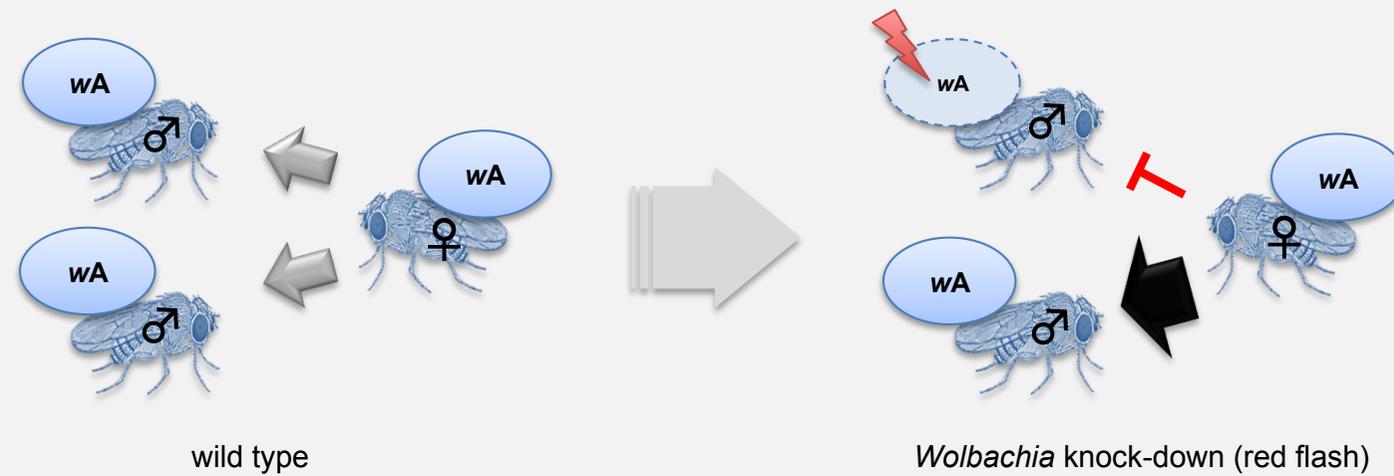
974 **Figure 5**

975 Fluorescence *in situ* hybridization (FISH) on *Drosophila* oenocytes. (A, A') *Wolbachia*
976 are present in *D. paulistorum* O11^{wt} females, and (B, B') in O11^{wt} males. (C) Symbionts
977 are lacking from oenocytes of O11 F₄^{kd} males and from F₄^{kd} females (C'). (D) In F₁₃
978 post kd, oenocytes seem recolonized by *Wolbachia*. (E, E') Oenocytes from
979 *Wolbachia*-infected *D. melanogaster* Harwich strain (H2) tested also negative. (F)
980 *Wolbachia*-uninfected *D. willistoni* (Willi3) was used as negative control. *Wolbachia* are
981 shown in red (16-23S rRNA-specific probe); *Drosophila* nuclei are stained in blue
982 (DAPI). Grey dashed lines mark borders of oenocyte cells. Abbreviations: *Wol*
983 *Wolbachia*, kd knockdown. Scale bar is 10µm.

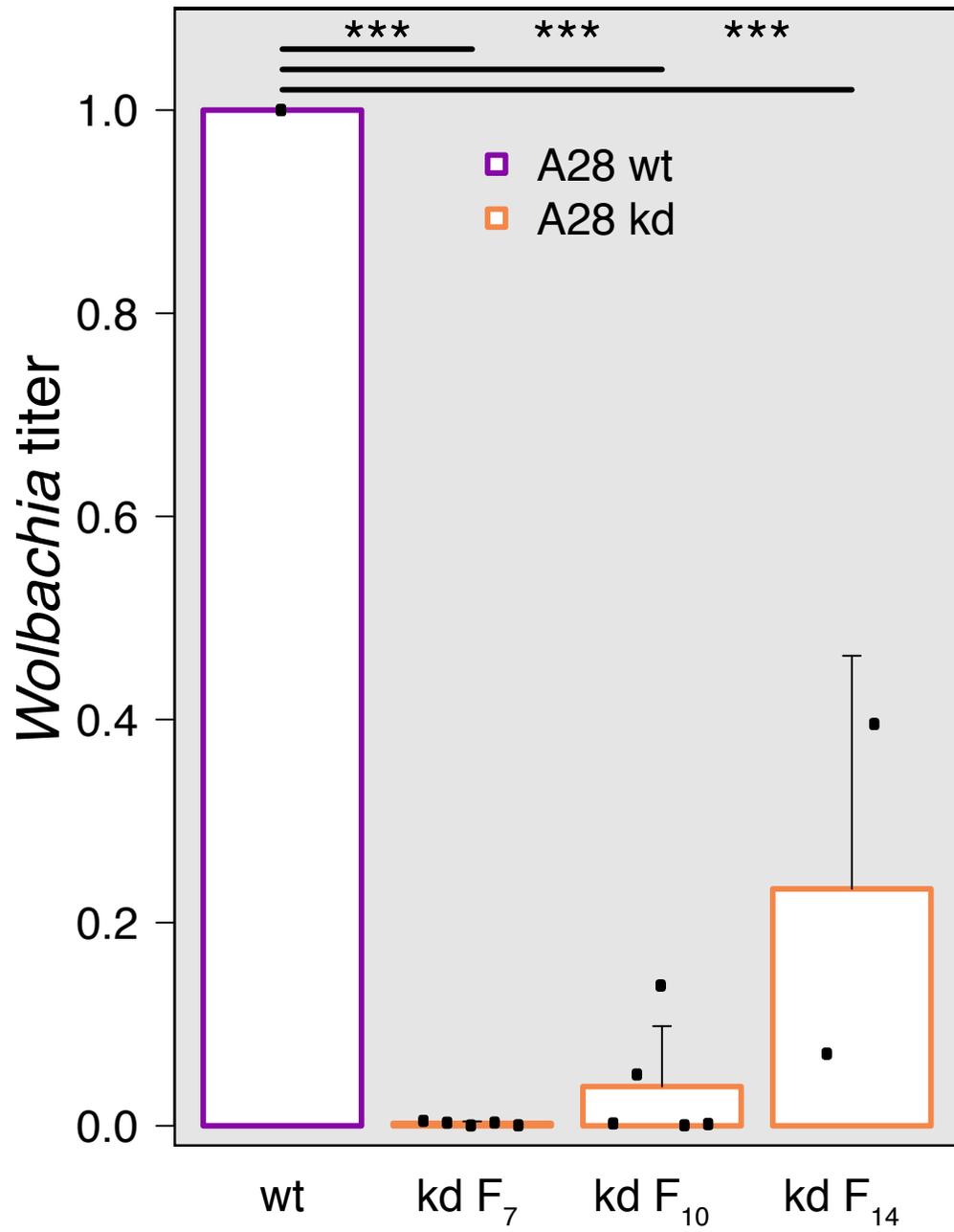
A Mate choice in *D. paulistorum*: *Wolbachia*-knockdown females lose assortative mating behavior against heterogamic wild type males (Miller et al. 2010)



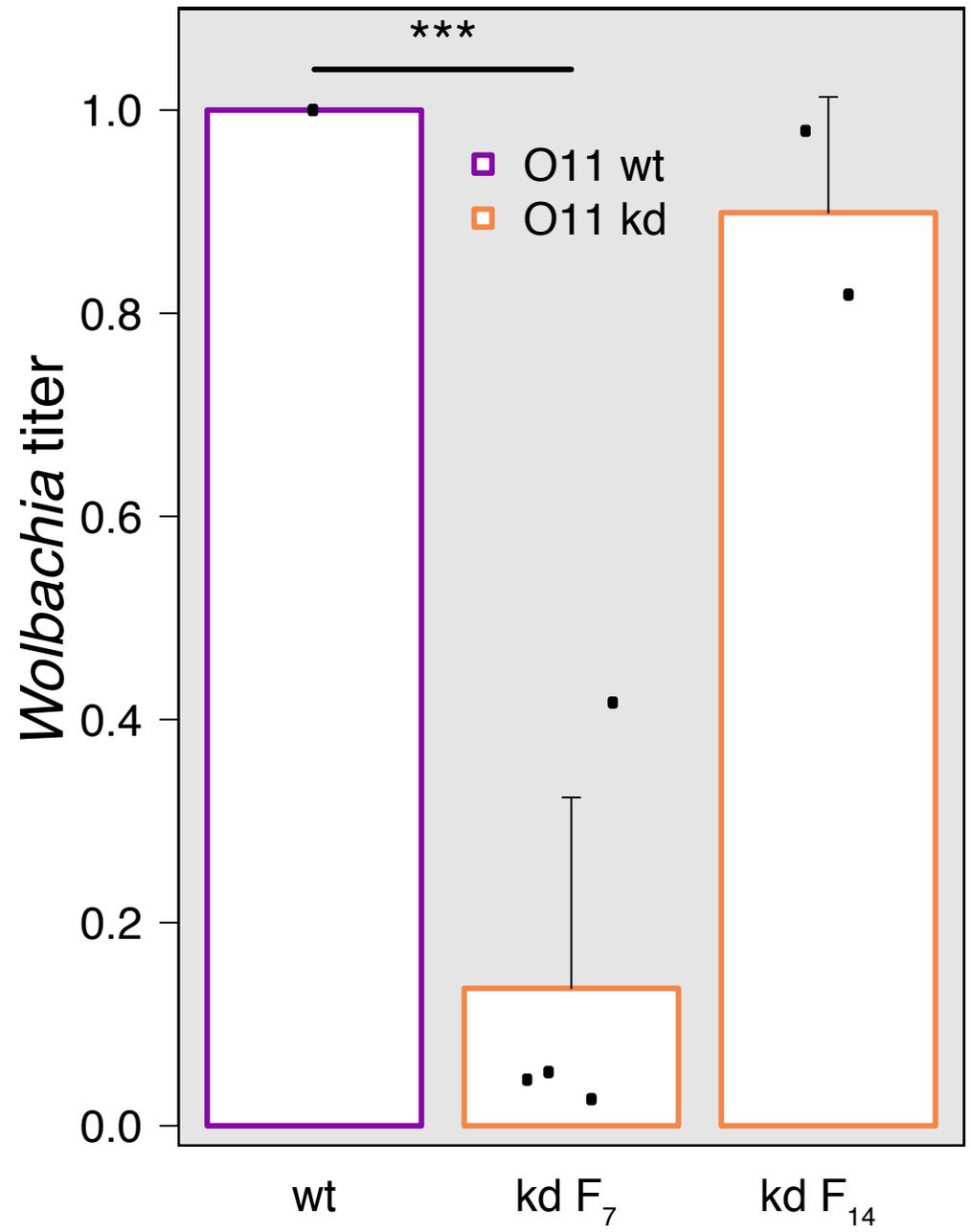
B Mate choice in *D. paulistorum*: *Wolbachia*-knockdown males are rejected by homogamic wild type females (this study)

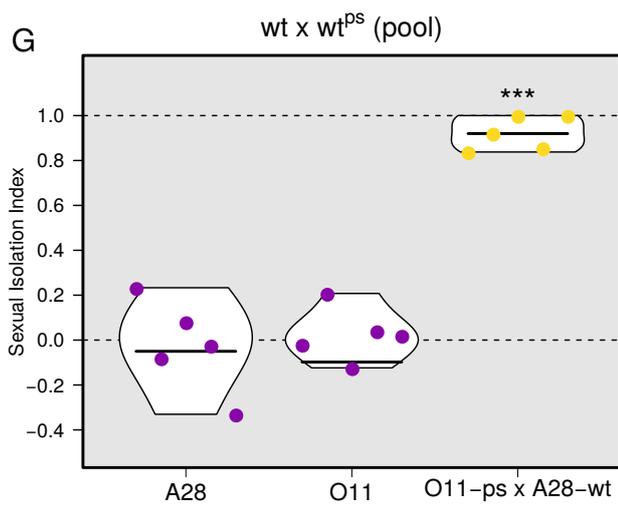
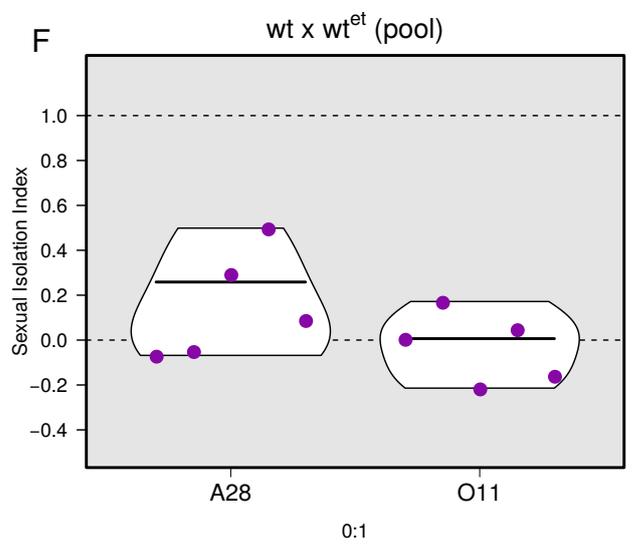
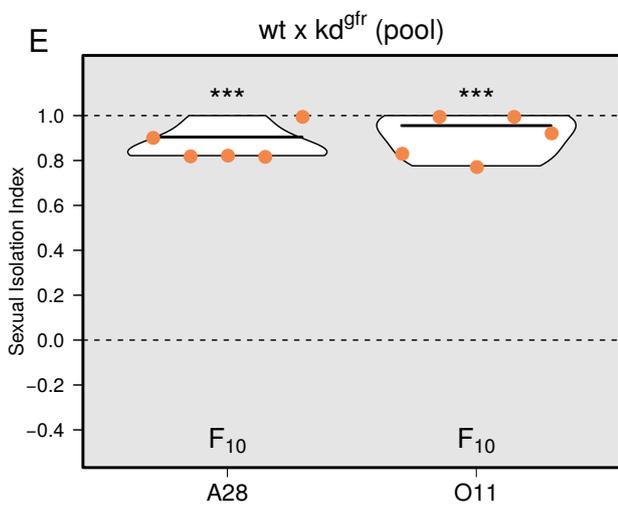
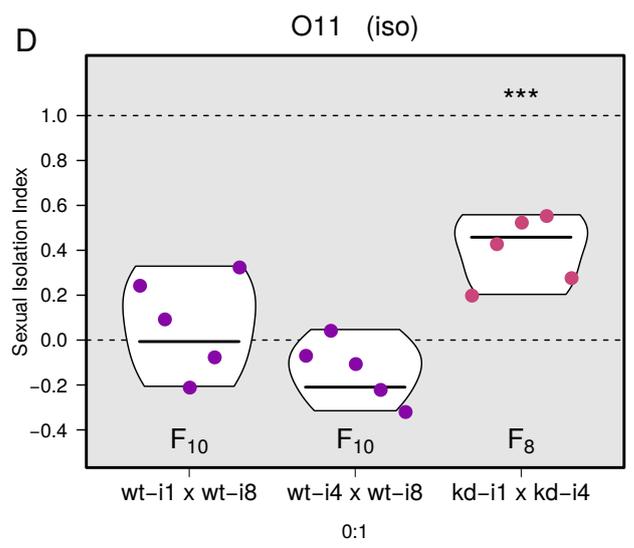
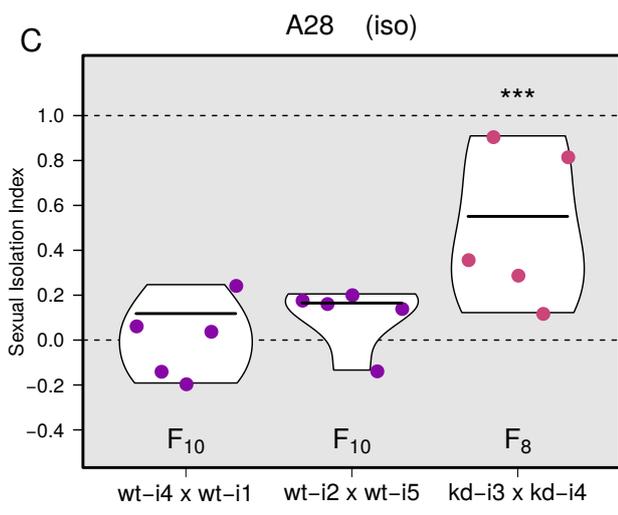
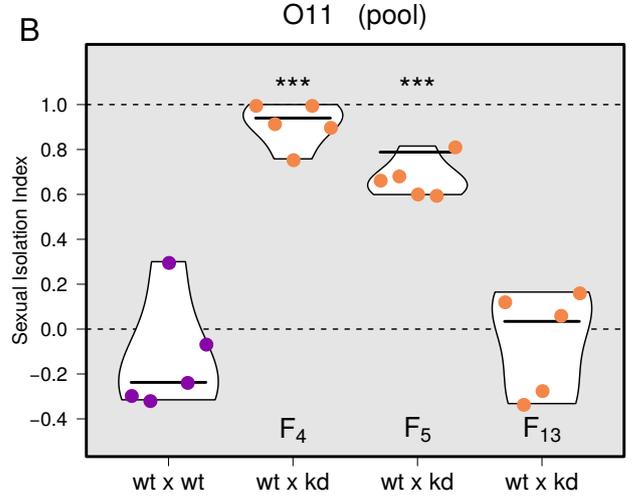
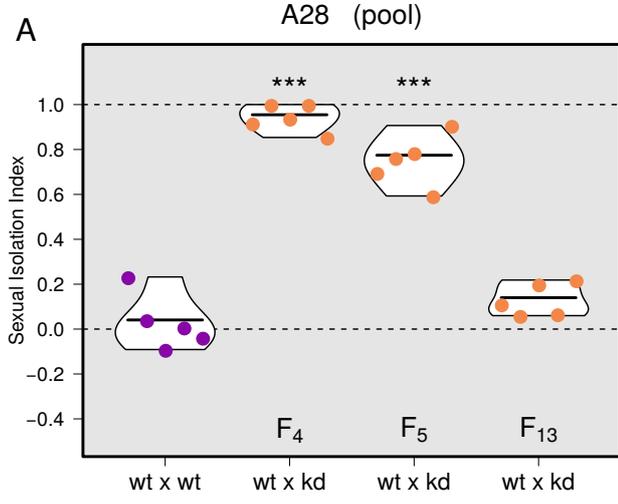


A

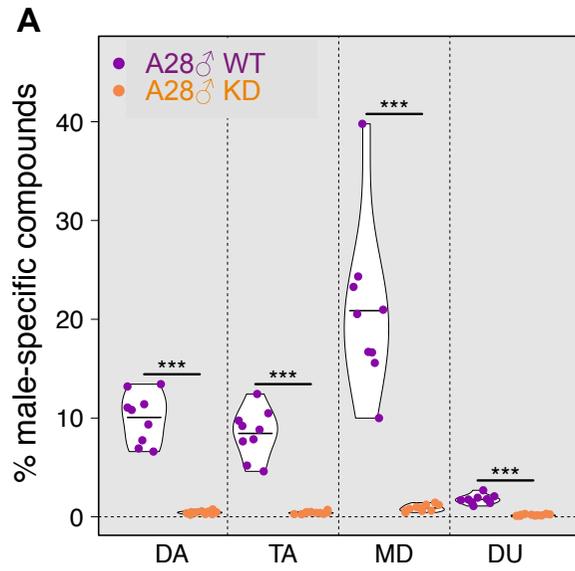


B

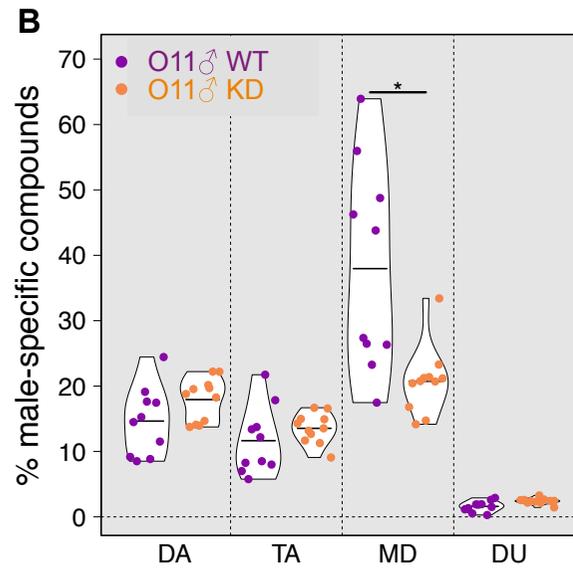




- intra-semispecies wildtype x wildtype
- intra-semispecies wildtype x knockdown
- intra-semispecies independent knockdown x knockdown
- inter-semispecies wildtype x knockdown



| CHC | A28 ^{wt} | A28 ^{kd} | fold change |
|-----|-------------------|-------------------|-------------|
| DA | 10.06% | 0.44% | 22.7 ↓ |
| TA | 8.44% | 0.41% | 20.6 ↓ |
| MD | 20.87% | 0.89% | 23.4 ↓ |
| DU | 1.76% | 0.19% | 9.3 ↓ |



| CHC | O11 ^{wt} | O11 ^{kd} | fold change |
|-----|-------------------|-------------------|-------------|
| DA | 14.65% | 17.94% | 1.2 ↑ |
| TA | 11.65% | 13.54% | 1.2 ↑ |
| MD | 97.22% | 20.74% | 4.7 ↓ |
| DU | 1.60% | 2.41% | 1.5 ↑ |

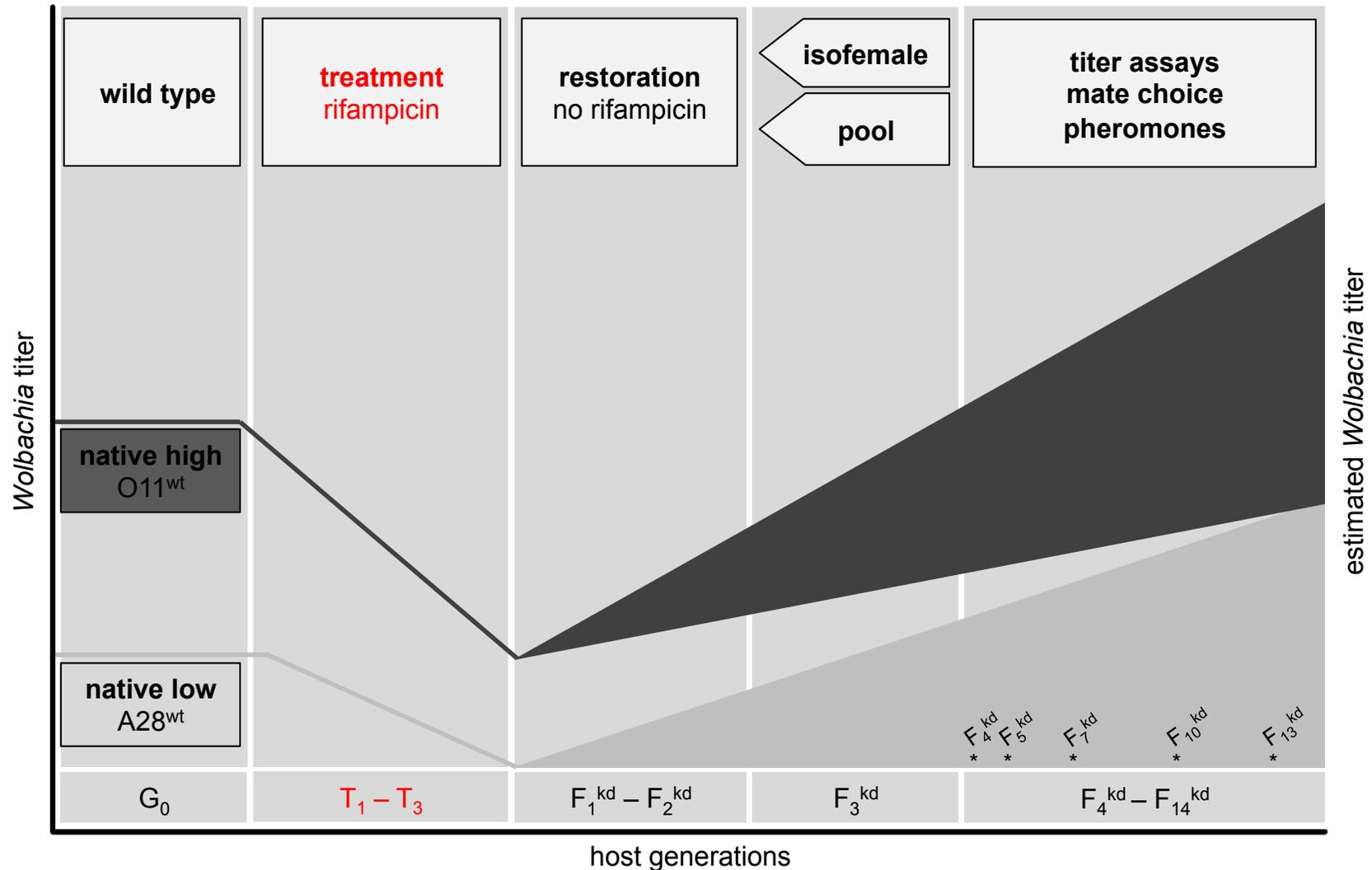


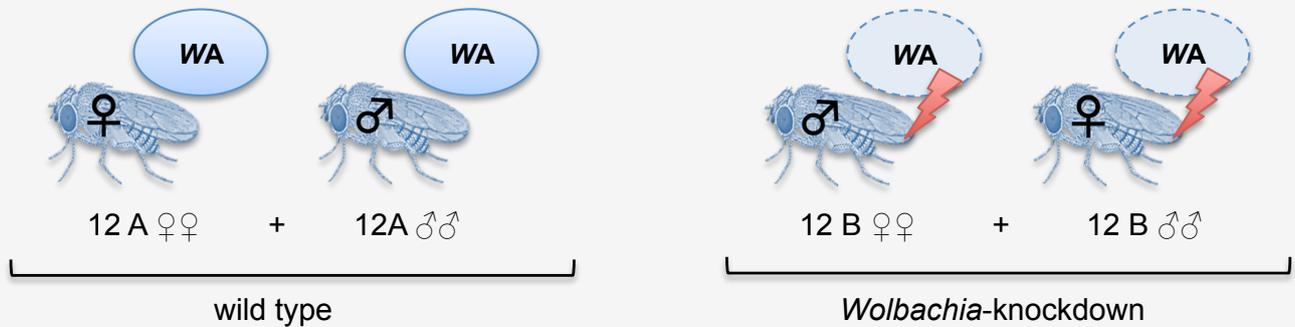
Figure S1. Generation of *D. paulistorum* knockdown (kd) lines. Wild type (wt) *D. paulistorum* individuals (A28^{wt}, O11^{wt}) were treated with 0.2% rifampicin during generations T₁-T₃ to reduce native *Wolbachia* titers followed by restoration phases during generations F₁^{kd}-F₂^{kd}. F₂^{kd} was used to start isofemale lines or proceed in mass-mated pool lines. Assays were then performed between generations F₄^{kd} and F₁₃^{kd}. Asterisks mark generations, which were actually tested in diverse assays. Post antibiotic treatment, *Wolbachia* titers are assumed to be variable, *i.e.*, remain decreased, drop back to or maybe even exceed native titer levels (gray triangles). Abbreviations: T₁-T₃ treatment generation 1-3, kd *Wolbachia*-knockdown.

A Set-up of a multiple-mating choice assay (wild type vs. knockdown)

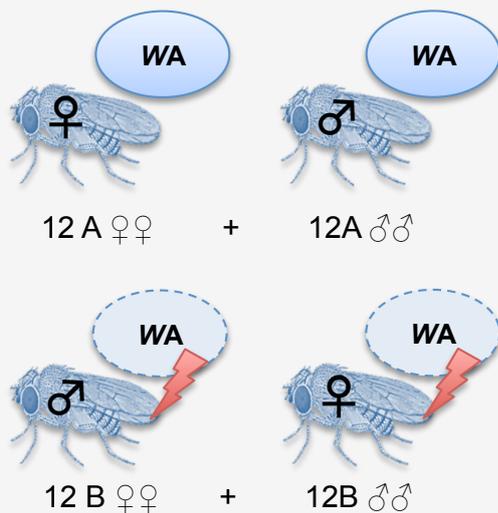
1 assay = 5 replicates (240 flies in total)



1 replicate = 48 flies



B HOMOgamic-like mating



HETEROgamic-like mating

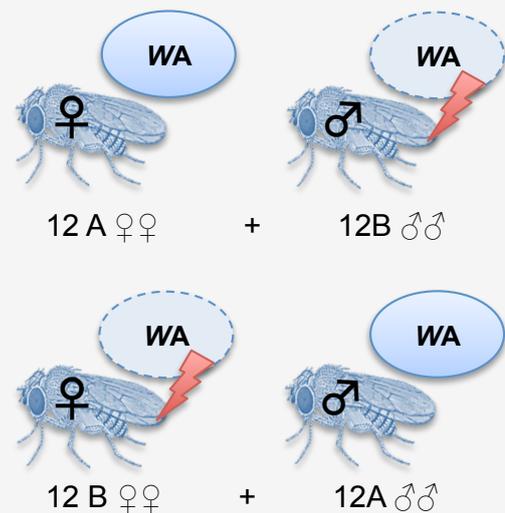
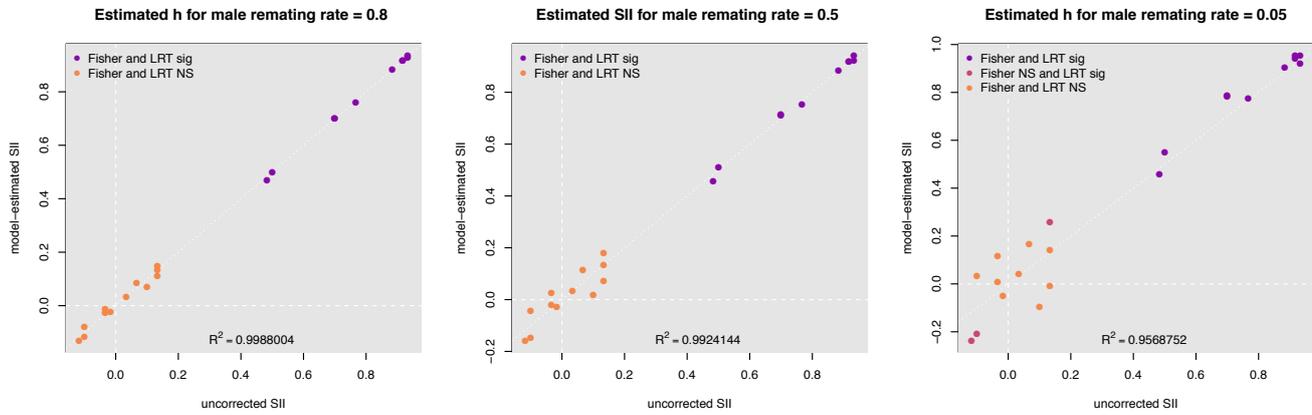


Figure S2. Schematic presentation of setting up multiple-mating choice assays. (A) Each completed assay consists of 5 replicates, totalling 240 individual flies. Each replicate consists of 48 individuals, *i.e.*, 12 females and 12 males from A (wild type); 12 females and 12 males from B (*Wolbachia*-knockdown). (B) In each replicate, scored mating events can be either homogamic-like (between A only and B only) or heterogamic-like (mixed between A and B). Flies are differentiated by alternated wing clips; ♀♀, ♂♂ represents female and male virgin flies (Dobzhansky and Pavlovsky 1966; Leonard and Ehrman 1983).

Figure S3 : Effect of the remating rate (male) on the estimation of the SII.

Application to the data. In all, a fixed rate of 0.01 was used for female remating rate. Fisher’s test have been used for uncorrected SII. Likelihood Ratio Test (LRT) have been used for estimated SII.



| Assay | Cross | Table | Assay | Uncorrected SII | Estimated SII (remating=0.8) | Estimated SII (remating=0.5) | Estimated SII (remating=0.05) |
|-------|---------------------|-------|-------|-----------------|------------------------------|------------------------------|-------------------------------|
| 1 | AxO_control | S2 | 2 | 0.7 | 0.70 | 0.71 | 0.78 |
| 2 | AxA_control | S2 | 3 | 0.03 | 0.03 | 0.03 | 0.04 |
| 3 | OxO_control | S2 | 4 | -0.12 | -0.13 | -0.16 | -0.24 |
| 4 | ISOA28_wt_vs_wt_I | S2 | 5 | -0.03 | -0.01 | 0.02 | 0.12 |
| 5 | ISOA28_wt_vs_wt_II | S2 | 6 | 0.07 | 0.08 | 0.11 | 0.16 |
| 6 | ISOO11_wt_vs_wt_I | S2 | 7 | 0.13 | 0.11 | 0.07 | -0.01 |
| 7 | ISOO11_wt_vs_wt_II | S2 | 8 | -0.1 | -0.12 | -0.15 | -0.21 |
| 8 | A28_wt_vs_kd_F4 | S3 | 1 | 0.93 | 0.94 | 0.94 | 0.95 |
| 9 | A28_wt_vs_kd_F5 | S3 | 2 | 0.77 | 0.76 | 0.75 | 0.77 |
| 10 | A28_wt_vs_kd_F13 | S3 | 3 | 0.13 | 0.13 | 0.13 | 0.14 |
| 11 | O11_wt_vs_kd_F4 | S3 | 4 | 0.92 | 0.92 | 0.92 | 0.94 |
| 12 | O11_wt_vs_kd_F5 | S3 | 5 | 0.7 | 0.70 | 0.71 | 0.79 |
| 13 | O11_wt_vs_kd_F13 | S3 | 6 | -0.1 | -0.08 | -0.05 | 0.03 |
| 14 | ISOA28_kd_vs_kd_F8 | S3 | 7 | 0.5 | 0.50 | 0.51 | 0.55 |
| 15 | ISOO11_kd_vs_kd_F8 | S3 | 8 | 0.48 | 0.47 | 0.46 | 0.46 |
| 16 | A28_wt_vs_gfr_F10 | S4 | 1 | 0.88 | 0.88 | 0.88 | 0.90 |
| 17 | O11_wt_vs_gfr_F10 | S4 | 2 | 0.92 | 0.92 | 0.92 | 0.96 |
| 18 | A28wtxA28et_control | S4 | 3 | 0.13 | 0.15 | 0.18 | 0.26 |
| 19 | O11wtxO11et_control | S4 | 4 | -0.03 | -0.03 | -0.02 | 0.01 |
| 20 | A28wtxA28ps_control | S4 | 5 | -0.02 | -0.02 | -0.03 | -0.05 |
| 21 | O11wtxO11ps_control | S4 | 6 | 0.1 | 0.07 | 0.02 | -0.10 |
| 22 | A28wtxO11ps | S4 | 7 | 0.93 | 0.93 | 0.92 | 0.92 |

At high remating rate, the estimated SII (eSII) fit quite well the uncorrected SII (uSII), since the last matings are not as constrained as in case of no remating. With decreasing remating rate, the estimated SII remain quite similar to uncorrected ones for high level of homogamy. For low level of homogamy (close to random mating), more discrepancies are observed between eSII and uSII.

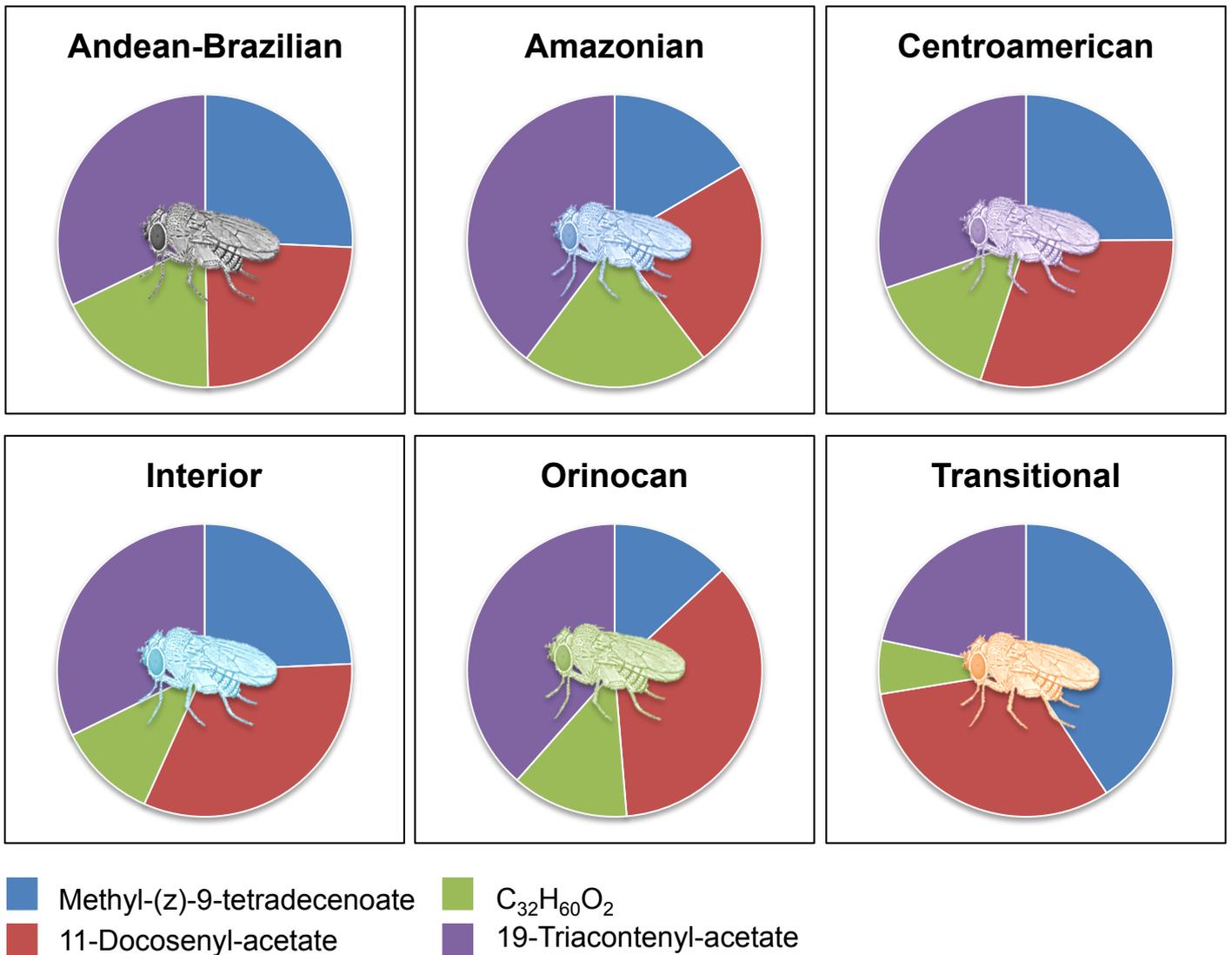


Figure S4. Male-specific long-chain ester compounds in the six *D. paulistorum* semispecies. Pie charts show presence of the same male specific compounds (Methyl-(z)-tetradecenoate, 11-Docosenyl-acetate, C₃₂H₆₀O₂, and 19-Triacontenyl-acetate), but in differing proportions in all six semispecies (Chao et al. 2010; Kim et al. 2014). Quantities are relative amounts in percent. Each semispecies is represented by a fly in a different color.

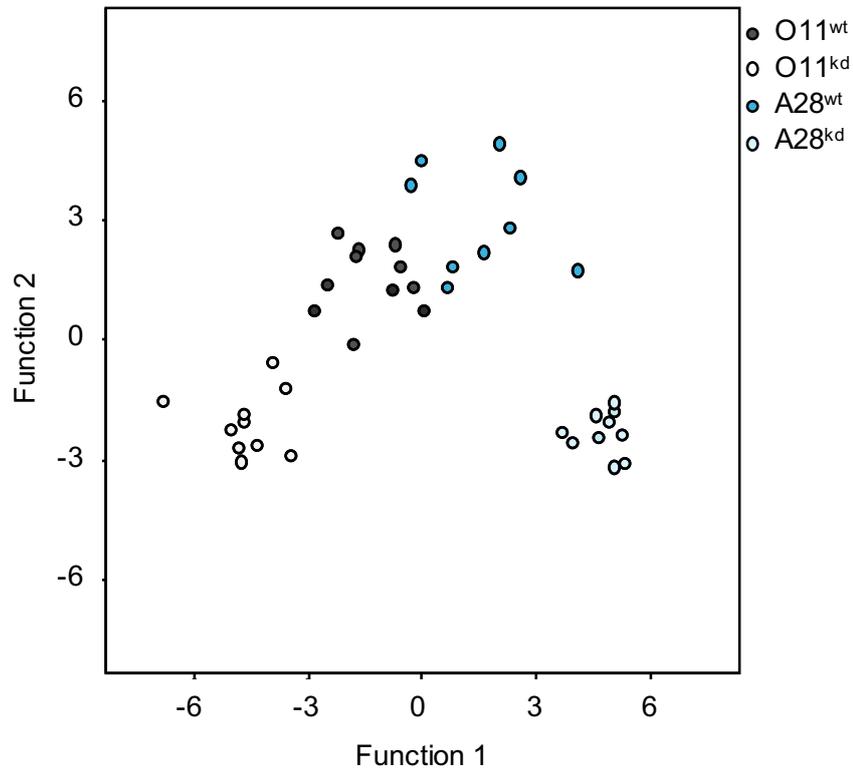


Figure S5. Discriminant analyses (DA) of CHC-profiles in *D. paulistorum* males upon symbiont knockdown based on six principal components derived from centered log-ratio transformed relative amounts of all 27 detected compounds. The plot of canonical discriminant functions shows that semispecies A28^{wt} and O11^{wt} are different in their CHC profiles and that the CHC profiles knockdown groups (A28^{kd}, O11^{kd}) differ from the corresponding wildtypes (A28^{wt}, O11^{wt}). Abbreviations: wt wild type, kd *Wolbachia*-knockdown. X-axis shows discriminant function, y-axis plots how data points are distributed in intervals of the discriminant function.

| Abbreviation | Line name | Semispecies | Description |
|---------------------|-----------------------------|-------------|----------------------------------------------------------------------------------------|
| A28 ^{wt} | control line A28 | Amazonian | wild type |
| O11 ^{wt} | control line O11 | Orinocan | wild type |
| A28 ^{wt-i} | control line A28 | Amazonian | wild type, isofemale line |
| O11 ^{wt-i} | control line O11 | Orinocan | wild type, isofemale line |
| A28 ^{kd} | knockdown line A28 | Amazonian | 0.2% rifampicin for 3 gen plus restoration for 3 gen, pool line |
| O11 ^{kd} | knockdown line O11 | Orinocan | 0.2% rifampicin for 3 gen plus restoration for 3 gen, pool line |
| A28 ^{kd-i} | knockdown line A28 | Amazonian | 0.2% rifampicin for 3 gen plus restoration for 3 gen, isofemale line |
| O11 ^{kd-i} | knockdown line O11 | Orinocan | 0.2% rifampicin for 3 gen plus restoration for 3 gen, isofemale line |
| A28 ^{gfr} | gut flora restored line A28 | Amazonian | 0.2% rifampicin for 3 gen plus restoration for 3 gen, gut flora restoration, pool line |
| O11 ^{gfr} | gut flora restored line O11 | Orinocan | 0.2% rifampicin for 3 gen plus restoration for 3 gen, gut flora restoration, pool line |
| A28 ^{et} | axenic line A28 | Amazonian | eggs washed with 70% ethanol before establishment of line |
| O11 ^{et} | axenic line O11 | Orinocan | eggs washed with 70% ethanol before establishment of line |
| A28 ^{ps} | pen/strep line A28 | Amazonian | 0.01% penicillin/streptomycin for 1 gen |
| O11 ^{ps} | pen/strep line O11 | Orinocan | 0.01% penicillin/streptomycin for 1 gen |

Table S1. Detailed information on experimental *Drosophila paulistorum* lines. Pool lines were maintained *via* sibling mating. Rifampicin and penicillin/streptomycin concentration is w/v final concentration in fly food. Abbreviations: gen generation(s), wt wildtype, kd *Wolbachia* knockdown, wt-i wildtype isofemale line, kd-i *Wolbachia* knockdown isofemale line, gfr gut flora restored, et axenic (ethanol-washed) flies, ps penicillin/streptomycin-treated flies.

| Assay | Semispecies | Strain | Year | eSII (CI 95%) | LRT <i>p</i> value | uSII ± SE | Fisher's test <i>p</i> value | |
|-------------------------------------|-------------|---------------------|---------------------------------------------|---------------|----------------------|-----------|------------------------------|--------------------|
| Wildtype control assays (pool) | | | | | | | | |
| 1* | inter | Amazonian/Orinocan | A28 ^{wt} x O11 ^{wt} | 1965 | NA | NA | +0.61 ± 0.07 | NA |
| 2 | | Amazonian/Orinocan | A28 ^{wt} x O11 ^{wt} | this study | +0.71 (0.58 – 0.82) | 0 | +0.70 ± 0.07 | < 10 ⁻⁴ |
| 3 | intra | Amazonian/Amazonian | A28 ^{wt} x A28 ^{wt} | this study | +0.03 (-0.15 - 0.21) | 0.7337 | +0.03 ± 0.09 | 0.8552 |
| 4 | | Orinocan/Orinocan | O11 ^{wt} x O11 ^{wt} | this study | -0.16 (-0.33 – 0.02) | 0.0796 | -0.12 ± 0.09 | 0.2716 |
| Wildtype control assays (isofemale) | | | | | | | | |
| 5 | intra | Amazonian/Amazonian | A28 ^{wt-i4} x A28 ^{wt-i1} | this study | +0.02 (-0.14 – 0.18) | 0.7661 | -0.03 ± 0.09 | 0.8523 |
| 6 | | Amazonian/Amazonian | A28 ^{wt-i2} x A28 ^{wt-i5} | this study | +0.11 (-0.07 – 0.29) | 0.2195 | +0.07 ± 0.09 | 0.5839 |
| 7 | | Orinocan/Orinocan | O11 ^{wt-i1} x O11 ^{wt-i8} | this study | +0.07 (-0.1 – 0.25) | 0.4239 | +0.13 ± 0.09 | 0.2001 |
| 8 | | Orinocan/Orinocan | O11 ^{wt-i4} x O11 ^{wt-i8} | this study | -0.15 (-0.32 – 0.03) | 0.1102 | -0.10 ± 0.09 | 0.3590 |

Table S2. Control mate choice assays between (inter) and within (intra) wild type *D. paulistorum* semispecies. Estimated and uncorrected Sexual Isolation Index (eSII and uSII) in intra or interspecific control assays. The eSII has been estimated for a male remating rate of 0.5. Other estimated parameters are shown in Table S5. The uSIIs (and associated Fisher's tests) are indicated for comparison. The assay marked with an asterisk (1) was performed by L. Ehrman in a previous study (Kim et al. 2004). Inter-specific assays (Amazonian (A28) vs. Orinocan (O11; assays 1,2)) show high SII. Assays 3-4 correspond to mate choice assays performed between members of the same semispecies (intra-semispecific assays). As expected, no isolation is observed when testing A28 vs. A28 (assay 3) and O11 vs. O11 (assay 4). Assays 5-8 are intra-semispecific assays between independent isofemale lines of A28 (assays 5, 6) and O11 (assays 7, 8), which show no sexual isolation. Abbreviations: wt wildtype, wt-i wildtype isofemale line, CI confidence interval, LRT Likelihood Ratio Test, ±SE standard error of the mean. Fisher's tests are two-tailed tests. Raw data are available in the supplementary file Raw_Data.

| Assay | Semispecies | Strain | Generation post treatment | eSII (CI 95%) | LRT <i>p</i> value | uSII ± SE | Fisher's test <i>p</i> value | |
|------------------------------|-------------|---------------------|---------------------------------------------|---------------|----------------------|--------------------|------------------------------|--------------------|
| knockdown assays (pool) | | | | | | | | |
| 1 | intra | Amazonian/Amazonian | A28 ^{kd} x A28 ^{wt} | 4 | +0.94 (0.87 - 0.98) | < 10 ⁻⁴ | +0.93 ± 0.03 | < 10 ⁻⁴ |
| 2 | | Amazonian/Amazonian | A28 ^{kd} x A28 ^{wt} | 5 | +0.75 (0.62 - 0.86) | < 10 ⁻⁴ | +0.77 ± 0.06 | < 10 ⁻⁴ |
| 3 | | Amazonian/Amazonian | A28 ^{kd} x A28 ^{wt} | 13 | +0.13 (-0.05 - 0.31) | 0.1473 | +0.13 ± 0.09 | 0.2008 |
| 4 | | Orinocan/Orinocan | O11 ^{kd} x O11 ^{wt} | 4 | +0.92 (0.83 - 0.97) | < 10 ⁻⁴ | +0.92 ± 0.04 | < 10 ⁻⁴ |
| 5 | | Orinocan/Orinocan | O11 ^{kd} x O11 ^{wt} | 5 | +0.71 (0.57 - 0.82) | < 10 ⁻⁴ | +0.70 ± 0.07 | < 10 ⁻⁴ |
| 6 | | Orinocan/Orinocan | O11 ^{kd} x O11 ^{wt} | 13 | -0.05 (-0.21 - 0.12) | 0.6008 | -0.10 ± 0.09 | 0.3571 |
| knockdown assays (isofemale) | | | | | | | | |
| 7 | intra | Amazonian/Amazonian | A28 ^{kd-i3} x A28 ^{kd-i4} | 8 | +0.51 (0.34 - 0.65) | < 10 ⁻⁴ | +0.50 ± 0.08 | 0.0000 |
| 8 | | Orinocan/Orinocan | O11 ^{kd-i1} x O11 ^{kd-i4} | 8 | +0.46 (0.28 - 0.61) | < 10 ⁻⁴ | +0.48 ± 0.08 | 0.0000 |

Table S3. Induction of *de novo* assortative mating in *D. paulistorum* semispecies upon *Wolbachia* knockdown. Estimated and uncorrected Sexual Isolation Index (eSII and uSII) in intraspecific assays between wildtype and knockdown strains or between different isofemale knockdown strains. The eSII has been estimated for a male remating rate of 0.5. Other estimated parameters are shown in Table S6. Assays were performed with pool lines (1-6) and isofemale lines (7-8) from A28 and O11. Intra-semispecific pool assays in generations F₄ and F₅ show high SIIs, whereas in F₁₃, SIIs are significantly lower. Intra-semispecific assays between kd-i lines show moderate levels of sexual isolation. Abbreviations: wt wildtype, kd *Wolbachia* knockdown, kd-i *Wolbachia* knockdown isofemale line, CI confidence interval, LRT Likelihood Ratio Test, ±SE standard error of the mean. Fisher's tests are two-tailed tests (on SII). Raw data are available in the supplementary file Raw_Data.

| Assay | Semispecies | Strain | Generation post treatment | eSII (CI 95%) | LRT p value | uSII \pm SE | Fisher's test p value | |
|---------------------------------------------|-------------|---------------------|----------------------------------------|---------------|----------------------|---------------|-------------------------|-------------|
| Gut flora-restored assays (gfr) | | | | | | | | |
| 1 | intra | Amazonian/Amazonian | A28 ^{gfr} x A28 ^{wt} | 10 | 0.88 (0.78 – 0.95) | $< 10^{-4}$ | +0.88 \pm 0.040 | $< 10^{-4}$ |
| 2 | | Orinocan/Orinocan | O11 ^{gfr} x O11 ^{wt} | 10 | 0.92 (0.83 – 0.97) | $< 10^{-4}$ | +0.92 \pm 0.040 | $< 10^{-4}$ |
| Axenic (ethanol-washed) assays (et) | | | | | | | | |
| 3 | intra | Amazonian/Amazonian | A28 ^{et} x A28 ^{wt} | 2 | 0.18 (0 – 0.35) | 0.0523 | +0.13 \pm 0.090 | 0.2008 |
| 4 | | Orinocan/Orinocan | O11 ^{et} x O11 ^{wt} | 2 | -0.02 (-0.18 – 0.15) | 0.8262 | -0.03 \pm 0.090 | 0.8564 |
| Penicillin/streptomycin-treated assays (ps) | | | | | | | | |
| 5 | intra | Amazonian/Amazonian | A28 ^{ps} x A28 ^{wt} | 1 | -0.03 (-0.21 – 0.15) | 0.7468 | -0.02 \pm 0.090 | 1.0000 |
| 6 | | Orinocan/Orinocan | O11 ^{ps} x O11 ^{wt} | 1 | 0.02 (-0.15 – 0.18) | 0.8272 | +0.10 \pm 0.090 | 0.3546 |
| 7 | inter | Amazonian/ Orinocan | A28 ^{wt} x O11 ^{ps} | 1 | 0.92 (0.83 – 0.98) | $< 10^{-4}$ | +0.93 \pm 0.030 | $< 10^{-4}$ |

Table S4. Mate choice assays with gut flora restored (gfr), axenic (et), and penicillin/streptomycin-treated *D. paulistorum* semispecies. Estimated and uncorrected Sexual Isolation Index (eSII and uSII) in assays between wildtype and treated lines. The eSII has been estimated for a male remating rate of 0.5. Other estimated parameters are shown in Table S5. Intra-semispecific assays with gfr lines (assays 1-1: gfr x wt) show high SIIs (similar to assays with knockdown lines). Gfr lines came from direct treatment with faeces of naturally infected flies to consequently restore the gut flora.

| | Cross | Table | Assay | eSII | <i>p</i> value (eSII) | pM | <i>p</i> value (pM) | pF | <i>p</i> value (pF) |
|----|---------------------|-------|-------|----------------------|-----------------------|--------------------|---------------------|--------------------|---------------------|
| 1 | AxO_control | S2 | 2 | 0.71 (0.58 - 0.82) | 0.0000 | 0.35 (0.17 - 0.57) | 0.1773 | 0.65 (0.54 - 0.74) | 0.0055 |
| 2 | AxA_control | S2 | 3 | 0.03 (-0.15 - 0.21) | 0.7337 | 0.46 (0.36 - 0.55) | 0.3750 | 0.57 (0.47 - 0.67) | 0.1660 |
| 3 | OxO_control | S2 | 4 | -0.16 (-0.33 - 0.02) | 0.0796 | 0.60 (0.50 - 0.70) | 0.0590 | 0.58 (0.47 - 0.68) | 0.1573 |
| 4 | ISOA28_wt_vs_wt_I | S2 | 5 | 0.02 (-0.14 - 0.18) | 0.7661 | 0.69 (0.61 - 0.78) | 0.0000 | 0.32 (0.23 - 0.41) | 3e-04 |
| 5 | ISOA28_wt_vs_wt_II | S2 | 6 | 0.11 (-0.07 - 0.29) | 0.2195 | 0.48 (0.38 - 0.58) | 0.6589 | 0.45 (0.35 - 0.55) | 0.3392 |
| 6 | ISOO11_wt_vs_wt_I | S2 | 7 | 0.07 (-0.1 - 0.25) | 0.4239 | 0.59 (0.49 - 0.68) | 0.0733 | 0.66 (0.56 - 0.75) | 0.0013 |
| 7 | ISOO11_wt_vs_wt_II | S2 | 8 | -0.15 (-0.32 - 0.03) | 0.1102 | 0.44 (0.33 - 0.54) | 0.2533 | 0.40 (0.31 - 0.50) | 0.0501 |
| 8 | A28_wt_vs_kd_F4 | S2 | 1 | 0.94 (0.87 - 0.98) | 0.0000 | 0.00 (0 - 0) | 0.0334 | 0.75 (0.66 - 0.82) | 0.0000 |
| 9 | A28_wt_vs_kd_F5 | S3 | 2 | 0.75 (0.62 - 0.86) | 0.0000 | 0.32 (0.14 - 0.57) | 0.1492 | 0.45 (0.35 - 0.55) | 0.2971 |
| 10 | A28_wt_vs_kd_F13 | S3 | 3 | 0.13 (-0.05 - 0.31) | 0.1473 | 0.48 (0.38 - 0.58) | 0.6915 | 0.50 (0.40 - 0.59) | 0.9352 |
| 11 | O11_wt_vs_kd_F4 | S3 | 4 | 0.92 (0.83 - 0.97) | 0.0000 | 0.78 (0.35 - 0.99) | 0.1985 | 0.35 (0.25 - 0.45) | 0.0034 |
| 12 | O11_wt_vs_kd_F5 | S3 | 5 | 0.71 (0.57 - 0.82) | 0.0000 | 0.65 (0.44 - 0.83) | 0.1609 | 0.57 (0.47 - 0.66) | 0.1749 |
| 13 | O11_wt_vs_kd_F13 | S3 | 6 | -0.05 (-0.21 - 0.12) | 0.6008 | 0.35 (0.26 - 0.44) | 0.0012 | 0.67 (0.57 - 0.75) | 7e-04 |
| 14 | ISOA28_kd_vs_kd_F8 | S3 | 7 | 0.51 (0.34 - 0.65) | 0.0000 | 0.48 (0.33 - 0.64) | 0.8103 | 0.58 (0.49 - 0.67) | 0.0970 |
| 15 | ISOO11_kd_vs_kd_F8 | S3 | 8 | 0.46 (0.28 - 0.61) | 0.0000 | 0.60 (0.45 - 0.74) | 0.1787 | 0.65 (0.55 - 0.74) | 0.0041 |
| 16 | A28_wt_vs_gfr_F10 | S4 | 1 | 0.88 (0.78 - 0.95) | 0.0000 | 0.29 (0.06 - 0.64) | 0.2447 | 0.59 (0.50 - 0.68) | 0.0599 |
| 17 | O11_wt_vs_gfr_F10 | S4 | 2 | 0.92 (0.83 - 0.97) | 0.0000 | 0.56 (0.18 - 0.91) | 0.7770 | 0.38 (0.28 - 0.48) | 0.0157 |
| 18 | A28wtxA28et_control | S4 | 3 | 0.18 (0 - 0.35) | 0.0523 | 0.45 (0.34 - 0.56) | 0.3547 | 0.74 (0.65 - 0.81) | 0.0000 |
| 19 | O11wtxA28et_control | S4 | 4 | -0.02 (-0.18 - 0.15) | 0.8262 | 0.66 (0.57 - 0.75) | 5e-04 | 0.52 (0.42 - 0.63) | 0.6406 |
| 20 | A28wtxA28ps_control | S4 | 5 | -0.03 (-0.21 - 0.15) | 0.7468 | 0.53 (0.43 - 0.62) | 0.5538 | 0.68 (0.59 - 0.77) | 3e-04 |
| 21 | O11wtxA28ps_control | S4 | 6 | 0.02 (-0.15 - 0.18) | 0.8272 | 0.67 (0.58 - 0.75) | 3e-04 | 0.73 (0.62 - 0.81) | 0.0000 |
| 22 | A28wtxA28ps | S4 | 7 | 0.92 (0.83 - 0.98) | 0.0000 | 0.00 (0 - 0) | 0.0098 | 0.51 (0.42 - 0.61) | 0.7707 |

Table S5. Estimation of SII (Sexual Isolation Index), pM ('Male preference') and pF ('Female preference'). pM and pF reflect the bias in the mating order. No bias corresponds to a value of 0.5. The *p* values are computed from likelihood ratio test, the null hypothesis being random mating (for e SII) and no bias in the mating order (for pM and pF).

| No | Compound name | Retention time (min) | Retention index | Specificity |
|-----|-------------------------------------------------------------------------------|----------------------|-----------------|---------------|
| 1 | 11-Docosenyl-acetate (C ₂₄ H ₄₆ O ₂) | 18.816 | 25.79 | male-specific |
| 2 | 19-Triacontenyl-acetate (C ₃₂ H ₆₂ O ₂) | 28.670 | 33.86 | male-specific |
| 3 | 2-Methyl-triacontane (C ₃₁ H ₆₄) | 24.465 | 30.63 | male-specific |
| 4 | a di-unsaturated acetate (C ₃₂ H ₆₀ O ₂) | 28.175 | 33.54 | male-specific |
| 5 | Methyl-(Z)-9-tetradecanoate (C ₁₅ H ₂₈ O ₂) | 6.5920 | 17.13 | none |
| 6 | Ethyl-9-tetradecenoate (C ₁₆ H ₃₀ O ₂) | 7.5420 | 17.80 | none |
| 7 | MethylC28 | 22.228 | 28.62 | none |
| 8 | MethylC29 | 23.349 | 29.61 | none |
| 9* | unknown compound | 26.797 | 32.60 | none |
| 10 | C31:1 | 24.619 | 30.78 | none |
| 11 | C33:1A | 26.577 | 32.43 | none |
| 12 | C33:1B | 26.683 | 32.51 | none |
| 13* | C33:2A+unknown | 26.795 | 32.60 | none |
| 14 | C33:2B | 26.915 | 32.69 | none |
| 15 | C33:2C | 27.038 | 32.78 | none |
| 16 | C33:2D | 27.173 | 32.88 | none |
| 17 | C33:2E | 27.320 | 32.99 | none |
| 18 | C35:1A | 29.581 | 34.37 | none |
| 19 | C35:1B | 29.736 | 34.45 | none |
| 20 | C35:2A | 29.922 | 34.55 | none |
| 21 | C35:2B | 30.069 | 34.63 | none |
| 22 | C35:2C | 30.250 | 34.73 | none |
| 23 | C35:2D | 30.409 | 34.81 | none |
| 24 | C35:2E | 30.590 | 34.91 | none |
| 25 | C37:2A | 34.569 | 36.57 | none |
| 26 | C37:2B | 34.783 | 36.64 | none |
| 27 | C37:2C | 35.017 | 36.73 | none |

Table S6. Full list of compounds from GC/MS analysis of *D. paulistorum* males. Retention times determined *via* gas chromatography coupled to mass-spectrometry are given in minutes for each of the 27 compounds. Retention indices were calculated based on an external alkane standard. Asterisks mark two additional compounds that were not included in the previous studies by (Kim et al. 2004; Chao et al. 2010).