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Virus evolution in *Wolbachia*-infected *Drosophila*

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Abstract

Wolbachia, a common vertically transmitted symbiont, can protect insects against viral infection and prevent mosquitoes from transmitting viral pathogens. For this reason, *Wolbachia*-infected mosquitoes are being released to prevent the transmission of dengue and other arboviruses. An important question for the long-term success of these programs is whether viruses can evolve to escape the antiviral effects of *Wolbachia.* We have found that *Wolbachia* altered the outcome of competition between strains of the DCV virus in *Drosophila.* However, *Wolbachia* still effectively blocked the virus genotypes that were favoured in the presence of the symbiont. We conclude that

25 *Wolbachia* did cause an evolutionary response in viruses but this has little or no impact on the
26 effectiveness of virus-blocking.

27

28 **Introduction**

29 *Wolbachia* is a maternally-transmitted intracellular bacterium found in many insects (1). Its ability to
30 rapidly spread through insect populations by inducing a sperm-egg incompatibility called cytoplasmic
31 incompatibility (2–4) coupled with its inhibitory effect on the replication of RNA viruses (5–8) make it
32 a promising control agent to prevent the transmission of mosquito-borne diseases (9). In several
33 parts of the world the bacterial symbiont is being introduced into natural populations of the
34 mosquito *Aedes aegypti*, the main vector of dengue and Zika viruses (10–13). Preliminary field
35 releases of *Wolbachia*-infected *Ae. aegypti* females have demonstrated that the bacterial infection is
36 able to spread and be stably maintained (4,10,11), turning susceptible populations of mosquitoes
37 into virus-resistant ones (14).

38 Like other control methods, there is a risk that the release of *Wolbachia*-infected mosquitoes may
39 not be evolution-proof. It may promote adaptive changes in the mosquito vector, *Wolbachia* or
40 virus that could hamper the long-term success of field interventions. Therefore, there is an urgent
41 need to understand and predict what genetic changes might follow the introduction of *Wolbachia*,
42 especially because such introductions are likely to be irreversible (15,16). For instance, high antiviral
43 resistance is associated with high densities of the symbiont within the insect tissues (17,18), and this
44 leads to reductions in the fecundity, lifespan and other fitness-related traits of the insect host
45 (11,19–22). These costs may lead to the evolution of lower *Wolbachia* densities and thus a reduction
46 or loss of the antiviral phenotype. A second concern is the evolution of the virus itself. Since
47 *Wolbachia* blocks the transmission of the virus by inhibiting its replication, virus populations should
48 be selected to overcome such inhibition. Potentially, virus strains that are able to replicate at a

49 higher rate in the presence of *Wolbachia* could be advantaged and spread. For example, the
50 intensity of *Wolbachia'*s effect on dengue transmission varies between virus serotypes (14), and the
51 magnitude of these differences is sufficiently large that it is predicted to alter the outcome of control
52 programs (23). Therefore, if viruses can escape the resistance conferred by *Wolbachia,* this would
53 threaten the sustainability of symbiont-based interventions.

54 Here we passaged Drosophila C virus (DCV) through *Wolbachia*-infected *Drosophila melanogaster*
55 and examined how the symbiont affected the evolution of the virus. DCV is a positive-strand RNA
56 virus of the family Discistroviridae that naturally infects *D. melanogaster* (24). It is highly pathogenic
57 in laboratory experiments, leading to fly death within a few days(25). *Wolbachia* leads to large
58 reductions in DCV titres and increases survival after DCV infection, which has led to DCV becoming a
59 common model to study *Wolbachia*-mediated antiviral protection (5,17,18). We found that
60 populations of the virus became genetically differentiated from controls in *Wolbachia*-free flies, with
61 the same viral genotype being favoured across replicate populations. However, despite these
62 parallel genetic changes providing evidence of adaptive evolution in the viral populations, we could
63 not detect any reduction in *Wolbachia'*s antiviral effect or any increase in DCV virulence.

64

65 **Methods**

66

67 **Fly husbandry and virus isolates**

68 *D. melanogaster* lines previously described in (21) were kindly provided by Luis Teixeira. The DrosDel
69 w^{1118} isogenic background was used as the *Wolbachia*-free control. The *Wolbachia*-infected line was
70 created in (21) by introgressing the DrosDel w^{1118} nuclear background into a cytoplasm infected with
71 the *Wolbachia* strain wMelCS_b through chromosome replacement using balancers for the first,
72 second and third chromosomes (the fourth chromosome was not replaced). Flies were maintained

73 on a cornmeal diet (1200ml water,13g agar,105g dextrose,105g maize,23g yeast,35ml Nipagin) at
74 25°C under a 12h light-dark cycle and 70% humidity.

75 DCV isolates were previously described in (26) and kindly provided by Karyn N. Johnson. Isolates
76 DCV-C and -G originate from France, DCV-EB and -CYG from Australia and DCV-M, -O, -T and -Z from
77 Morocco.

78

79 **Virus production**

80 All DCV isolates were passaged once in Schneider Drosophila Line cells (DL2) before the series of
81 experiments. Cells were cultured at 26.5°C in Schneider's Drosophila medium with 10% foetal bovine
82 serum, 100 U/ml penicillin and 100 mg/ml streptomycin (all Invitrogen, UK). Cells were then freeze-
83 thawed twice to lyse cells and centrifuged at 4,000 g for 10 min at 4°C to remove cellular debris.
84 Finally, the supernatant containing DCV was aliquoted and frozen at -80°C.

85 For infection assays, aliquots of virus solutions were defrosted on the day of infection. Virus aliquots
86 were diluted in Ringer's solution (27) to standardise the concentration of DCV RNA measured by
87 quantitative PCR (qPCR, see section on DCV titer below for primers and amplification cycles). The
88 concentration of DCV RNA was used instead of the TCID50 method (28) as the cytopathic effects of
89 the eight DCV isolates differed considerably. Total RNA from the eight virus solutions was extracted
90 and a standard *Wolbachia* spike-in added during the RNA extraction. The DCV critical threshold
91 values were then normalized relative to the *Wolbachia* gene *atpD* using primers atpDQALL_F (5'-
92 CCTTATCTTAAAGGAGGAAA-3') and atpDQALL_R (5'-AATCCTTTATGAGCTTTTGC-3').

93

94

95

96 **Sequencing of DCV genome and phylogenetic analysis**

97 Viral RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed with the Maxima
98 H Minus Reverse Transcriptase (ThermoFisher Scientific) and oligo dT primers. The DCV genome was
99 then amplified by PCR using a set of 22 primers distributed along the 9,264 bp genome (Table S1).
100 For each genome, the eleven PCR products were Sanger-sequenced and the reads assembled into a
101 consensus genomic sequence using the Sequencher v4.5 software (GenBank accession numbers:
102 MK645238-MK645245). DCV genomes were aligned with ClustalW in BioEdit v7.0.9 (29). A maximum
103 likelihood phylogenetic tree was built using the aligned genomes and node supports were assessed
104 with 1,000 bootstrap replications.

105

106 **Selection experiment 1 on genetically diverse virus populations**

107 To investigate virus adaptation from standing genetic variation, we created a virus population
108 composed of equal proportions of each of the eight DCV isolates based on the relative amount of
109 DCV RNA in the original virus solutions. The DCV mixture was then passaged in flies with or without
110 *Wolbachia* by infecting 3-6 day old female flies (Passage 1). For this, flies were anaesthetized on CO_2
111 and stabbed on one side of the thorax with a steel needle (Austerlitz Insect Pins) dipped into the
112 virus solution as explained in (30). Ten flies were then transferred to a vial containing cornmeal food.
113 Twelve vials per *Wolbachia* treatment were prepared, representing twelve biological replicates.
114 Virus populations were harvested three days post-infection by homogenizing the ten virus-infected
115 flies from each vial in 25 µl of Ringer solution. The homogenate was then centrifuged at 12,000g and
116 10 µl of the supernatant was frozen at -80°C and later used as the inoculum for further passage. The
117 remaining 20 µl containing the fly tissues were diluted in 250 µl of TRIzol reagent and frozen at -80°C
118 for later RNA extraction. The virus populations were serially passaged two more times (Passages 2
119 and 3) by repeating the steps above and infecting new flies from the respective *Wolbachia*

120 treatment. Note the amount of virus in the inoculum was only controlled at the start of selection
121 (Passage 1). In order to avoid cross-contamination between virus populations, different tools
122 (needles, handling brushes) were used for each replicate population at each passage.
123

124 **Pool-Sequencing, read processing, mapping and variant identification**

125 Total RNA was extracted from flies in Passage 3 of the selection experiment 1. Since the DCV
126 genome contains a poly(A) tail, we isolated the virus genomic RNA along with fly transcripts by
127 capturing the polyadenylated RNAs from the extracted total RNA using the KAPA Stranded mRNA-
128 Seq kit (Kapa Biosystems) and a different indexed adapter for each RNA library. Twenty-four libraries
129 were prepared (one per virus population), quantified by qPCR and pooled in equal proportions into a
130 multiplexed library. The pool was sequenced in one lane of Illumina HiSeq4000 to generate single-
131 end 50bp reads (SRA study accession number PRJEB21984).

132 We used Trimmomatic v0.32 (31) to trim reads. We first removed three bases from 3’ end of the
133 read. Reads were quality trimmed from the 3’ end, cutting when average quality scores in sliding
134 windows of 4 bases dropped below 15. We required reads have a minimum length of 36. Using *BWA*
135 *MEM* (32), we mapped reads to the genome of a genetically homogenous DCV population (DCV-ref)
136 previously produced from the isolate DCV-C by endpoint dilution (33). We removed optical
137 duplicate reads using Picard tools (https://broadinstitute.github.io/picard/). We realigned reads
138 close to indels using GATK (34). Following that, we used Samtools (35) to remove reads with
139 mapping quality scores lower than 40. We also used Samtools to generate a multi-pileup file to assist
140 with variant identification. Finally, we used PoPoolation2 to identify the allele frequency of the
141 variants in the pooled genomic library samples (36). To make the statistical power and influence of
142 different variants and libraries similar, we down-sampled sites to a maximum coverage of
143 50x/library.

144

145 **Recombination analysis**

146 In order to investigate the extent of recombination of the DCV genome in selection experiment 1, we
147 resequenced the 24 RNA libraries using MiSeq to generate paired-end 250 bp reads (SRA study
148 accession number PRJEB21984). The software bcl2fastq on BaseSpace (Illumina Inc, San Diego) was
149 used to demultiplex the base call files to Fastq files, trim adaptor sequences using a sliding window
150 with an adapter stringency of 0.9. We then used Trimmomatic (31) to cut reads at the first base
151 where the quality score (Q) dropped below 30, retaining only reads with a minimum length of 35 bp.
152 These were mapped to the genome of isolate DCV-C (Genbank accession number MK645242) using
153 *BWA MEM* (32). We used Picard tools (https://broadinstitute.github.io/picard/) to reorder reads,
154 add read groups and sort them by coordinates. Mapped reads were converted into a table of
155 variants where each row is a separate sequence read using sam2tsv in JVarkit (37). The reads from
156 all libraries were combined. We kept properly paired reads, as identified by their SAM flags, with at
157 least one SNP of interest in each of the forward and reverse reads using custom scripts that are
158 deposited in the Dryad Data Repository
159 (https://datadryad.org/review?doi=doi:10.5061/dryad.18j31ch). When a site had the DCV-C allele,
160 we then counted the proportion of read-pairs where the other SNP either had the DCV-C allele (non-
161 recombinant) or the alternate allele (recombinant). As polymorphisms segregating in the founding
162 viral populations can give a spurious signal of recombination in this analysis (38), we removed any
163 pairs of SNPs where the ratio of reads carrying the two possible products of recombination deviated
164 from the expected 50:50 ratio (binomial test, $p < 0.01$). We tested whether the fraction of
165 recombinant reads between pairs of SNPs increased with distance between the SNPs using logistic
166 regression, accounting for over dispersion using a quasibinomial model. Retaining only pairs of SNPs
167 that were represented by at least 25 read pairs, we regressed the proportion of recombinant reads

168 against the distance between the SNPs to estimate the fraction of recombinants between adjacent
169 nucleotides.
170

171 **DCV titer**

172 Total RNA was extracted three days post-infection from pools of ten DCV-infected flies. Five
173 biological replicates (10 flies each) were performed per *Wolbachia* treatment, DCV isolate (single
174 infection experiment) or DCV population (selection experiments). The extracted RNA was reverse-
175 transcribed with Promega GoScript reverse transcriptase (Promega) and random hexamers. The
176 amount of virus RNA was quantified with qPCR by amplifying a 135 bp region of the DCV genome
177 with primers DCV_S (5'-GACACTGCCTTTGATTAG-3') and DCV_AS (5'-CCCTCTGGGAACTAAATG-3')
178 targeting regions that are conserved among the DCV isolates used in this study. Additionally, we
179 quantified the fly gene *actin 5C* in a separate reaction (forward: 5'-
180 GACGAAGAAGTTGCTGCTCTGGTTG-3' ; reverse: 5'-TGAGGATACCACGCTTGCTCTGC-3' ; 193 bp
181 product). The qPCR cycle was 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°Cfor 30 s.
182 Two reactions per sample and per target gene were performed and the mean *Ct* value of the two
183 technical replicates was used to calculate the relative amount of DCV RNA per fly as $2^{\Delta Ct}$, with $\Delta Ct =$
184 $Ct_{fly\ gene} - Ct_{DCV}$.
185

186 **Virus-induced mortality**

187 Flies were infected with each DCV isolate or with Ringer's solution (mock-infected controls) as above
188 except that biological replicates consisted of vials with 20 females. Following infection, flies were
189 transferred onto fresh food every three days and survival was recorded daily for 15 days post-
190 infection.

191

Selection experiment 2 on genetically homogenous virus populations

193 In order to study virus evolution from *de novo* mutation, we used a similar protocol as in the
194 selection experiment on genetically diverse virus populations. We passaged the homogenous DCV
195 population DCV-ref (33) for ten passages in either *Wolbachia*-free or *Wolbachia*-infected flies (25
196 replicate populations in each treatment). The only difference was that male flies were used instead
197 of females and viruses were harvested two days post-infection instead of three. Flies were initially
198 infected in passage 1 with a virus concentration of 6.32×10^8 TCID50/mL.

199

Statistical analysis

201 All statistical analysis were done in the *R* software v3.2.3 (39) and R scripts deposited in the Dryad
202 Data Repository (https://datadryad.org/review?doi=doi:10.5061/dryad.18j31ch). Pairwise genetic
203 distances between virus populations of the selection experiment 1 were calculated as the probability
204 of drawing different alleles from two different populations $P_{diff} = 1 - (p_{pop1} \times p_{pop2} + q_{pop1} \times q_{pop2})$
205 where p_{pop1} and p_{pop2} are the mean frequencies of reference alleles in population 1 and 2 and q_{pop1}
206 and q_{pop2} the mean frequencies of the alternative allele. We conducted a Mantel test with 1,000
207 permutations by randomly attributing *Wolbachia* treatments to populations. The *p*-value was
208 obtained by comparing the observed mean genetic distance between treatments with the null
209 distribution of mean genetic distances obtained by permutation.

210 The principal component analysis was performed with the function *dudi.pca* (*R* package *ade4*). For
211 each *Wolbachia* treatment, 95% confidence ellipses were computed with the assumption of
212 multivariate normal distribution of the data using the function *stat_ellipse* (*R* package *ggplot2*). The
213 discriminant analysis of principal components was performed with the function *dapc* (*R* package
214 *adegenet* (40)).

215 Viral titers were analyzed with a linear model after $\log_{10}$ transformation of the data to meet the
216 assumptions of normality and homoscedasticity. For the experiment measuring viral titers of evolved
217 populations, the virus population was treated as a random effect in a linear mixed effect model
218 (package *LmerTest*). For the time-course analysis of DCV titer, 3 parameter asymptotic exponential
219 growth curves were fitted to the $\log_2$ viral titer using the function *nls* (*R* package *stats*). To test
220 whether there was an effect of *Wolbachia* on the growth curve, we used a likelihood ratio test to
221 compare the fit of a single curve to all the data with the fit of separate curves to the data from
222 *Wolbachia*-infected and *Wolbachia*-free flies. To test whether there were either differences in the
223 growth of the three viruses or there were virus-specific effects of *Wolbachia,* we compared our two-
224 curve model (*Wolbachia* + and -) to a six-curve model (a separate curve for each of the three viruses,
225 with and without *Wolbachia*). When plotting the curves, 95% confidence intervals were estimated
226 by Monte Carlo simulation.

227 Fly survival was analyzed with a Cox's proportional hazard mixed-effect model (*R* package *coxme*).
228 Flies that were alive at the end of the experiment were treated as censored data. Multiple pairwise
229 comparisons were performed with the function *glht* (*R* package *multcomp*, (41)).

230

231 **Results**

232

233 **Virus populations evolve in response to *Wolbachia***

234 In order to test whether DCV adapts to the presence of *Wolbachia*, we passaged a genetically
235 diverse population of viruses through *Wolbachia*-infected or *Wolbachia*-free flies (selection
236 experiment 1). The viral population was founded by mixing eight DCV isolates collected from both
237 laboratory stocks and wild *D. melanogaster* from around the world (26). The mixture initially
238 contained equal concentrations of viral RNA from each DCV isolate and after three passages, we

239 sequenced the polyadenylated RNA from twelve independent replicates per *Wolbachia* treatment.
240 The mean depth of coverage of each replicate ranged from 65 to 89x (Table S2). After filtering out
241 variants with a mean minor allele frequency below 5% there were 167 Single Nucleotide
242 Polymorphisms (SNPs). Among these, 161 were found among the genomes of the eight DCV isolates
243 used to found the viral population. There were 703 SNPs among the founding DCV genomes,
244 suggesting that ~77% had minor allele frequencies below 5% by the end of the selection experiment.

245 Parallel evolution, where the same genetic changes evolve independently in response to the same
246 selection pressure, provides evidence of adaptation. To test for parallel evolution in our experiment
247 we calculated the genetic distance between all possible pairs of populations. We found that the
248 mean genetic distance between DCV populations from the same *Wolbachia* treatment (*Wolbachia*-
249 infected or *Wolbachia*-free flies) was less than between populations from different *Wolbachia*
250 treatments (Mantel test, $P = 0.028$, Figure S1A). Parallel evolution of the DCV populations was also
251 apparent in a Principal Component Analysis (PCA) on DCV allele frequencies, where the second
252 principle component separated DCV populations depending on whether or not they had evolved in
253 flies infected with *Wolbachia* (Figure 1A).

254

255 ***Wolbachia* alters the frequency of viral genotypes in the population**

256 The evolutionary response to *Wolbachia* could either involve changes in the frequency of the eight
257 founding viral genotypes or selective sweeps of specific SNPs through a recombining population of
258 viruses. We calculated the difference in the frequency of SNPs between the populations that had
259 evolved in flies with and without *Wolbachia* (Figure 1B). Across the viral genome, alleles from the
260 DCV-C isolate consistently showed higher frequencies in the presence of *Wolbachia* (Figure 1B),
261 while the DCV-EB, -CYG and -G alleles had lower frequencies. All of the variants that were at a
262 substantially higher frequency in the *Wolbachia*-infected flies were present in DCV-C, and these

263 were scattered across the genome (Figure 1B). Alleles specific to the DCV-C isolate have increased in
264 frequency in both the *Wolbachia*-infected and *Wolbachia*-free flies, reaching mean frequencies of
265 77% and 67% respectively (based on DCV-C specific alleles, Figure S1). Therefore, DCV-C was
266 successful in all the populations, but its competitive advantage has been increased with *Wolbachia.*

267 These analyses suggest that there may have been limited recombination in the population, and the
268 frequency of the founding viruses has changed in response to selection by *Wolbachia.* To confirm
269 this we generated longer sequence reads from the same samples and used read-pairs containing
270 multiple SNPs to examine the rate at which DCV-C had recombined with other viral isolates. We
271 found that there were more putative recombination events between SNPs that were further apart in
272 the genome, as expected if there is recombination (logistic regression: $t = 4.45$, $p = 0.0001$). By
273 estimating a per bp recombination rate and extrapolating this to the whole genome, we estimate
274 that 86% of DCV-C genomes will have survived intact without recombination by the end of the
275 experiment. This supports the conclusion that we are primarily looking at changes in the frequency
276 of the founding viruses.

277 As there is limited recombination between DCV isolates, we can use SNPs as markers to track
278 changes in the frequency of different viral isolates. We first reconstructed the phylogeny of the DCV
279 isolates using their Sanger-sequenced genomes (Figure 1C), finding similar relationships to published
280 analyses of restriction fragment length polymorphisms (26). In the evolved populations, we
281 identified isolate-specific alleles for four isolates (Figure 1D; DCV-C, -E, -M and -T), while DCV-G and
282 DCV-O are polymorphic since their genomes contain high numbers of ambiguous bases (Table S3).
283 Larger numbers of SNPs were found that defined clades of viruses on the phylogeny (Figure 1C and
284 D). Comparing the frequency of these SNPs among our evolved populations confirmed that DCV-C
285 was favoured in *Wolbachia*-infected flies, while alleles specific to the clade containing DCV-EB, -CYG
286 and -G decreased in frequency by around 10% in the presence of *Wolbachia*. There was little

287 difference between *Wolbachia* treatments in the frequency of isolates in the other clade on the
288 phylogeny (DCV-M, -Z, -T and -O; Figure 1C and D).

289 To further examine the genetic basis of differentiation between the DCV populations in *Wolbachia*-
290 infected and *Wolbachia*-free flies, we used discriminant analysis on the principal components
291 (DAPC). The virus populations showed a bimodal distribution which separates the *Wolbachia*
292 treatments (Figure S2A). The genetic differentiation is driven by SNPs across the viral genome, which
293 is consistent with there being limited recombination (Figure S2B). Alleles specific to the isolate DCV-
294 C and the EB-CYG-G clade consistently contributed the most to the genetic differentiation between
295 *Wolbachia* treatments (Figure S2C). This confirms that DCV-C was favoured in the presence of
296 *Wolbachia* and that this was at the expense of viruses in the EB-CYG-G clade.

297

298 **The DCV isolate favoured in *Wolbachia*-infected flies does not evade the symbiont's antiviral**
299 **effects**

300 *Wolbachia* may be selecting for viruses that evade its antiviral effects. In order to investigate this we
301 inoculated *Wolbachia*-free and *Wolbachia*-infected flies with the DCV isolate that increased in
302 frequency in the presence of *Wolbachia* (DCV-C), one isolate that decreased in frequency (DCV-EB)
303 and one isolate which frequency was little affected by *Wolbachia* (DCV–M). Viral titers were
304 measured over 6 days to allow an asymptotic exponential growth curve to be fitted to the data. We
305 found that *Wolbachia* altered the growth curve of DCV (Figure 2A; χ^2=36.8, df=4, p<0.0001), reducing
306 both the viral growth rate and the final viral titer. However, there was no difference in the growth
307 curves of the three viral isolates, regardless of whether the flies were infected with *Wolbachia*
308 (Figure 2A; χ^2=20.6, df=16, p=0.20). Therefore, we found no evidence that *Wolbachia* has favoured
309 viral isolates that overcome the symbiont's antiviral properties.

310 We also measured the ability of the DCV isolates to kill *Wolbachia*-free and *Wolbachia*-infected flies.

311 Levels of mortality varied among virus isolates, with flies infected with DCV-CYG showing the lowest

312 mortality, regardless of whether the flies carried *Wolbachia* (Figure 2B ; Main effect virus: χ_i^2 =

313 125.29, d.f. = 10, $P < 0.0001$). *Wolbachia* had no effect on the survival of mock-infected flies (control

314 in Figure 2B), but in all cases increased survival of DCV-infected flies (Figure 2B and C ; Main effect

315 *Wolbachia*: χ_i^2 = 132.7, d.f. = 6, $P < 0.0001$). The magnitude of the protective effects of *Wolbachia*

316 depended on the DCV isolate (Figure 2B and C ; *Wolbachia*-by-Virus interaction: χ_i^2 = 16.7, d.f. = 2, P

317 = 0.005). However, there was no association between the extent to which *Wolbachia* protected flies

318 against the virus (Figure 2B) and whether that virus increased in frequency in the presence of

319 *Wolbachia* (Figure 1).

320

321 **Virus populations evolved with *Wolbachia* did not adapt to counteract its antiviral effect**

322 To test whether virus had adapted to overcome the antiviral effects of *Wolbachia*, we randomly

323 chose five virus populations from each of the selection treatment and infected flies with equal

324 concentrations of viral RNA (see methods). The viral titer three days post-infection was lower in

325 *Wolbachia*-infected flies, regardless of the selection regime (*Wolbachia* effect: χ_i^2 = 158.68; P <

326 0.0001; Figure S3 for pairwise comparisons). Surprisingly, the effect of *Wolbachia* on viral titers was

327 slightly greater for the viral populations that had been passaged through *Wolbachia*-infected flies

328 (*Wolbachia*-by-selection effect: χ_i^2 = 4.34; P = 0.04; Figure S3). Therefore, viruses evolved with the

329 symbiont were still susceptible to the inhibitory effect of *Wolbachia*.

330

331

332

333 **A genetically homogenous virus population did not evolve to escape the antiviral effects of**
334 ***Wolbachia***

335 The high mutation rates, replication rates and population sizes of many viruses mean that they can
336 frequently evolve to overcome host resistance during selection experiments through *de novo*
337 mutations. To test whether this was the case for *Wolbachia* and DCV, we serially passaged a
338 genetically homogenous population of the DCV-C isolate through *Wolbachia*-free and *Wolbachia*-
339 infected flies (selection experiment 2). While DCV was maintained at high titers in *Wolbachia*-free
340 flies, titers tended to progressively decrease in the presence of the symbiont and frequently became
341 undetectable (Figure 3A). After ten passages, only two of 25 replicates in *Wolbachia*-infected flies
342 had DCV titers close to those observed in the absence of the symbiont (Figure 3A). We tested
343 whether these two virus populations had maintained high titres by evolving to counter the antiviral
344 effects of *Wolbachia* by infecting new flies with equal concentration of viral RNA. There was no
345 significant difference in the effects of *Wolbachia* on these viruses compared to control populations
346 passaged through *Wolbachia*-free flies (Figure 3B ; *Wolbachia* effect: $F_{1,44}$ = 113.95 ; P < 0.0001 ;
347 Selection effect: $F_{1,44}$ = 0.72 ; P = 0.4 ; *Wolbachia*-by-selection interaction: $F_{1,44}$ = 3.25 ; P = 0.08).

348

349 **Discussion**

350 *Wolbachia* is able to block the replication of RNA viruses and this is being harnessed by public health
351 programmes to control mosquito-borne diseases (42). The impact of such interventions on evolution
352 of the viruses is unknown. If viruses could evolve to escape *Wolbachia*'s antiviral effects, or
353 *Wolbachia* selects for increased viral virulence in the mosquito or human host, this would have
354 important implications for control programs. For example, interventions that reduce the growth rate
355 of a pathogen without clearing the infection can select for compensatory increases in replication
356 rates that in turn increase virulence (43). We found that *Wolbachia* can alter the evolution of a

357 virus—when DCV evolved in the presence of *Wolbachia* there were replicable genetic changes in
358 viral population. This was mediated by *Wolbachia* modifying competition between virus strains
359 within the insect. However, these changes neither allow the virus to escape *Wolbachia's* blocking
360 effect nor alter the virus's virulence.

361 In our first experiment, we passaged a genetically diverse viral population through *Wolbachia*-
362 infected flies in an attempt to select for viruses that escaped the symbiont's antiviral effects. At the
363 genetic level there was a clear response to selection, with the DCV-C genotype reaching higher
364 frequencies in the presence of *Wolbachia.* However, we were unable to find any evidence that DCV-
365 C either had higher virulence or escaped *Wolbachia's* antiviral effects. It is possible DCV-C was
366 favoured because of *Wolbachia* altering the strength of competition between viruses. While the
367 mechanisms of the *Wolbachia*-mediated antiviral effect remain elusive, previous studies have shown
368 that competition between the symbiont and viruses for resources such as cholesterol might be
369 involved (44,45). By reducing the availability of these resources, *Wolbachia* might exacerbate fitness
370 differences that already exist between virus isolates. Consistent with this, DCV-C increased in
371 frequency in *Wolbachia*-free flies, but to a lesser extent than in the presence of *Wolbachia.*

372 In the second selection experiment we tested virus adaptation from de novo mutation. Selection
373 was strong, leading to the loss of most viral populations across serial passages. The few virus
374 populations that managed to persist in the presence of the symbiont still suffered large reductions in
375 titer in *Wolbachia*-infected flies. Therefore, we were unable to select for major-effect *Wolbachia*-
376 escape mutations in the virus, and it is possible these viruses simply persisted due to stochastic
377 processes. This is similar to a recent study where dengue virus populations evolved in *Wolbachia*-
378 infected mosquito cell lines showed rapid decline and frequently went extinct (46). Strikingly, the
379 few dengue-infections that persisted in the presence of *Wolbachia* showed an almost complete
380 inability to replicate in both *Wolbachia*-free and *Wolbachia*-infected cells following selection. We did
381 not observe this with DCV, but our mixed DCV populations evolved with *Wolbachia* showed slightly

382 lower replication in *Wolbachia*-infected flies (Figure S3), i.e. they appeared to be more affected by
383 *Wolbachia* than populations evolved without symbiont. It is possible that the presence of *Wolbachia*
384 leads to the production of more defective viral genomes (47,48). However the size of this effect was
385 modest, and whether this is a general pattern remains to be investigated.

386 The reason why viruses do not readily evolve to escape the antiviral effects of *Wolbachia* is unclear.
387 However, clues come from two patterns that are recurrently observed. First, strong antiviral
388 protection is associated with high symbiont density within cells (17,18,21). Second, *Wolbachia*
389 provides protection against a diverse array of distantly related RNA viruses, including viruses whose
390 natural hosts are *Wolbachia*-infected (17,49,50). This is reminiscent of 'quantitative' plant defenses
391 against insect herbivores (51). These are secondary metabolites that are produced in large
392 quantities, and protect against a broad array of herbivores by reducing the digestibility of the plant.
393 These contrast with 'qualitative' toxin defenses that are produced in low quantities (51). While
394 specialist herbivores frequently evolve to escape qualitative defenses, quantitative defenses are
395 thought to be more 'evolution proof' (51,52). As discussed above, one of the leading hypotheses to
396 explain the antiviral effects of *Wolbachia* is that the symbiont competes with the virus for resources
397 such as cholesterol (44,45). If these resources are essential to viral replication, such a mechanism
398 may be a form of quantitative defense which viruses cannot readily evolve to escape from. This
399 contrasts with 'qualitative' forms of antiviral protection, such as restriction factors, changes to
400 surface receptors or drugs, where virus escape may evolve by altering the molecular target of the
401 antiviral effector.

402 The final verdict on whether *Wolbachia*-based control of vector-borne disease is more 'evolution-
403 proof' than drugs or insecticides awaits its long-term deployment in the field. However, there are
404 grounds for optimism. *Wolbachia* is maintained at high frequency and retains its antiviral properties
405 years after it is released into populations (11,14). RNA viruses, including DCV, frequently show
406 considerable responses to selection in short-term laboratory experiments (33,53). Therefore our

results together with similar work in cell culture demonstrate that viruses do not readily evolve to escape *Wolbachia's* effects (14). Nonetheless, in the wild viral population sizes are larger and selection will act for longer periods of time, so our results may not reflect the outcome of this interaction in nature. However, *Wolbachia* effectively protects against viruses that will have likely experienced many decades of selection because their natural hosts are *Wolbachia*-infected, suggesting that our experiments may reflect the outcome of evolution in nature (17,49,50) (although it is unclear whether the symbiont is an important selection pressure in natural populations of *D. melanogaster* (54,55)). Long-term monitoring of field populations will be essential to test whether this reflects fundamental biological constraints that prevent viruses evolving to escape from the effects of *Wolbachia*.

Authors' contributions

JM, GB, SCLS, JD and BL carried out the experimental work. JM, RA, GB and FMJ analysed the data. JM and FMJ designed the study and drafted the manuscript. All authors gave final approval for publication.

Competing interests

The authors have no competing interests.

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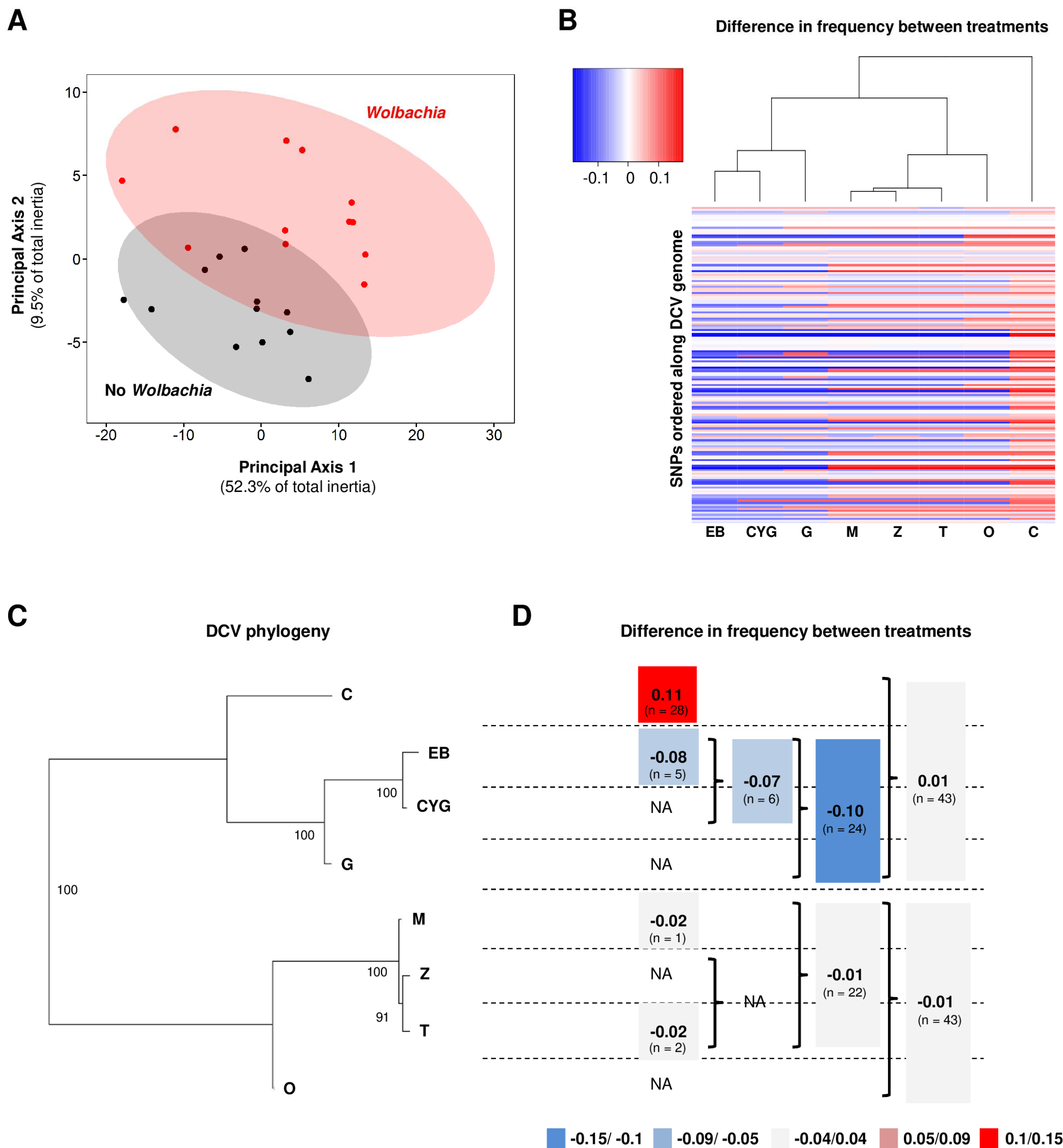
586 **Figures and Tables**



587

588 **Figure 1. Effect of *Wolbachia* on virus allele frequencies when selecting on a genetically diverse**
589 **viral population.** A genetically diverse population of DCV was passaged through *Wolbachia*-infected
590 or *Wolbachia*-free flies and then sequenced. (A) Principal components analysis on allele frequencies
591 of SNPs, where each point is an independent virus population (biological replicate). (B) Differences in
592 the frequency of SNPs along the viral genome. The sequence reads were mapped to the DCV-C
593 reference genome, and the heatmap shows the difference in the frequency of the allele carried by a

594 given DCV strain between *Wolbachia* treatments (frequency in *Wolbachia*-infected flies minus

595 frequency in *Wolbachia*-free flies; red is a higher allele frequency in *Wolbachia*-infected flies). The

596 tree was computed from the Euclidian distance computed from these differences in allele

597 frequencies. (C) DCV phylogeny with bootstrap support for the nodes. (D) Differences in the

598 frequency of isolate- or clade-specific variants between viral populations that had evolved in

599 *Wolbachia*-infected and *Wolbachia*-free flies (positive numbers are more common in the presence of

600 *Wolbachia*). The number of SNPs is shown in parentheses, and the number in bold is the mean

601 difference in frequency across all those SNPs. NA stands for cases where no isolate- or clade-specific

602 SNPs could be found.

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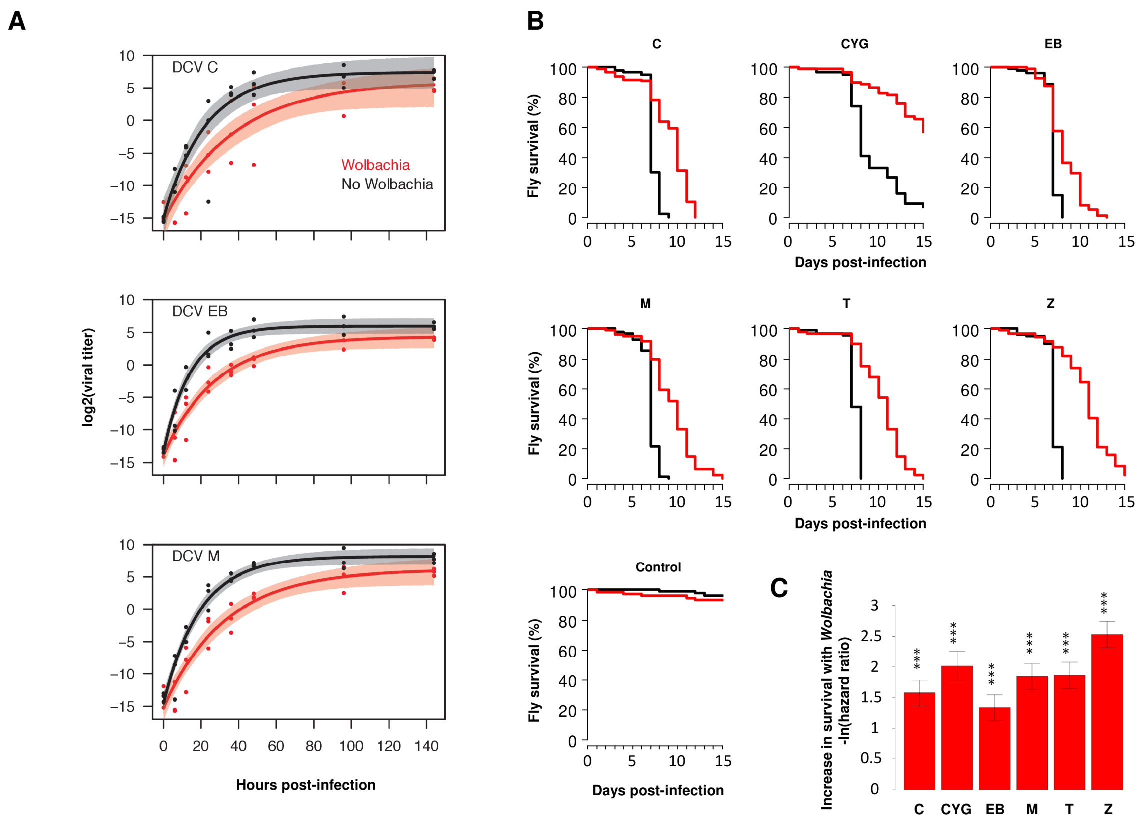

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605 **Figure 2. *Wolbachia*'s effect on viral titers and virus-induced mortality in single virus infections.** (A)

606 Growth curves of three DCV isolates in *Wolbachia*-free and *Wolbachia*-infected flies. The lines are

607 asymptotic exponential curves and the shaded area 95% confidence intervals. (B) Survival curves

608 following infection with DCV. (C) *Wolbachia* effect on virus-induced mortality expressed as –

609 ln(hazard ratio) where the hazard ratio is the probability of flies dying in *Wolbachia*-infected flies

610 relative to their *Wolbachia*-free counterpart. Error bars are standard errors and *** $P < 0.001$.

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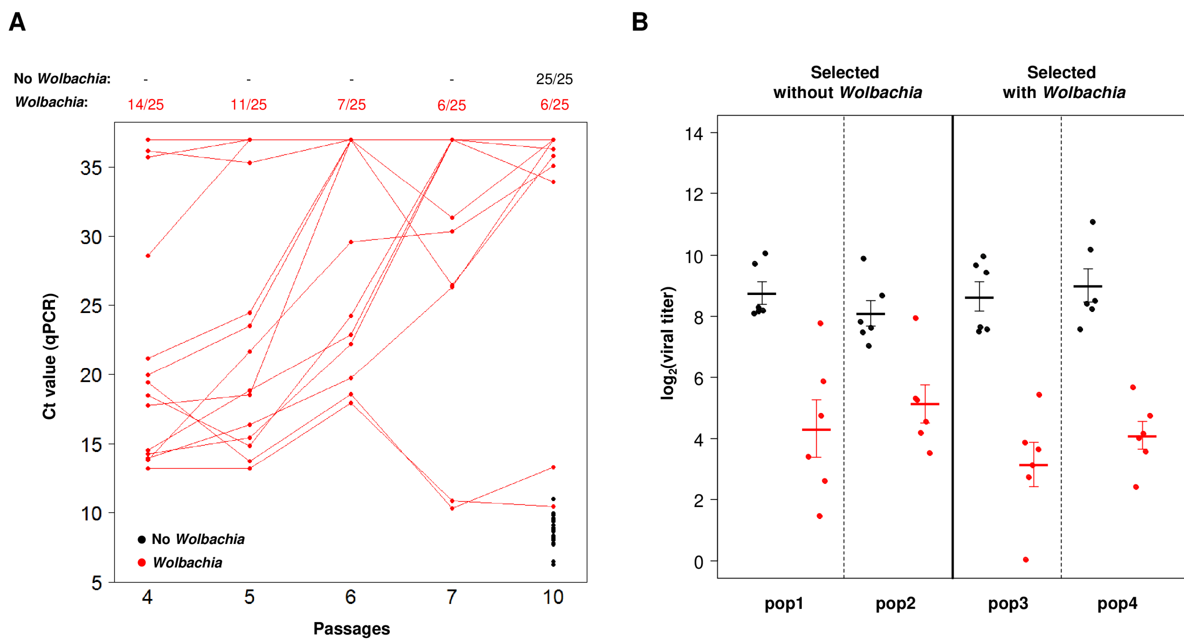

612

613 **Figure 3. Presence of DCV and *Wolbachia* effect on viral titers in selection experiment 2.** (A) Ct

614 values obtained from qPCR reactions targeting DCV RNA in virus populations at different passages

615 during selection. Populations passaged in *Wolbachia*-free flies were only checked for DCV infection

616 at passage 10. Fractions at the top of the plot indicate the number of biological replicates for which

617 DCV was detected relative to the total number of replicates (n = 25 in each selection treatment). (B)

618 Virus titer of the DCV populations three-days post-infection in *Wolbachia*-free (black) and –infected

619 (red) flies. Horizontal bold lines and dots indicate mean titers and values per biological replicate

620 respectively. Error bars are standard errors.

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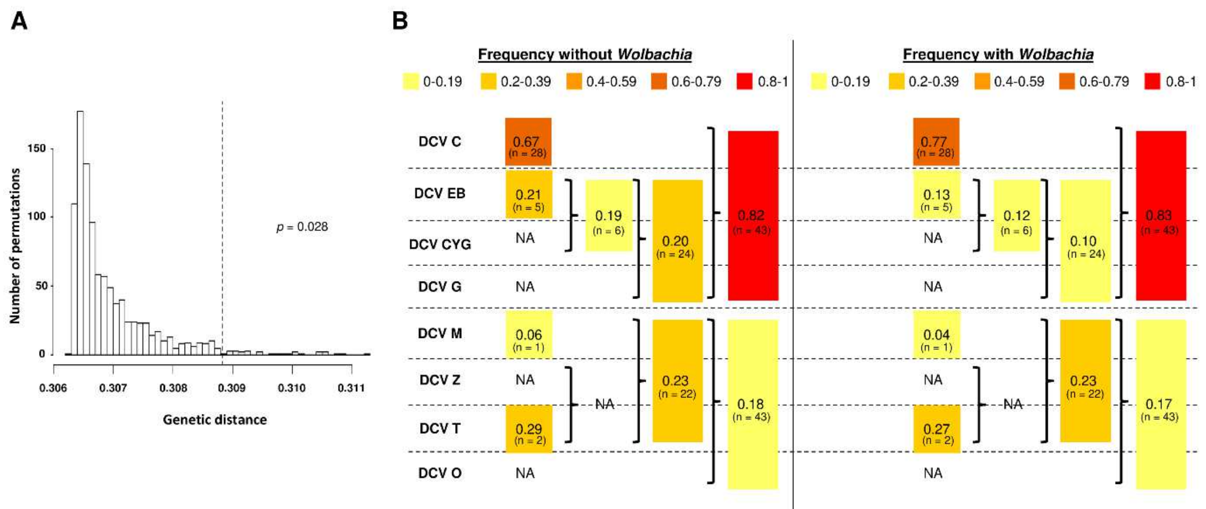

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625 **Figure S1. Genetic differentiation of genetically diverse virus populations evolved in *Wolbachia*-**
626 **free and *Wolbachia*-infected *Drosophila* (selection experiment 1).** (A) The vertical dashed line
627 shows the observed mean genetic distance between DCV populations that had evolved in flies with
628 different *Wolbachia* infection statuses. The bars show the null distribution of this statistic obtained
629 by randomizing the *Wolbachia* treatments across the viral populations 1000 times. (B) Frequency of
630 polymorphisms found in different DCV isolates or clades (see DCV phylogeny in Figure 1C). The
631 number of SNPs is shown in parentheses, and the number in bold is the mean frequency across all
632 the SNPs. NA stands for cases where no isolate-specific SNPs could be found.

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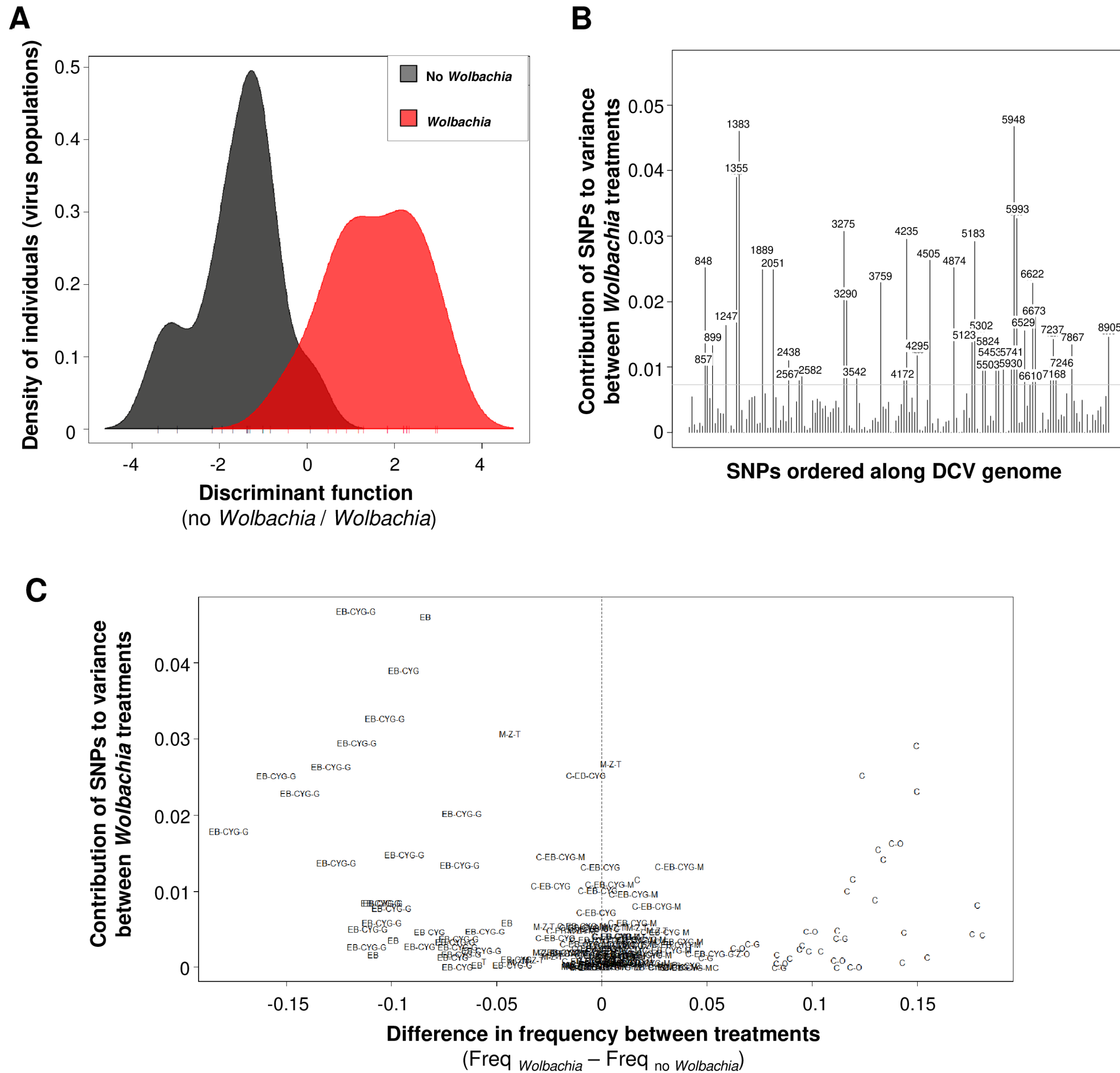

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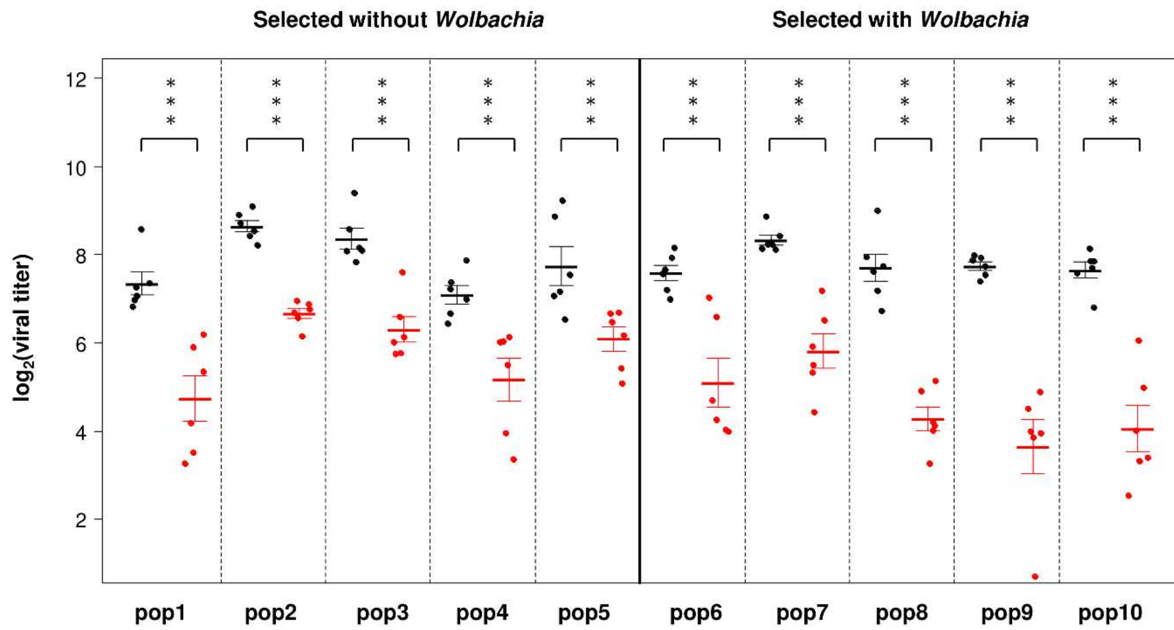
636 **Figure S2. Discriminant Analysis of Principal Components.** (A) Distribution of virus populations
637 along the discriminant function. (B) Contribution of DCV SNPs to the discriminant function. Numbers
638 indicate SNP positions along the genome. The horizontal line is a threshold calculated using the
639 Ward's minimum variance clustering method. The positions of SNPs above this threshold which
640 contribute most to the separation between *Wolbachia* treatments are indicated. (C) Relationship
641 between changes in reference allele frequencies and the contribution of the respective SNPs to the
642 discriminant function. The names of the DCV isolates or clades for a given allele are plotted.



643

644 **Figure S3. *Wolbachia* effect on viral titers in selection experiment 1.** Virus titer of the DCV
645 populations three-days post-infection in *Wolbachia*-free (black) and –infected (red) flies. Horizontal
646 bold lines and dots indicate mean titers and values per biological replicate respectively. Error bars
647 are standard errors. *** indicate $P < 0.001$ in pairwise comparison tests between *Wolbachia*
648 treatments.

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657 **Table S1. PCR and sequencing primers used to Sanger-sequence DCV genomes.**

Primer name	Sequence
DCV26F	5'-TGTACACACGGCTTTTAGGTAG-3'
DCV874F	5'-TCTACCCTTAAGATGGTTGCTGA-3'
DCV1046R	5'-TGACGTGCAGCTTCCTGTTT-3'
DCV1478F	5'-AGTATCGTGCTTCTCTGTGTGT-3'
DCV1774R	5'-CAGTGATCTTTTTAGCTCCCTCA-3'
DCV2192F	5'-ATGTTCTTCGGGAAATGGGGA-3'
DCV2350R	5'-GGTTAGCTGCTGTTTTGTCATC-3'
DCV2875F	5'-GTCGATGATATTGCCAAACGC-3'
DCV3044R	5'-TCGCTCAAACAAATGTCCATCC-3'
DCV3920F	5'-ATTGTGTGCGCTTGCCATTT-3'
DCV4022R	5'-AAATGCCGAACCAAATCACG-3'
DCV4659F	5'-ATGTGGTGTAGACACTGCGG-3'
DCV4857R	5'-TCCTGGTGACGTTGTACGAT-3'
DCV5640F	5'-TGATGCAAAGGTTGTGGAATGG-3'
DCV5732R	5'-CCAGTTTTAGCTTCGTCCGT-3'
DCV6423F	5'-ACTACTCGTGAAGATCGTATCCA-3'
DCV6666R	5'-GCATCAATCGTCCTTGCTGG-3'
DCV7325F	5'-TGGTCAAGTTCGAATGGCGA-3'
DCV7508R	5'-GGCATCGGTTGTGTTCCAAG-3'
DCV8271F	5'-CCGGAAGCGCATTGTATTGG-3'
DCV8444R	5'-AAGGGACATGGGTTCAGCAG-3'
DCV9205R	5'-CGAAAAACCTGGTAGCCCCT-3'

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660 **Table S2. Depth of coverage per RNA library.** There is no significant difference in coverage between

661 *Wolbachia* treatments (*t*-test: t = -0.53 ; P = 0.6)

Selection treatment	Replicate population	Mean depth of coverage
No *Wolbachia*	1	72x
No *Wolbachia*	2	76x
No *Wolbachia*	3	71x
No *Wolbachia*	4	84x
No *Wolbachia*	5	87x
No *Wolbachia*	6	83x
No *Wolbachia*	7	84x
No *Wolbachia*	8	74x
No *Wolbachia*	9	75x
No *Wolbachia*	10	71x
No *Wolbachia*	11	70x
No *Wolbachia*	12	68x
Wolbachia	1	70x
Wolbachia	2	71x
Wolbachia	3	77x
Wolbachia	4	84x
Wolbachia	5	87x
Wolbachia	6	89x
Wolbachia	7	89x
Wolbachia	8	70x
Wolbachia	9	68x
Wolbachia	10	75x
Wolbachia	11	81x
Wolbachia	12	79x

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664 **Table S3. Nucleotide composition of the sequenced genome of DCV isolates used in this study.**

DCV isolates	A	C	G	T	K (T/G)	M (C/A)	R (A/G)	S (G/C)	W (A/T)	Y (C/T)	Total
C	2712	1487	1856	3006	-	-	-	-	-	-	9061
EB	2720	1481	1856	3003	-	-	-	-	-	1	9061
CYG	2721	1484	1855	2998	-	-	-	-	-	3	9061
G	2690	1425	1819	2949	4	4	50	1	8	110	9060
M	2740	1469	1828	3022	-	-	-	-	-	-	9059
Z	2741	1474	1826	3019	-	-	1	-	-	-	9061
T	2740	1468	1829	3024	-	-	-	-	-	-	9061
O	2706	1444	1818	3007	1	3	35	-	6	40	9060

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