Title

Wolbachia-conferred antiviral protection is determined by developmental temperature

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Abstract

Wolbachia is a maternally transmitted bacterium widespread in arthropods and filarial nematodes, and confers strong antiviral protection in *Drosophila melanogaster* and other insects. *Wolbachia*-transinfected *Aedes aegypti* are currently being deployed to fight transmission of dengue and Zika viruses. However, the mechanism of antiviral protection and factors influencing it are still not fully understood. Here we show that temperature modulates *Wolbachia*-conferred protection in *Drosophila melanogaster*. Temperature after infection directly impacts *Drosophila* C virus replication and modulates *Wolbachia* protection. At higher temperatures virus proliferates more and is more lethal, while *Wolbachia* confers lower protection. Strikingly, host developmental temperature is a determinant of *Wolbachia*-conferred antiviral protection. While there is a strong protection when flies are raised from egg to adult at 25°C, the protection is highly reduced or completely abolished when flies develop at 18°C. However, *Wolbachia*-induced changes during development are not sufficient to limit virus-induced mortality, as *Wolbachia* is still required to be present in adults at the time of infection. This developmental effect is general, since it was present in different host genotypes, *Wolbachia* variants and upon infection with different viruses. Overall, we show that *Wolbachia*-conferred antiviral protection is temperature dependent, being present or absent depending on the environmental conditions. This interaction likely impacts *Wolbachia*-host interactions in nature and, as a result, frequencies of host and symbionts in different climates. Dependence of *Wolbachia*-mediated pathogen blocking on developmental temperature could be used to dissect the mechanistic bases of protection and should be considered by programmes deploying *Wolbachia* as an antiviral agent in the field.

Significance Statement

Insects are often infected with beneficial intracellular bacteria. The bacterium *Wolbachia* can protect insects from pathogenic viruses. This effect can be used to prevent transmission of dengue and Zika viruses by *Wolbachia*-infected mosquitoes. To deploy *Wolbachia* in the field successfully and understand the biology of insects in the wild we need to discover which factors affect *Wolbachia*-conferred antiviral protection. Here we show that the temperature in which insects develop from eggs to adults can determine presence or absence of antiviral protection. The environment, therefore, influences this insect-bacterium interaction. Our work may help to provide insights into the mechanism of viral blocking by *Wolbachia* and inform programs using *Wolbachia* in mosquito-borne disease control.

Main Text

Introduction

Wolbachia is a maternally inherited intracellular bacterium infecting a wide range of arthropods and some nematodes. This endosymbiont induces strong phenotypes in many of its hosts, which may contribute to its success in invading and being maintained in host populations. *w*Mel, the *Wolbachia* strain present in *Drosophila melanogaster,* confers a strong protection to a wide range of RNA viruses, which can be a fitness benefit in nature (1, 2). This protection extends to non-native *Wolbachia*-hosts associations, including mosquito vectors of human disease (3). Recently, *Wolbachia* has become one of the most promising approaches to control dengue and Zika viruses in the wild. It has been shown that the releases of *Wolbachia*-infected *Aedes aegypti* can reduce the number of dengue cases in both, endemic and non-endemic areas (4–7). Although molecular mechanisms of *Wolbachia*-conferred antiviral protection are not yet known, identification of factors influencing protection can contribute to understanding the association of *Wolbachia* and hosts in natural population, as well as guide *Wolbachia*-based field interventions.

Wolbachia-conferred antiviral protection is influenced by host and bacterial genetics. Different *Wolbachia* strains in the same host genetic background vary in protection (8–12). In general, differences in *Wolbachia* titres are correlated with differences in protection, with higher titres conferring higher antiviral protection (8–12). However, some *Wolbachia* strains do not provide protection despite high titres (13). Host genetic variation can also contribute to the strength of *Wolbachia*-conferred protection, as seen in *Aedes aegypti* (14).

Environmental factors also affect this symbiont-mediated protection, either through modulation of *Wolbachia* titre or by titre-independent mechanisms. Host diet rich in cholesterol reduces antiviral protection in *Drosophila melanogaster* (15). Similarly, antibiotic treatment reducing *Wolbachia* titre reduces the antiviral protection (16). Finally, temperature was shown to modulate *Wolbachia*-conferred protection to parasites in an engineered *Wolbachia*-host association; in *Anopheles stephensi*, temperature and somatic *Wolbachia* infection determined *Plasmodium* titre in mosquitoes (17). On the other hand, in *Aedes aegypti* stably transinfected with *w*Me*l,* the comparison of a constant and two fluctuating temperature regimes concluded, that the antiviral protection is robust across conditions and unlikely to be compromised in the antiviral field trials (18). However, different temperatures regimes affect dengue transmission independently of *Wolbachia* (19).

110 Despite two studies tackling temperature effect on *Wolbachia*-conferred protection in artificial
111 associations, the impact of temperature on antiviral protection in natural *Wolbachia*-host symbiosis
112 remains unknown. Yet, it is known that temperature can affect the interaction between *Wolbachia*
113 and *D. melanogaster.* For instance, higher temperature leads to higher proliferation and cost of the
114 pathogenic variant *w*MelPop (20–22). Also, flies with different *w*Mel variants prefer different
115 temperatures, probably related with cost of these variants to the host (23). Geographic distribution
116 of *Wolbachia* in *D. melanogaster* populations could be explained by their relative fitness effects at
117 varying thermal conditions (24, 25). Antiviral protection could be a temperature-dependent fitness
118 benefit, which would be balanced against the potential cost of highly protective, high titre symbionts.
119
120 Here we tested how temperature affects *Wolbachia* protection to viruses in natural *Wolbachia*-
121 *Drosophila* associations. First, we asked how different virus doses and post-infection temperatures
122 affect *Wolbachia* densities and antiviral protection. Then, we dissected the effect of developmental
123 temperature on *Wolbachia*-carrying *Drosophila melanogaster*. We show strong interaction between
124 this environmental variable and *Wolbachia*-conferred protection against viruses.
125
126 **Results**
127
128 To test how different infection temperatures affect *Wolbachia*-conferred protection to *Drosophila* C
129 virus (DCV), we used the *Drosophila melanogaster* Drosdel w^{1118} isogenic line (*iso)* carrying a
130 natural *Wolbachia* variant, *w*MelCS_b (*Wolb*+), and a matching *Wolbachia*-free control (*Wolb*-) (1,
131 8). Flies were raised at 25°C and 3-6-day-old flies were challenged with serial dilutions of DCV and
132 subsequently placed at either 18°C or at 25°C (Fig. 1A, B). Virus-induced mortality was higher at
133 25°C than 18°C at all doses except the lowest one, where there is almost no mortality associated
134 with the infection (Temperature effect, $p \leq 0.001$ for all comparisons, except with infection dose of
135 10^5 $TCID_{50}$/ml). Importantly, *Wolbachia* protection against DCV varies with temperature
136 (*Wolbachia**Temperature interaction effect, $p < 0.001$), and it is stronger when temperature of
137 infection is 18°C (Cox hazard ratio (CHR) = -1.84, $p < 0.001$) than 25°C (CHR = -0.75 $p < 0.001$,
138 at 18°C). Overall, the temperature of infection affects the survival of the flies and *Wolbachia*
139 protects more at lower infection temperature (Fig. 1A,B).

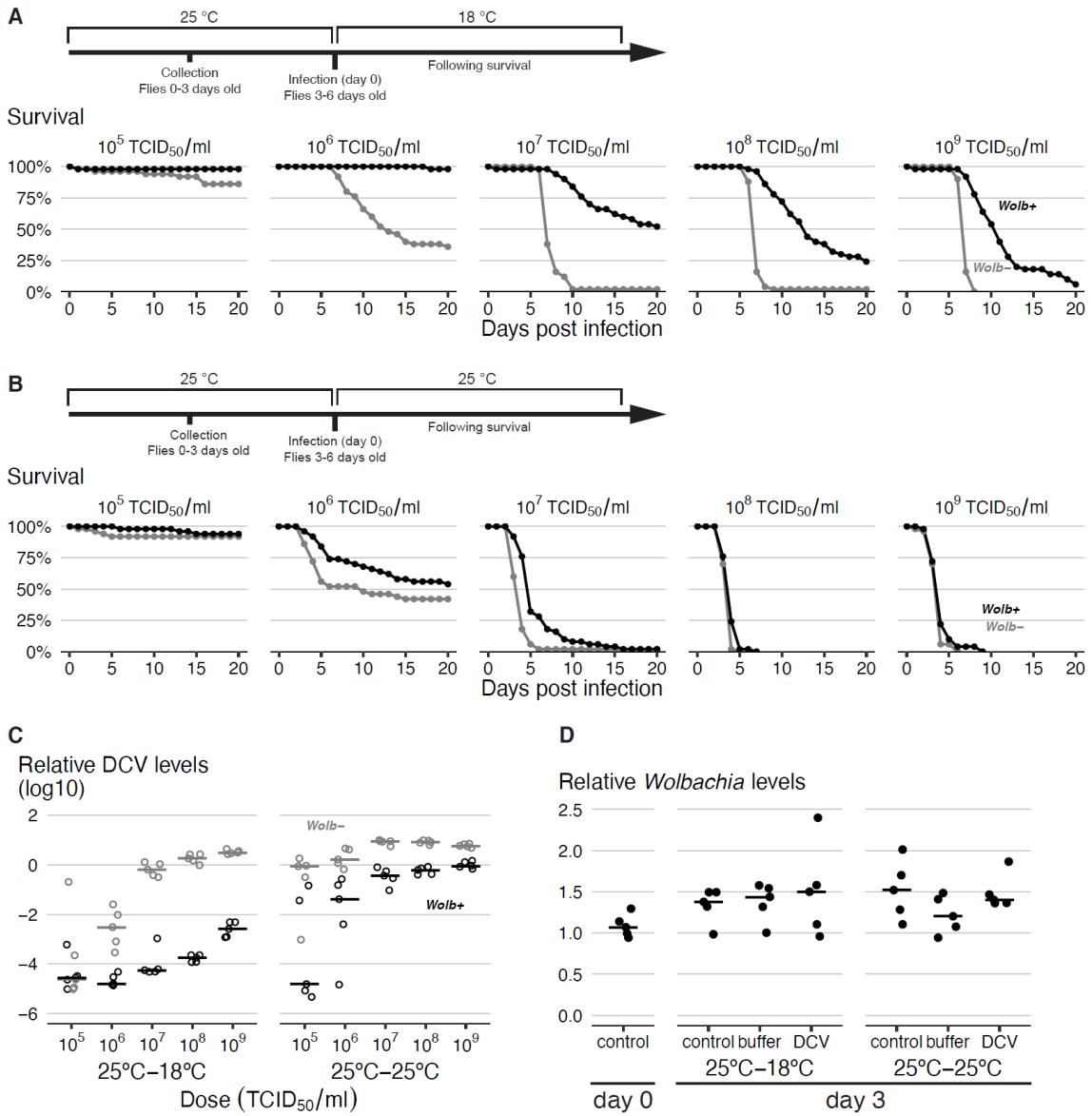


Figure 1. Post-infection temperature modulates the strength of *Wolbachia*-conferred antiviral protection. (A,B) Survival curves of flies infected at different temperatures, with the schemes of the experimental designs shown above. *Wolb+* and *Wolb-* flies, 50 per *Wolbachia* status and condition, were raised at 25ºC, infected with different doses of DCV and placed at either 18°C (A) or 25°C (B) after DCV infection. Mortality was recorded daily. C) DCV titres in DCV-infected flies 3 dpi measured by RT-qPCR. Flies were kept at 25°C before infection and either at 18°C or 25°C after infection. (D) *Wolbachia* levels measured by qPCR at the day of infection (3-6 days old), or 3 days after in unchallenged (control), buffer- or DCV-challenged (10^7 $TCID_{50}$/ml) flies. Flies were kept at 25°C before and placed at either 18°C or 25°C after DCV infection. In (C) and (D) each dot is a sample of ten flies and horizontal lines are medians.

To test if the interaction of *Wolbachia* protection with temperature is also reflected in viral loads, we measured viral titres in flies three days post infection (dpi) by quantitative reverse transcription PCR (RT-qPCR) (Fig. 1C). We detected a strong interaction between *Wolbachia* presence,

154 temperature of infection, and dose of the virus ($p < 0.001$). At the lower temperature *Wolbachia*
155 confers more resistance at higher viral doses, as viral titres stay very low at lower doses in both
156 *Wolb+* and *Wolb-* flies. At the higher temperature, it confers more resistance at the lower doses, as
157 virus titres are very high in *Wolb+* and *Wolb-* flies exposed to high virus doses. The mean viral load
158 was higher at 25ºC than at 18ºC ($p < 0.001$) and was lower in the presence of *Wolbachia* ($p <$
159 0.001). On average, *Wolbachia* induces higher resistance at 18°C than at 25°C. There was a 550-
160 fold reduction in viral load at 18°C ($p < 0.001$) and 50-fold reduction at 25°C ($p < 0.001$), producing
161 approximately 11-fold difference between these two conditions ($p = 0.003$). These results show
162 that the strength of *Wolbachia*-conferred protection to DCV, in terms of both, survival and viral
163 loads, depends on the temperature of infection. Protection is higher at a lower temperature. Since
164 post-infection temperature affects viral infection independently of *Wolbachia*, the lower capacity of
165 *Wolbachia* to protect at higher post-infection temperatures may be related with a higher replication
166 of the virus.
167
168 Antiviral protection is also usually positively correlated with *Wolbachia* levels (8, 9, 11, 16, 26, 27),
169 however, so we tested *Wolbachia* levels at the day of infection and after three days in DCV-infected,
170 buffer-pricked (control), or unmanipulated flies, at both temperatures (Fig. 1D). *Wolbachia* levels
171 were not affected by virus, buffer or temperature of infection ($p > 0.425$ for effect of temperature or
172 treatment). Thus, the difference in protection is likely independent of *Wolbachia* levels, as these
173 are not significantly affected within three days of the viral challenge.
174
175 We extended our analysis by testing how pre-infection temperature affects *Wolbachia* protection
176 (Fig. 2). We followed survival of infected flies in all four possible combinations of pre- and post-
177 infection temperatures of 18°C and 25°C (Fig. 2A, B). There is a strong interaction between pre-
178 infection temperature and *Wolbachia* ($p = 0.009$). Remarkably, in flies raised at 18°C *Wolbachia*
179 does not protect against DCV infection ($p = 0.207$), while in flies raised at 25°C it does (CHR = -
180 1.38, $p < 0.001$), irrespectively of the post-infection temperature. Also, in the absence of *Wolbachia*
181 the pre-infection temperature has no effect on the survival after DCV infection ($p = 0.276$). This
182 strong interaction between *Wolbachia*-conferred protection to DCV and pre-infection temperature
183 is also reflected in DCV loads (Fig. 2C, S1, $p < 0.001$). On average, *Wolbachia* reduced viral titres
184 14-fold at pre-infection temperature of 18°C and 1900-fold at 25°C (difference $p < 0.001$). In these
185 assays, pre-infection temperature also did not affect viral loads in the absence of *Wolbachia* ($p =$
186 0.534). Therefore, in contrast to post-infection temperature, pre-infection temperature does not
187 directly impact virus performance and modulates only *Wolbachia* protection. In summary, in flies
188 raised at lower temperature *Wolbachia* confers a reduced resistance to DCV in terms of viral titres
189 and no protection in terms of survival.

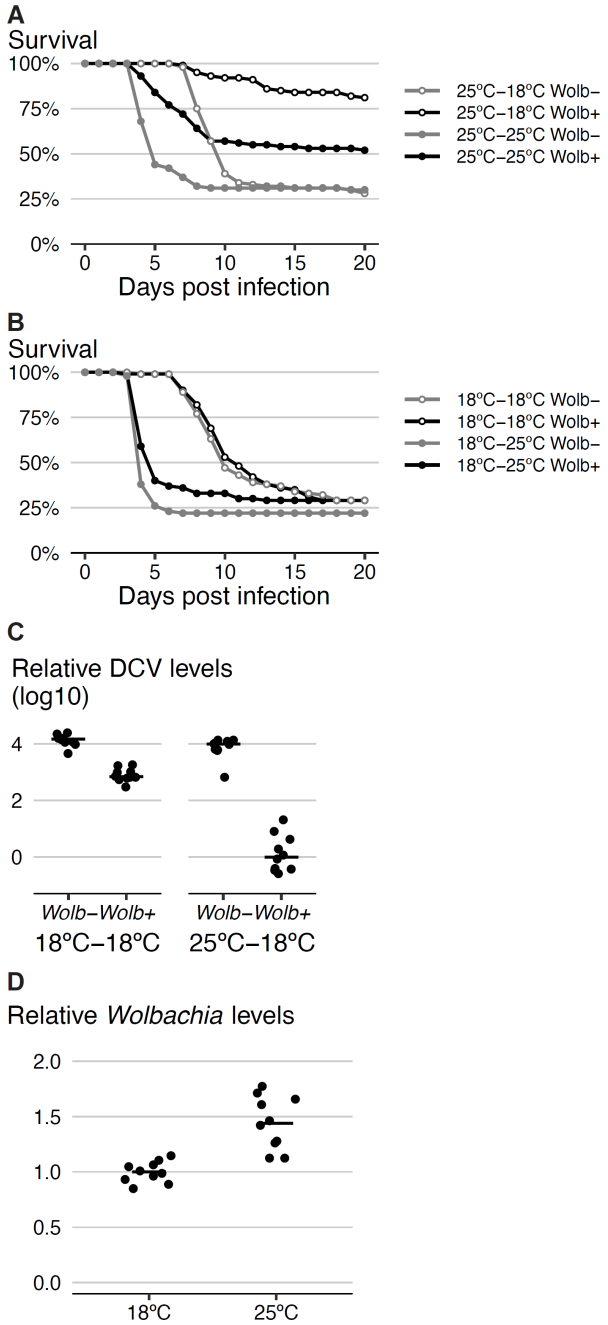


Figure 2. Pre-infection temperature determines *Wolbachia*-conferred antiviral protection. (A,B) *Wolb+* and *Wolb-* flies, fifty per *Wolbachia* status per condition, were infected with DCV (10^8 $TCID_{50}$/ml) and checked for survival every day. Flies were raised and kept at 25°C (A) or 18°C (B) before DCV infection and placed at either 18°C or 25°C after infection. (C) DCV titres in flies 3 dpi measured by RT-qPCR. Flies were raised and kept at either 18°C or 25°C before infection and at 18°C after infection. Replicate of this experiment is shown in Fig. S1. (D) *Wolbachia* levels measured by qPCR in 3-6 days old flies raised at either 18°C or 25°C. For (C) and (D) each dot is a sample of ten flies. Horizontal lines are medians of the samples.

199 Next, we measured *Wolbachia* levels in flies raised at 18°C and 25°C (Fig 2D). Flies raised at 18°C
200 had approximately 33% less *Wolbachia* than the flies raised at 25°C ($p < 0.001$). Although this
201 difference may contribute to the difference in protection, a larger, 50% difference in *Wolbachia* titres
202 between *w*Mel- and *w*MelCS-harbouring flies did not abolish the protection (8). Thus, differences
203 in *Wolbachia* titres do not seem to fully explain the difference in protection between flies raised at
204 different temperatures.
205
206 To test the robustness of pre-infection temperature effect on *Wolbachia*-conferred protection, we
207 tested flies with distinct host genetic backgrounds, Aljezur1 and Oregon-R W-20 (1), each
208 harbouring its original *w*Mel-like *Wolbachia* strain (Fig. 3A,B and S2A,B). In agreement with the
209 previous results, there was a significant effect of pre-infection temperature on *Wolbachia*-conferred
210 antiviral protection against DCV in both lines ($p<0.001$ for both). *Wolbachia* in Aljezur1 line
211 conferred protection only when the pre-infection temperature was 25°C ($p = 0.763$ for 18°C, and
212 CHR = -1.19, $p < 0.001$ for 25°C). In the Oregon-R W-20 line, *Wolbachia* still conferred some
213 protection when pre-infection temperature was 18°C, although it was significantly less than at 25°C
214 (CHR = -1.21, $p < 0.001$ for 18°C, and CHR = -3.02, $p < 0.001$ for 25°C, CHR difference = 1.8, $p <$
215 0.001). Next, we investigated if the pre-infection temperature effect was specific to DCV or if it was
216 also present upon challenge with another RNA virus, Flock house virus (FHV) (Fig. 3C and S2C),
217 There was an interaction between *Wolbachia*, pre-infection temperature, and dose in this assay (p
218 $= 0.013$). *Wolbachia* protection was significantly higher at flies raised at 25°C for all doses of virus
219 except for the lowest one (contrasts of CHR < -2.84, $p < 0.001$ for 10^7 – 10^9 $TCID_{50}$/ml). At the
220 lowest dose, 10^6 $TCID_{50}$/ml, there was an overall low mortality of flies and the difference in survival
221 was not statistically significant (contrast of CHR = -1.00 but $p = 0.421$). The pre-infection
222 temperature had no effect on survival to FHV in the absence of *Wolbachia* ($p = 1$, in all
223 comparisons), as observed for DCV. In conclusion, the strong influence of pre-infection
224 temperature on *Wolbachia*-conferred protection is a general effect observed in different host
225 genetic backgrounds, *Wolbachia* variants and with the different viruses used.
226
227 Since there is a strong interaction between protection and constant pre-infection temperature, we
228 asked if protection exists when temperature is cycling daily between 18ºC and 25ºC (Fig. S3).
229 *Wolbachia* protects against DCV infection at the cycling temperature (CHR = - 0.67, $p = 0.014$).
230 This protection seems to be intermediate between the protection seen in flies developed at 18ºC
231 or 25ºC. This result shows that in daily cycling temperatures, which occur in natural conditions,
232 *Wolbachia* can protect against viruses.

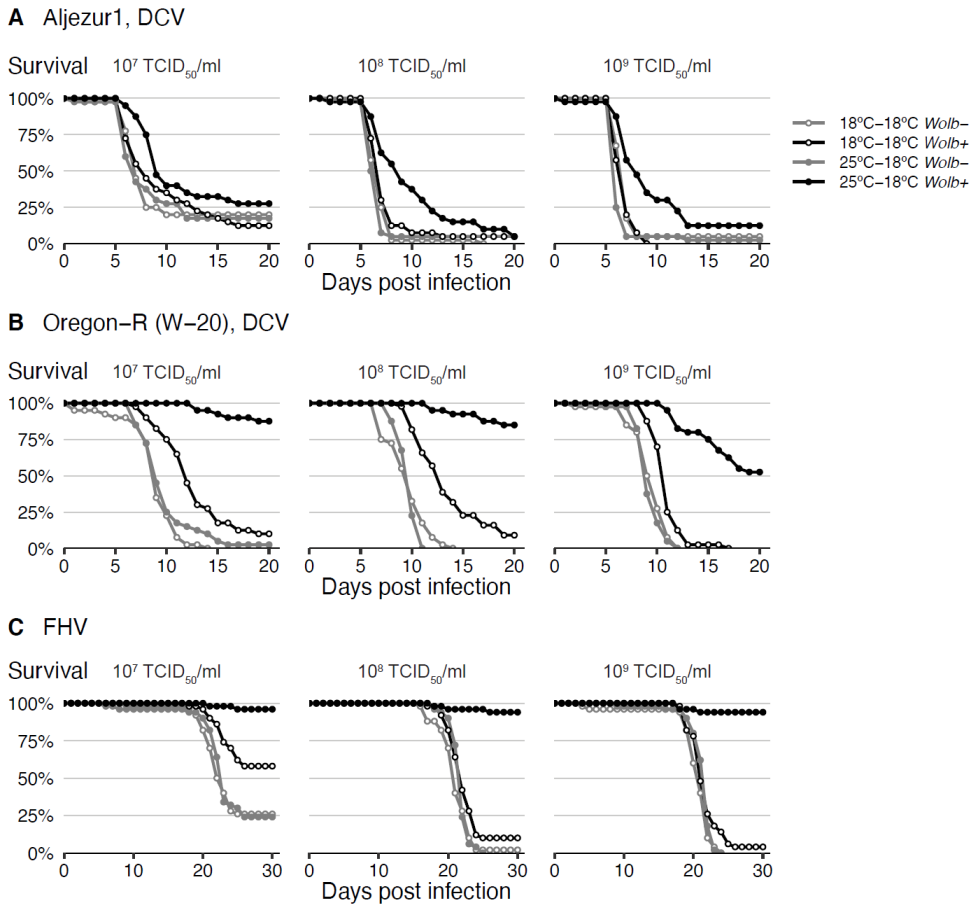
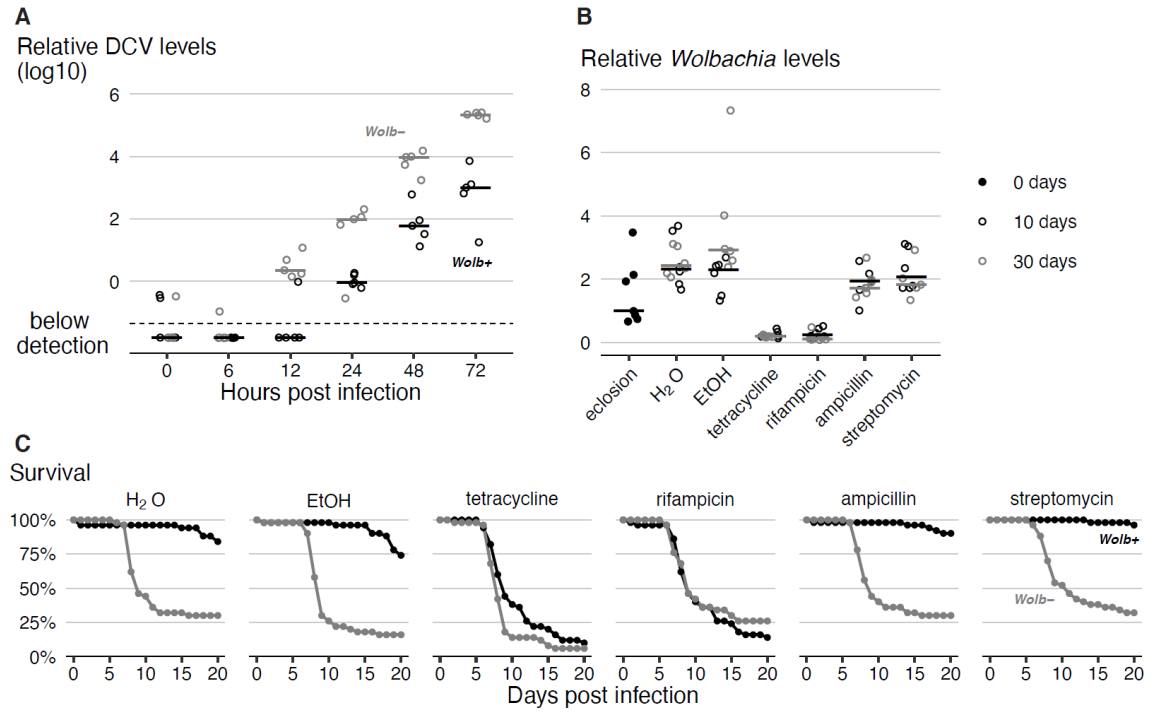


Figure 3. Pre-infection temperature determines *Wolbachia*-conferred antiviral protection in different *Wolbachia* and *Drosophila* genotypes and against different viruses. (A) Aljezur 1 and (B) Oregon-R (W-20) *Wolb*+ and *Wolb*- flies, fifty per *Wolbachia* status per condition, were pricked with three dilutions of DCV. (C) w^{1118} *iso* flies, fifty per *Wolbachia* status per condition, were infected with three doses of FHV. Flies were kept at 25°C or 18°C before the infection, and at 18°C after the infection. Mortality was recorded daily. A replicate of this experiment is shown in Fig. S2.

In the above protocols, flies developed from egg to adult at a given temperature and after collection they were aged for three more days at the same temperature, before infection with viruses. To dissect at which of these stages, development or aging, temperature influences *Wolbachia* protection, we tested the four possible combinations of the two different temperatures for these two stages (Fig. S4). Developmental temperature strongly influenced *Wolbachia* protection, which was higher at 25°C (CHR = -1.16, $p < 0.001$). Aging the flies at 25°C also interacted with *Wolbachia* protection, but increased it only slightly compared to aging at 18ºC (CHR = -0.431, $p = 0.011$). Therefore, *Wolbachia* protection to viruses is mainly dependent on the temperature of development form egg to adult.

251 Since pre-infection events are crucial for *Wolbachia*-conferred resistance we asked how fast the
252 difference in virus titres arise between flies with and without *Wolbachia* (Fig. 4A). We observed a
253 *Wolbachia*-induced resistance of approximately 120-fold as soon as we can detect viral RNA, at
254 12h after infection, ($p < 0.001$ at 12h, and $p < 0.002$ for all posterior time points in Fig 4A and S5A).
255 Thus, *Wolbachia* lowers viral titres very early in the course of DCV infection. We observed a similar
256 pattern of early FHV blocking by *Wolbachia*, from day one post infection onwards, but with smaller
257 viral titre differences (*Wolbachia*-infected flies had 2.3 to 5.5 times less FVH from the first day
258 onwards, $p > 0.028$ for all these comparisons, Fig. S5B).
259



260
261 **Figure 4. *Wolbachia* presence in adults is required for antiviral protection.** (A) Time course analysis of
262 DCV titres in *Wolb+* and *Wolb-* flies raised at 25°C and kept at 18°C after DCV infection. Relative DCV levels
263 were determined by RT-qPCR. Time 0 corresponds to the time of infection. Each dot is a sample consisting
264 of 10 flies, lines are medians. (B) *Wolbachia* levels in single flies raised at 25°C, at eclosion (day 0), after 10
265 days of different antibiotic and control treatments at 25°C (day 10), and after additional 20 days of treatment
266 at 18°C (day 30), measured by qPCR. A replicate of this experiment is shown in Fig. S5C. (C) Survival of
267 *Wolb+* and *Wolb-* flies, fifty per *Wolbachia* status per treatment, developed at 25°C, collected at eclosion, fed
268 antibiotics-containing food for 10 days at 25°C, infected with DCV (10^8 $TCID_{50}$/ml), and placed at 18°C on
269 antibiotic-containing food. Mortality was recorded daily. A replicate of this experiment is shown in Fig. S5E.
270

271 Developmental temperature determines protection and the protection can be detected as early as
272 we detect viral replication in flies. Thus, we asked if changes in the fly caused by *Wolbachia* during
273 development are sufficient to block viral infection in adults. To answer this, adult flies developed at
274 25°C were subsequently treated with antibiotics for ten days to remove *Wolbachia* before viral
275 infection (Fig. 4B, S5C). *Wolbachia* levels were reduced approximately 10-fold in ten days of

276 treatment with tetracycline and rifampicin ($p < 0.001$ for both) and stayed low until the end of the
277 experiment at 30 days ($p < 0.001$ for both). These treatments eliminated antiviral protection from
278 the flies ($p = 1.00$ for both, Fig. 4C, S5E). The control treatments with the antibiotics ampicillin and
279 streptomycin did not affect *Wolbachia* levels ($p > 0.254$ for both, Fig. 4B, S5C) and did not affect
280 endosymbiont-mediated protection (in both treatments *Wolbachia* protection is significant, $p <$
281 0.001 and not different from *Wolbachia* protection in controls, $p > 0.301$, Fig. 4C, S5E). As bacteria
282 in the fly gut were efficiently cleared by all antibiotic treatments (Fig. S5D), we conclude that it is
283 *Wolbachia* loss that leads to the loss of protection and not differential effect of the antibiotics on
284 gut-associated bacteria. These data show that *Wolbachia* presence in adults is required for the
285 *Wolbachia*-conferred antiviral protection, even though the presence or absence of protection is
286 determined by the temperature during development, before the challenge occurs.
287
288 **Discussion**
289
290 Here we show that temperature is a strong modulator of *Wolbachia*-conferred antiviral protection
291 in the natural host *D. melanogaster*. The temperature at which the infection progresses influences
292 this interaction, with *Wolbachia* giving more resistance and increasing survival at lower
293 temperatures. However, the most striking phenotype we report is the effect of host developmental
294 temperature on this interaction. While development at 25ºC leads to a strong antiviral protection in
295 terms of survival and resistance to DCV, development at 18ºC abrogates, or strongly reduces
296 protection. This is observed with different genotypes of *D. melanogaster,* different variants of
297 *Wolbachia* (*w*Mel and *w*MelCS), and different viruses, and is, therefore, likely to be a general
298 phenomenon.
299
300 This complex interaction between temperature and *Wolbachia* protection to viruses may play a role
301 in the natural environment. The outcome of *Wolbachia*-host interactions, in terms of antiviral
302 protection, may vary in regions with different climates or across seasons in the same region.
303 Therefore, *Wolbachia* may behave as a parasite or a mutualist, depending on the conditions.
304 *Wolbachia*'s placement along the parasitism-mutualism continuum at any given time will depend
305 on the cost of harbouring *Wolbachia* and the benefit of antiviral protection to the host. Our results
306 lead to a prediction that *Wolbachia* would protect more in warmer conditions, given the strong
307 reduction in antiviral protection with a low developmental temperature. We have suggested before
308 that the antiviral protection is a fitness advantage that may explain *Wolbachia* prevalence in *D.*
309 *melanogaster* populations (1). Selection for hosts carrying *Wolbachia* would, therefore, be stronger
310 at higher temperatures. Consequently, geography and seasonality could impact the frequency of
311 *Wolbachia* in a population. Interestingly, there is a high variation in the frequency of *Wolbachia* in

312 *D. melanogaster* natural populations and a clinal distribution of this frequency. At lower latitudes,
313 and therefore warmer climates, the frequency *of Wolbachia* is higher (28). Although, the
314 relationship between *Wolbachia* frequency, temperature and other environmental parameters in *D.*
315 *melanogaster* is more complex at a smaller geographic scale, *Wolbachia* frequency is the highest
316 in regions with mean annual temperature of 22-26ºC (29). In insects, in general, there is also a
317 positive correlation between temperature and *Wolbachia* frequency but only in temperate climates
318 (25).
319
320 These results are also relevant for the deployment of *Wolbachia*-carrying mosquitoes to block
321 dengue, Zika, chikungunya and other arboviruses transmission (3, 30–32). It is already known that
322 heat stress impacts *w*Mel in transinfected *Aedes aegypti* , reducing its titres (33), heatwaves can
323 impact titres and frequency of *Wolbachia* in these mosquito populations (34) and at low cycling
324 temperatures *Wolbachia* titers decrease (35). On the other hand, temperatures varying between
325 25 and 28ºC do not seem to affect protection to viruses in this system (18). It would be important,
326 however, to assess how lower temperatures influence *Wolbachia*-induced pathogen blocking in
327 mosquitoes. Determining the temperature range of effectiveness of *Wolbachia*-deploying antiviral
328 field interventions will be crucial to plan where to use it and when to combine it with other
329 complementary approaches (e.g. insecticides).
330
331 Pre-infection temperature has a drastic and enigmatic effect on *Wolbachia*-conferred antiviral
332 protection. Lower temperatures during host development determine the level of protection in adult
333 life. Understanding the molecular mechanism of this effect will elucidate how environment interacts
334 with host-microbe symbioses and may be key to understanding *Wolbachia* antiviral protection. The
335 effect of development temperature acts solely on the interaction between virus and *Wolbachia,* and
336 does affect viral infection by itself. The protection conferred by *Wolbachia* seems onset immediately
337 or very soon after viral infection. We observe a difference in viral titres as soon as we detected viral
338 replication in *Wolbachia*-free flies, at 12h after infection. Thus, *Wolbachia* likely inhibits the viral
339 entry or first replication cycle *in vivo*. *Wolbachia* interference in early stages of viral infection is in
340 agreement with cell culture data (36–38). However, this is not a simple pre-set antiviral state of the
341 host because *Wolbachia* is still required at the time of infection. The lower *Wolbachia* titres in flies
342 developed at 18ºC, when compared with flies developed at 25ºC, could partly explain a reduction
343 in protection. However, this difference should not be enough for the complete lack of protection
344 when flies develop at 18ºC. We have previously observed significant protection to DCV in flies
345 carrying even lower titres of other *w*Mel variants (8). Nonetheless, tissue-specific differences in
346 titres, set during development, may underlie the phenotypic differences. It would, therefore, be
347 interesting to characterize in detail the spatial characterisation of *Wolbachia* tissue tropism and viral

348 infection, in flies developed at the different temperatures. Comparative transcriptomic and
349 metabolomic analysis of *D. melanogaster* and *Wolbachia* in these two conditions could not only
350 elucidate how temperature affects protection, but also the mechanism of *Wolbachia* antiviral
351 protection itself.
352
353 Temperature affects many insect-symbiont interactions and their phenotypes (39), including
354 protective symbiosis (40–42). Therefore, this environmental factor may play a general critical role
355 determining the outcome of complex host-endosymbiont-pathogen interactions and shape the
356 geographic distribution of insects and their symbionts. The phenotypic variation we report here,
357 ranging from no protection to strong protection to viruses, indicates that temperature could be a
358 crucial determinant of cost/benefit ratio of carrying *Wolbachia*. Therefore, temperature could
359 deeply impact the *D. melanogaster* – *Wolbachia* interaction in natural populations. Moreover, these
360 results may have important implications for the deployment of *Wolbachia*-carrying mosquitoes to
361 fight arboviruses transmission and may lead to new approaches to dissect the mechanism of
362 *Wolbachia* antiviral protection.
363
364 **Materials and Methods**
365
366 **Fly strains and husbandry**
367 DrosDel w^{1118} *isogenic D. melanogaster* (*iso*) with *w*MelCS_b *Wolbachia* (*Wolb*+) and the matching
368 control without *Wolbachia* (*Wolb*-) were described elsewhere (1, 8, 43). Aljezur 1 and W-20 *D.*
369 *melanogaster* lines (*Wolb*+ and *Wolb*-) were also described before (1). We determined that the
370 *Wolbachia* variants in both these lines lack an IS5 transposon insertion in gene WD1310, based
371 on the primers described in (44). This insertion is present in all *w*MelCS-like variants, but not in
372 wMel variants (8, 44), therefore Aljezur-1 and W-20 are both *w*Mel-like *Wolbachia* variants. Stocks
373 were maintained at a constant temperature of 25°C on a diet consisting of: 45 g molasses, 75 g
374 sugar, 70 g cornmeal, 20 g yeast extract, 10 g agar, 1100 ml water and 25 ml of 10% Nipagin, with
375 addition of live yeast (Sigma).
376
377 **Virus infection experiments**
378 DCV and FHV were produced and titrated in cell culture as described before (1, 8). Flies for
379 experiments were produced by placing 12 females and 6 males per bottle for 4 days to produce
380 offspring at either 25°C, 18°C or at fluctuating temperature (18°C to 25°C gradual increase during
381 12 h, and 25°C to 18°C decrease during the subsequent 12 h). After 10 days (25°C), 15 days
382 (fluctuating temperature) or 20 days (18°C) the flies started to eclode. Unless specified otherwise,
383 0-3 days old flies were collected from the bottles and placed in the vials, ten males per vial, on food

384 without live yeast. Flies were aged for 3 more days at the developmental or otherwise indicated
385 temperature. These 3-6 days old flies were pricked intrathoracically with virus diluted in 50 mM
386 Tris-HCl, pH 7.5. After infection, flies were placed at the indicated temperatures. Survival was
387 monitored daily and vials were changed every 5 days.
388
389 **Nucleic acids extractions and real-time qPCR**
390 DNA for the quantification of *Wolbachia* was extracted from pools of 10 flies using DrosDel protocol
391 (http://www.drosdel.org.uk/molecular_methods.php) (43) or from single flies with the protocol
392 described before (45). RNA for assessment of viral titres was extracted using Trizol (Invitrogen)
393 and cDNA was prepared using M-MLV Reverse Transcriptase (Promega), as described previously
394 (8). Real-time qPCR reactions were carried out in 7900HT Fast Real-Time PCR System (Applied
395 Biosystems) with the iQ™ SYBR® Green supermix (Bio Rad) or in QuantStudio™ 7 Flex Real-
396 Time PCR System (Applied Biosystems) with iTaq™ universal SYBR® Green supermix (Bio Rad).
397 *Wolbachia* was quantified using *wsp* as the target gene and *Drosophila Rpl32* as the reference
398 gene. DCV was quantified with primers for DCV as the target gene and *Drosophila rpl32* as the
399 reference gene. Primers used were: *Wolbachia wsp,* 5'-CATTGGTGTTGGTGTTGGTG-3' and 5'-
400 ACCGAAATAACGAGCTCCAG-3'; DCV, 5'-TCATCGGTATGCACATTGCT-3' and 5'-
401 CGCATAACCATGCTCTTCTG-3'; FHV, 5'-ACCTCGATGGCAGGGTTT-3' and 5'-
402 CTTGAACCATGGCCTTTTG-3'; and *Drosophila rpl32*, 5'-CCGCTTCAAGGGACAGTATC-3' and
403 5'-CAATCTCCTTGCGCTTCTTG-3'. Thermal cycling protocol for *Wolbachia* amplification was:
404 initial 50°C for 2 min, denaturation for 10 min at 95°C followed by 40 cycles of 30 s at 95°C, 1 min
405 at 59°C and 30 s at 72°C. For DCV and FHV the same conditions were used, except for an
406 annealing temperature of 56°C. Relative levels of *Wolbachia* or DCV were calculated by the Pfaffl
407 method (46).
408

409 **Antibiotic treatment of flies**

410 *Wolb*+ and *Wolb*- flies were raised at 25°C, collected as 0-1 days old adults and placed on fly food
411 with 100 mg/ml of tetracycline hydrochloride, rifampicin, ampicillin sodium or streptomycin sulphate
412 (all Sigma) or control food with antibiotics solvent (water or ethanol). At day 10 of treatment, flies
413 were challenged with DCV, placed at 18°C and survival was followed for additional 20 days. Food
414 vials were changed every three days. At day 0, day 10 and day 30 flies were collected to assay
415 *Wolbachia* levels by qPCR. At day 31, guts from three *Wolb*+ flies per condition were dissected to
416 assess the effect of antibiotics on the gut-associated bacteria. Guts were homogenized in 250 µl
417 of sterile LB and 30 µl was plated on mannitol agar plates. This medium sustains growth of the
418 main gut-associated bacteria in lab *D. melanogaster*, *Acetobacter* and *Lactobacillus* species (47).
419 CFUs were counted after five days of incubation at 26°C.

420

421 **Statistical analysis**

422 All the statistical analysis was performed in R (48). The script of the statistical analysis and output
423 of this analysis are presented in supplementary texts 1 and 2, respectively. All data for statistical
424 analysis and presented in figures are available as supplementary files.

425 Analysis of survival data was performed with the Cox proportional hazard mixed effect models.
426 Fixed effects, depending on the experiment, included temperature, dose of DCV,
427 presence/absence of *Wolbachia,* and antibiotic treatment, while replicate vials within the same
428 experiment or full experimental replicates were considered as random effects. Model fitting was
429 performed using the coxme package in R version 2.2-16 (49).

430 *Wolbachia* and DCV titres were analysed with log-transformed qPCR data and linear models or
431 general linear models. Model fitting was performed using lm or the lme4 package in R (50).

432 The effect of interaction between factors in the models was determined by ANOVA. Post hoc
433 analysis of marginal (least square) means were compared between the conditions of interest using
434 the lsmeans package in R (51).

435 Figures were produced using the ggplot2 package (52).

436

437

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439

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444
445

446 **References**
447

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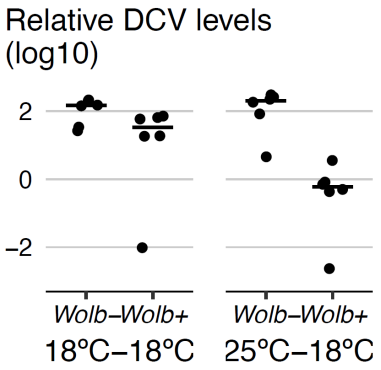

572
573
574 **Fig. S1. Pre-infection temperature determines viral titres.** DCV titres measured by RT-qPCR 3
575 dpi in *Wolb*+ and *Wolb*- flies infected with 10^8 $TCID_{50}$/ml DCV. Flies were kept at 18°C or 25°C
576 before the infection and at 18°C after the infection. Each dot is a sample, each sample consists of
577 ten flies. Horizontal lines are medians of the replicates. This is a replicate of experiment shown in
578 Fig. 2C.
579

580

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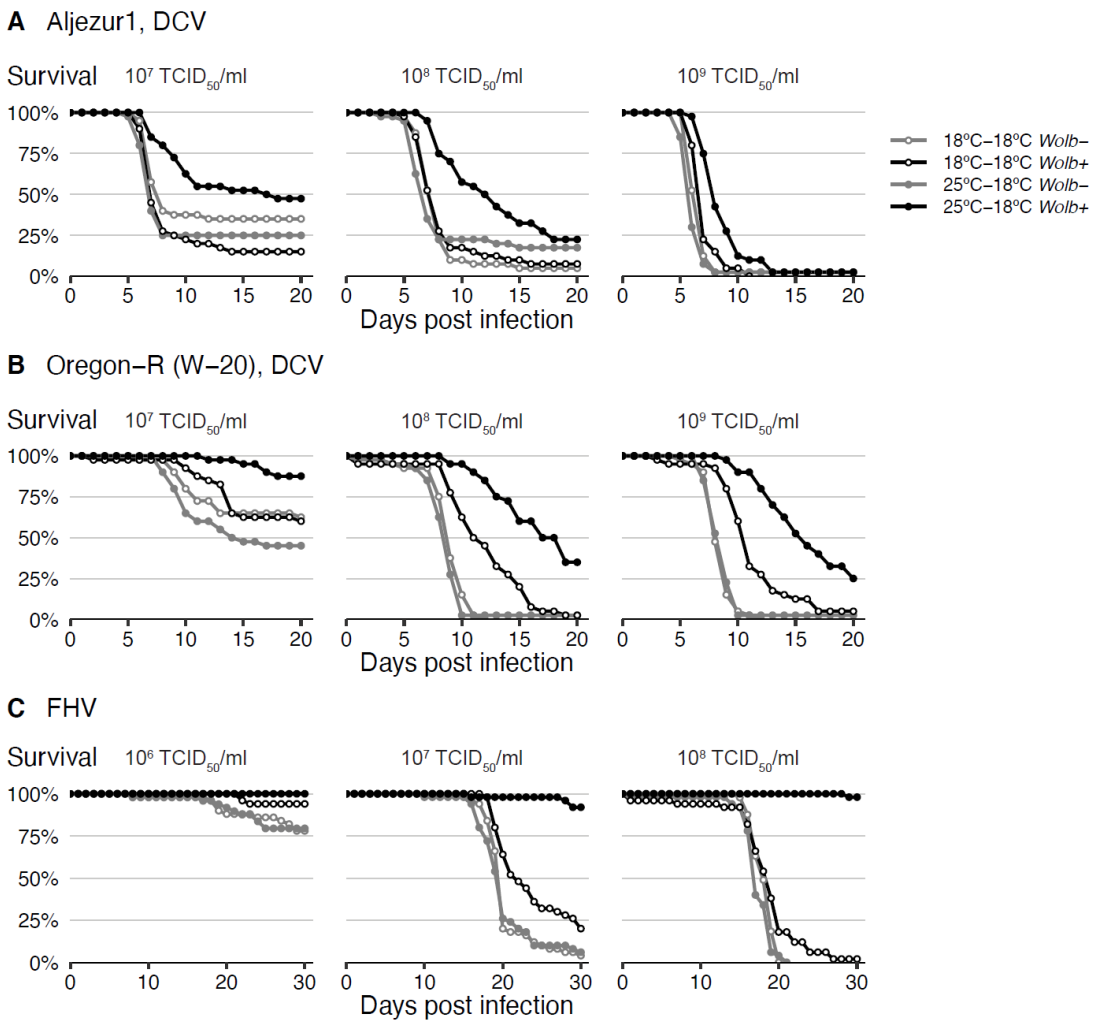
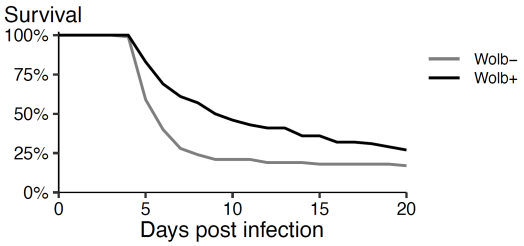


Figure S2. Pre-infection temperature determines *Wolbachia*-conferred antiviral protection in different *Wolbachia* and *Drosophila* genotypes and against different viruses. (A) Aljezur 1 and (B) Oregon-R (W-20) *Wolb*+ and *Wolb*- flies, fifty per *Wolbachia* status per condition, were pricked with three dilutions of DCV. (C) w^{1118} *iso* flies, fifty per *Wolbachia* status per condition, were infected with three doses of FHV. Flies were kept at 25°C or 18°C before the infection, and at 18°C after the infection. Mortality was recorded daily. This is a replicate of experiment shown in Fig. 3.

591



592
593
594 **Fig. S3. Wolbachia protects flies kept at a temperature cycling daily between 18 and 25ºC.**
595 Fifty *Wolb+* and *Wolb-* flies were raised with the temperature cycling daily between 25ºC (midday)
596 and 18ºC (midnight), infected with DCV (10^8 $TCID_{50}$/ml) and placed at the same cycling temperature
597 after infection. Mortality was recorded daily.
598

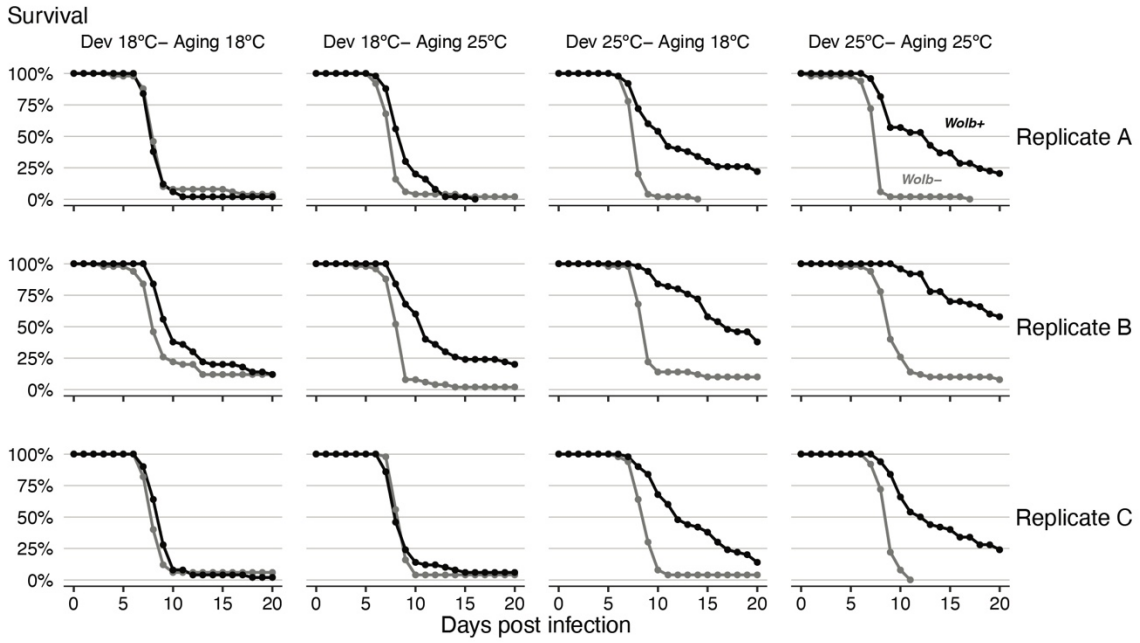


Figure S4. Developmental temperature determines *Wolbachia*-conferred antiviral protection. *Wolb+* and *Wolb-* flies, fifty per *Wolbachia* status per condition per replicate, were raised from egg to adult at 18 or 25ºC (Dev), collected at the age of 0-1 days, aged for 3 days at an 18 or (Aging), pricked with DCV (10^8 $TCID_{50}$/ml), and placed at 18ºC. Mortality was recorded daily. Three replicates of the same experiment are shown.

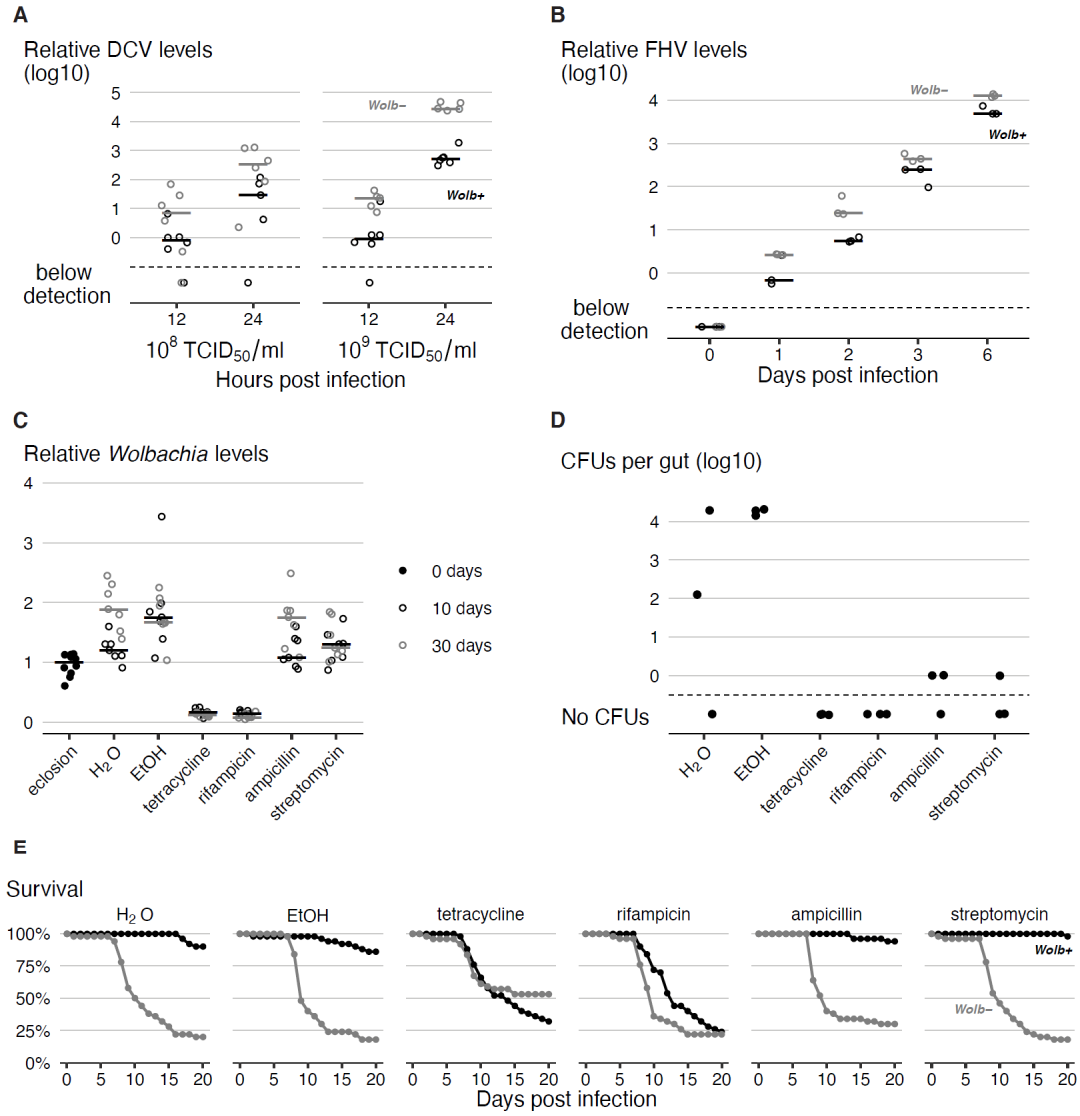


Figure S5. *Wolbachia* presence in adults is required for antiviral protection. (A) DCV titres in *Wolb+* and *Wolb-* flies raised at 25°C and kept at 18°C after DCV infection with two doses, 10^8 and 10^9 $TCID_{50}$/ml, and at two time points post infection, 12 and 24h. Relative DCV levels were determined by RT-qPCR. Each dot is a sample consisting of 10 flies, lines are medians. (B) FHV titres in *Wolb+* and *Wolb-* flies raised at 25°C and kept at 25°C after infection. Each dot is a sample consisting of 10 flies, lines are medians, and time 0 corresponds to the time of infection. (C) *Wolbachia* levels in single flies raised at 25°C, at eclosion (day 0), after 10 days of different antibiotic/ control treatments at 25°C (day 10), and after additional 20 days of treatment at 18°C (day 30) measured by qPCR. This is the replicate of the experiment shown in Fig. 4B. (D) Colony forming units (CFUs) in dissected *Wolb+* fly guts at the day 30 of each antibiotic or control treatment. Each dot is a pool of three guts. (E) Survival of *Wolb+* and *Wolb-* flies, fifty per *Wolbachia* status per treatment, developed at 25°C, collected at eclosion, fed antibiotics-containing food for 10 days at 25°C, infected with DCV (10^8 $TCID_{50}$/ml), and placed at 18°C on antibiotic-containing food. Mortality was recorded daily. This is the replicate of the experiment shown in Fig. 4C.

Supplementary Information

SI Text S1 (text_s1.txt)
Rmd script for statistical analysis

SI Text S2 (text_s2.pdf)
Output of statistical analysis

SI Dataset S1 (dataset_s1.txt)
Data for figures 1A, B

SI Dataset S2 (dataset_s2.txt)
Data for figure 1C

SI Dataset S3 (dataset_s3.txt)
Data for figure 1D

SI Dataset S4 (dataset_s4.txt)
Data for figures 2A, B, and S3

SI Dataset S5 (dataset_s5.txt)
Data for figures 2C and S1

SI Dataset S6 (dataset_s6.txt)
Data for figure 2D

SI Dataset S7 (dataset_s7.txt)
Data for figures 3A, B, and S2A, B

SI Dataset S8 (dataset_s8.txt)
Data for figures 3C and S2C

SI Dataset S9 (dataset_s9.txt)
Data for figure S4

SI Dataset S10 (dataset_s10.txt)
Data for figure 4A

SI Dataset S11 (dataset_s11.txt)
Data for figure S5A

SI Dataset S12 (dataset_s12.txt)
Data for figure S5B

SI Dataset S13 (dataset_s13.txt)
Data for figures 4B and S5C

SI Dataset S14 (dataset_s14.txt)
Data for figures 4C and S5E

SI Dataset S15 (dataset_s15.txt)
Data for figure S5D