Title

Regulation of *Wolbachia* proliferation by the amplification and deletion of an addictive genomic island

Authors

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Abstract

Wolbachia is one of the most prevalent bacterial endosymbionts, infecting approximately 40% of terrestrial arthropod species. *Wolbachia* is often a reproductive parasite but can also provide fitness benefits to its host, as for example protection against viral pathogens. This protective effect is currently being applied to fight arboviruses transmission by releasing *Wolbachia*-transinfected mosquitoes. Titre regulation is a crucial aspect of *Wolbachia* biology. Higher titres can lead to stronger phenotypes and fidelity of transmission but can have a cost to the host. Since *Wolbachi*a is maternally transmitted, its fitness depends on host fitness, and, therefore, its cost to the host needs to be controlled. Understanding this and other aspects of *Wolbachi*a biology has been hampered by the lack of genetic tools. Here we developed a new forward genetic screen to identify *Wolbachia* over-proliferative mutant variants. We characterized in detail two of these new mutants, *w*MelPop2 and *w*MelOctoless, and show that the amplification or loss of the Octomom genomic region causes their over-proliferation. These results confirm previous

data and expand on the complex role of this genomic region in the control of *Wolbachia* proliferation. Both new mutants shorten the host lifespan and increase antiviral protection. Moreover, we show that *Wolbachia* proliferation rate in *Drosophila melanogaster* depends on the interaction between Octomom copy number, the host developmental stage, and temperature. Our analysis also suggests that the life shortening phenotype and antiviral protection of *Wolbachia* are dependent on related, but different, properties of the endosymbiont; the rate of proliferation and the titres at time of infection, respectively. Altogether, we demonstrate the feasibility of a novel and unbiased experimental approach to study *Wolbachia* biology, which can be further adapted to characterize other genetically intractable bacterial endosymbionts.

Introduction

Intracellular maternally-transmitted bacterial symbionts (bacterial endosymbionts) are widespread in insects [1]. These bacteria can be mutualistic by complementing the diets of their hosts, or by increasing fitness of their hosts in other particular ecological contexts, and may expand the range of ecological niches of their insect hosts [1]. Alternatively, bacterial endosymbionts can be parasitic, many times manipulating the reproduction of their hosts and providing a benefit to infected females over uninfected counterparts [1]. Understanding the interaction of endosymbionts with their host is crucial to understand much of insect biology. A key aspect of this interaction is the regulation of endosymbiont titres which influence the strength of induced phenotypes and cost to the hosts [2,3].

Wolbachia is one of the most prevalent bacterial endosymbionts in arthropods, being found in approximately 40% of terrestrial arthropod species [4]. *Wolbachia* is broadly known as a host reproduction manipulator [5]. However it can also be mutualistic, by, for example, providing a vitamin to its host [6] or protection against viral pathogens [7,8].

Since insects are vectors of many viruses, the discovery of *Wolbachia* antiviral phenotype, in *Drosophila melanogaster*, boosted its use as a vector control agent [9]. *Aedes aegypti* mosquitoes trans-infected with *Wolbachia* have increased resistance to viruses, including dengue, chikungunya, Zika, and yellow fever viruses, and, therefore, reduced vectorial capacity [10–13]. Release of *Wolbachia*-infected mosquitoes in dengue-endemic areas is likely to reduce dengue burden [14,15]. Despite the preliminary successful results of this strategy we still lack knowledge on several fundamental aspects of *Wolbachia* biology and interaction with viral pathogens, which hinders predicting the long-term outcome of *Wolbachia*-based interventions in vector control.

Wolbachia titres are a critical factor regulating its biology and interaction with the host [3]. Titres correlate positively with transmission fidelity and the strength of induced phenotypes, including the *Wolbachia* pathogen blocking phenotype [3,16–20]. In contrast, higher titres are associated with a reduction in host lifespan [16,17,21,22]. This may have a cost to *Wolbachia* because, since it is vertically transmitted, its fitness depends on the host fitness. Thus, *Wolbachia* titres regulation by the symbiont or the host may be under selection. The interaction between titres, cost, and strength of antiviral resistance is also relevant for the deployment of *Wolbachia*-carrying mosquitoes to block arboviruses transmission. Although several host and environmental factors (e.g. temperature) have been shown to affect *Wolbachia* titres, less is known about *Wolbachia* genetic factors [3].

Comparison of *Wolbachia* variants in controlled conditions (e.g. same host genetic background) shows the role of *Wolbachia* genetic factors in titre regulation [16,16,23]. So far, a single *Wolbachia* genetic factor, the Octomom region, has been shown to influence proliferation [16,17]. This region, comprised of eight predicted genes, is amplified in the pathogenic *w*MelPop. The degree of the Octomom region amplification determines the proliferation rate of *w*MelPop and the extent of its life shortening phenotype [17].

The genetic intractability of *Wolbachia*, which remains unculturable so far, hampers the identification of more genetic modifications altering *Wolbachia* proliferation. Hence, unbiased approaches such as genetic screen could contribute to our understanding of the genetic bases of *Wolbachia*-host interactions. Here, we developed a screening strategy in *Wolbachia* to isolate novel over-proliferating variants. The strategy was based on random mutagenesis, which has been applied before to other unculturable bacteria [24]. We fed the mutagen ethyl methanesulfonate (EMS) to *D. melanogaster* females carrying *Wolbachia* and screened for over-proliferative *Wolbachia* in their progeny. This approach allowed us to isolate new mutant over-proliferating *Wolbachia* variants. We identified the causative genetic changes in *Wolbachia* for this over-proliferation and made a detailed phenotypical characterization in terms of growth and antiviral protection. We identified a new mutation leading to *Wolbachia* over-proliferation and revealed a complex role for the Octomom region in the regulation of *Wolbachia* growth. Moreover, we demonstrated the feasibility of a novel and unbiased experimental approach to study *Wolbachia* biology.

Results

Isolation of over-proliferative *Wolbachia* in an unbiased forward genetic screen

We implemented a classical forward genetic screen in order to isolate new over-proliferative *Wolbachia* variants. We attempted to mutagenize *Wolbachia* by feeding the mutagen EMS to *Wolbachia*-carrying *D. melanogaster* females. EMS is extensively used in *D. melanogaster* [25] and has been previously used to mutagenize intracellular bacteria in cell culture [24]. We then tested *Wolbachia* titres, by real-time quantitative PCR (qPCR), in the progeny of treated females, since this bacterium is maternally transmitted. We used flies with the variant *w*MelCS_b as our starting variant because of its genetic proximity to the over-proliferative and virulent *w*MelPop variant [16,22,26] and its potential to easily become over-proliferative [17].

Putative mutagenized *Wolbachia* cells within the host would be in a mixed population, which would make it harder to assess their specific phenotype. However, we hypothesized that over-proliferating *Wolbachia* cells would overtake the population and that the resulting higher titres could be detectable. Moreover, we decided to pre-treat some of the EMS exposed females with tetracycline to reduce the *Wolbachia* population in these females and their progeny. This *Wolbachia* titre reduction should decrease competition for any new mutated *Wolbachia*, increase drift, and maybe facilitate fixation of new variants. To set up the conditions for tetracycline treatment we tested different doses of this antibiotic on females, without EMS. The titre of *Wolbachia* in the progeny of treated females are reduced to approximately 0 to 90% of the titres in controls (S1 Fig, $p < 0.001$ for all doses compared with control, at generation 1). We then followed the subsequent progeny of these flies to test how many fly generations it takes to recover normal *Wolbachia* titres. Except for higher tetracycline doses which lead to infection loss, *Wolbachia* titres recovered to normal within four fly generations (S1 Fig; linear mixed model [lmm], $p > 0.05$ for all doses compared with control at generation 4).

To optimize the screen, we also tested for the effect of different EMS doses on the fecundity of *Drosophila* females and *Wolbachia* titres. We observed that increasing doses of EMS reduce female fecundity (S2A-B Fig, linear model [lm], $p < 0.001$ for both egg number and adult progeny per female). Moreover, we found that EMS feeding strongly reduces *Wolbachia* titres in the next generation, in a dose-dependent manner (S2C-D Fig, non linear model [nls] fit, $p < 0.001$). Titres were reduced by up to 90% when 8,000 mM EMS was supplied, leading to the loss of *Wolbachia* in the next generation in some lines (S2C-D Fig). This titre reduction due to the EMS treatment could hamper the identification

of over-proliferating *Wolbachia* in a screen in the first generation after treatment. Given these results and the recovery time after tetracycline treatment detailed above, we quantified *Wolbachia* titres at the first generation (F1), the immediate progeny of EMS-treated females, and at the fourth generation after treatment (F4) were we would expect *Wolbachia* titres to recover after the severe reduction due to EMS treatment.

We screened approximately 1000 F1 progeny of EMS-treated females, in a range of experimental conditions, and at least one F4 female descendent. We varied EMS dose from 10 mM to 8,000 mM, and tetracycline dose from 0 μg/ml to 12.5 μg/ml, in different combinations (S1 Table). The relative *Wolbachia* titre was determined when females were ten days old after they laid eggs so that any putatively interesting progeny could be followed up.

In three independent batches of EMS-treated flies, we detected females with 3 to 14-fold more *Wolbachia* than controls, suggesting the presence of over-proliferative variants (Fig 1 and S3 Fig). In two batches, over-proliferating *Wolbachia* was identified in the F1 and in the other batch in the F4. We assessed Wolbachia titres in the next generation and found that the over-proliferative phenotype was inherited. Subsequent selections allowed us to establish *Drosophila* lines carrying new potentially over-proliferative *Wolbachia* variants.

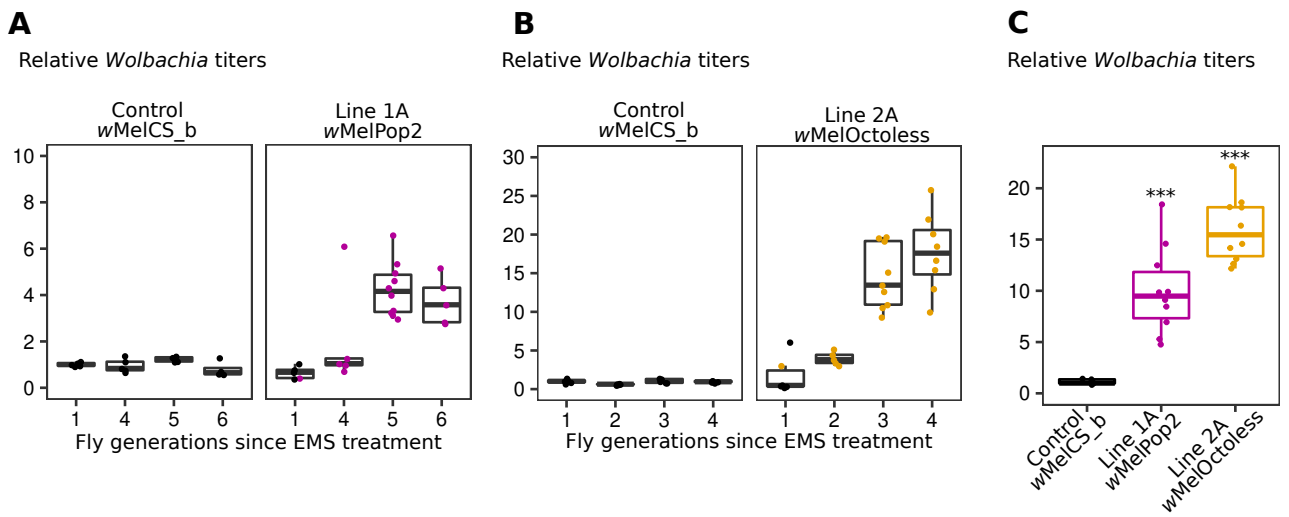

Fig 1. Isolation of over-proliferative *Wolbachia* variants by a forward genetic screen. (A and B) Relative *Wolbachia* titres in a control (*w*MelCS_b) and EMS-treated flies (Lines 1A and 2A). 5–10 virgin females were randomly collected each generation for egg-laying and *Wolbachia* titre measured using qPCR. Bacterial titres are normalized to that of control flies. The progeny of the female with the higher *Wolbachia* titre was used to set up the next generation. The selection of the other putative over-proliferating Wolbachia line in panel B in shown in S3A Fig. (C) Relative titres of over-proliferating *Wolbachia* variants in an isogenic genetic background. Both lines kept the over-proliferative phenotype ($p < 0.001$). Each dot represents Wolbachia titre of a single female.

158 We designed the screen to find new mutants of *Wolbachia,* and not host mutants, that lead
159 to the endosymbiont proliferation. However, EMS will most likely also induce mutations in
160 the host germline, in the nuclear or mitochondrial genomes, that can be transmitted. To
161 minimize the influence of new host nuclear mutations on our screen, we backcrossed the
162 EMS-treated females and their progeny, at every generation, with males from the control
163 isogenic line. To verify that new mutations in the host were not the cause of *Wolbachia*
164 over-proliferation, we replaced the first, second and third chromosomes of *Drosophila*
165 females carrying the over-proliferating *Wolbachia* variants, in line 1A and line 2A, with the
166 chromosomes of the control line, through the use of balancer chromosomes (S4 Fig.). We
167 repeated *Wolbachia* titres quantification and found that the over-proliferative phenotypes
168 were maintained (Fig 1C; lmm, p-value< 0.001 for both).

169 Since mitochondria are maternally transmitted, the experiments described above cannot
170 exclude the possibility that *Wolbachia* over-proliferation is mitochondria-determined
171 (directly or indirectly). Thus, the mitogenome of the lines showing higher *Wolbachia* titres
172 was Illumina sequenced and aligned to the mitochondrial reference genome release 6
173 (GenBank: KJ947872.2:1–14,000). A summary of the alignment features is given in S2
174 Table. We did not find SNPs or indels unique to the mitochondria of EMS-fed flies, which
175 shows that flies with over-proliferative *Wolbachia* did not inherit mutated mitochondria (S3
176 Table). Therefore, we concluded that the observed *Wolbachia* over-proliferative
177 phenotypes did not result from mutations in the host genome, neither nuclear nor
178 mitochondrial.

179

180 **Identification of genetic basis of the new over proliferative variants**

181 To identify the mutations associated with over-proliferation, we sequenced and assembled
182 the genomes of these over-proliferative *Wolbachia*. A summary of the sequencing results
183 is given in S2 table. We performed a hybrid assemble with short (Illumina) and long-reads
184 (Nanopore) and obtained single and circular *Wolbachia* chromosomes differing in length
185 (S4 Table).

186 To test our assembly pipeline we assembled a previously characterized Cluster III *w*Mel
187 *Wolbachia* variant, named *w*Mel [16], which derives from the line used for the original
188 *w*Mel reference genome (GenBank: AE017196.1) [27]. The new *w*Mel genome (GenBank:
189 CP046925.1) was also circular and comparable in size, structure and number of ORFs
190 with previously published *w*Mel genomes [27,28], including the *w*Mel reference genome
191 (S4 Table). We found, however, nine differences (2 SNPs and seven indels) relative to

192 *w*Mel reference genome, which we confirmed using Sanger sequencing (S5 Table). These
193 results validate our sequencing pipeline.

194 The only difference between the genome of the over-proliferative *Wolbachia* variant in Line
195 1A and *w*MelCS_b was an amplification of the Octomom region (Fig 2A and S1 Text).
196 There were three more copies of this region, giving a genome size difference of 62,814bp.
197 The Octomom region amplification, and lack of other differences, was also confirmed by
198 the mapping of the Illumina sequencing reads of Line 1A on the genome of wMelCS_b
199 (GenBank: CP046924.1) (Fig 2B). These results show that Octomom amplification is the
200 cause of over-proliferation, consistently with previous findings with the variant *w*MelPop
201 [16,17,29]. Given the nature of the genomic change inducing the over-proliferation we
202 named this variant *w*MelPop2.

203 As shown before for *w*MelPop [17], we observed variation in the Octomom copy number in
204 *w*MelPop2-carrying flies. For further analyses of the phenotype of these variant we
205 established, through selection (as in [17]), *Drosophila* lines carrying *w*MelPop2 with
206 specific low (2-3) and high Octomom (8-9) copy number (S5 Fig).

207 Interestingly, the genome of the over-proliferative *Wolbachia* variant in line 2A only differs
208 from *w*MelCS_b by a deletion of a 20,938bp genomic fragment that includes the full-length
209 Octomom region and one of its flaking direct repeats (Fig 2A and S1 Text). Mapping the
210 Illumina sequencing reads of this variant on the genome of *w*MelCS_b (GenBank:
211 CP046924.1) also confirmed this deletion has the only difference between the two (Fig
212 2B). The absence of all Octomom genes in this line is also confirmed by qPCR (S6 Fig).
213 These results identify Octomom loss as the cause of its over-proliferative phenotype.
214 Thus, we named this variant *w*MelOctoless.

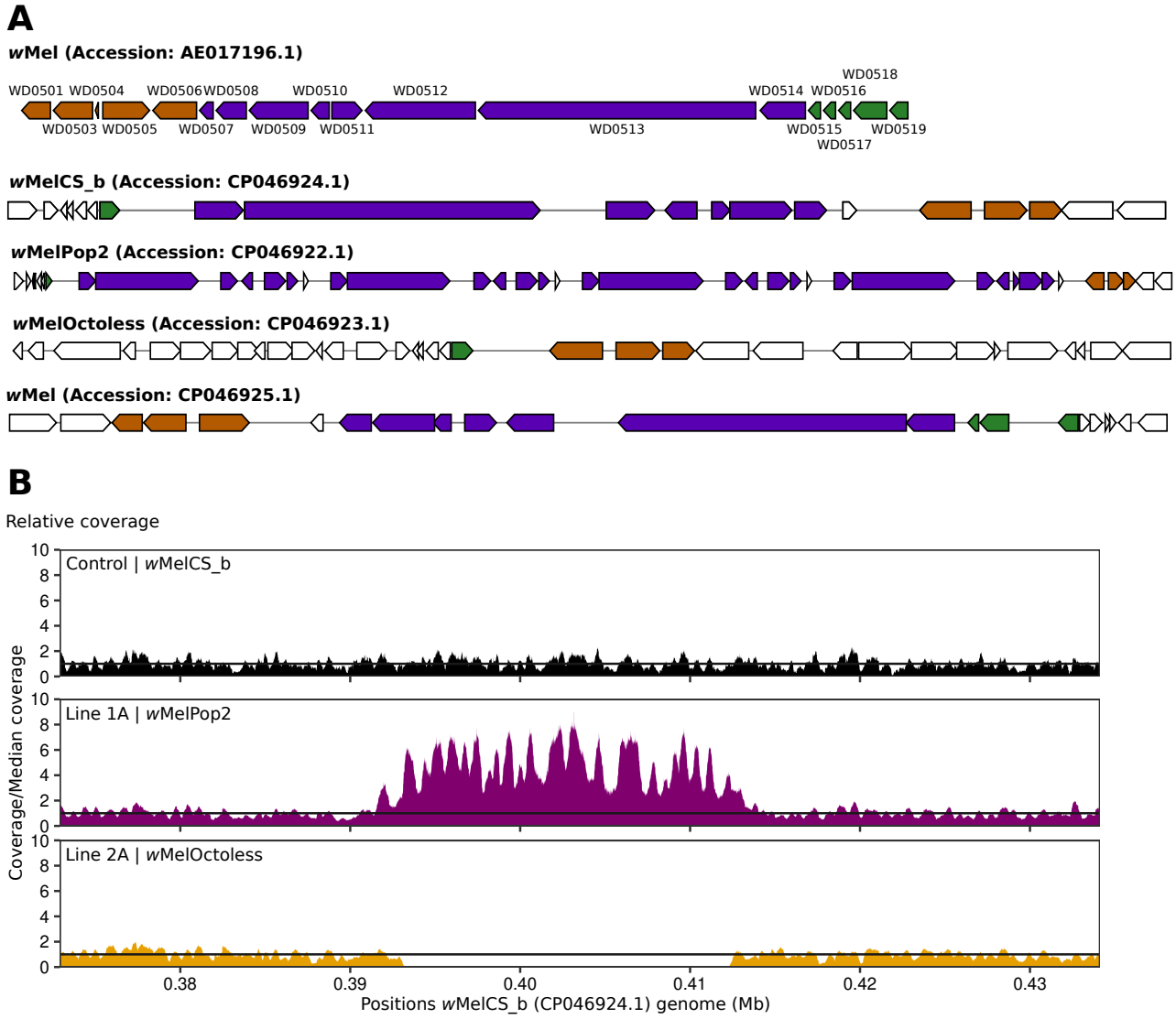

Fig 2. Both amplification and deletion of Octomom lead to *Wolbachia* over-proliferation. (A) Gene synteny across Octomom region and its flanking region. Assembled genomes of *w*MelCS_b, *w*MelOctoless and *w*MelPop2 were aligned using Mauve v2.4.0 to identify mutations causing over-proliferation. The three-fold amplification of Octomom in Line 1A (*w*MelPop2) and its deletion in Line 2A (*w*MelOctoless) were the only mutations identified when compared to *w*MelCS_b. For illustrative purposes, we used MultiGeneBlast v1.1.13 (http://multigeneblast.sourceforge.net/) to find, in Line 1A, 2A and a newly assembled *w*Mel genome, homologous proteins to those of *w*Mel locus WD0501–WD0519 (GenBank: AE017196.1). Genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline v4.10. (B) The relative coverage in a genomic region, including the Octomom region. Illumina paired-end reads were mapped to *w*MelCS_b genome (GenBank: CP046924.1). We confirmed the amplification or deletion of Octomom as the only difference between over-proliferating *Wolbachia* and *w*MelCS_b.

A second line isolated together with *w*MelOctoless, line 2B, also lost the Octomom region (S7 Fig). And this was the only observed difference with *w*MelCS_b when mapping the Illumina reads on *w*MelCS_b (S7 Fig). No differences were observed when the Illumina reads were mapped to the *w*MelOctoless genome (GenBank: CP046923.1). Since *w*MelOctoless and line 2B were identified in the same batch they may be not independent. However, and importantly, we obtained the same results with another independent over-

235 proliferative line, isolated in a different batch of treatment, line 3A (S3 and S7 Fig).
236 Mapping the Illumina sequence reads of this line identifies the loss of Octomom as the
237 only difference with *w*MelCS_b, and identifies no difference to *w*MelOctoless. We named
238 this line *w*MelOctoless2. These results further confirm that loss of the Octomom region
239 leads to an over-proliferative phenotype in *Wolbachia*.

240 We also sequenced by short and longs reads and made a hybrid assembly of *w*MelPop
241 genome and compared it to wMelPop2. We only detected and confirmed the two SNPs
242 previously identified between *w*MelCS_b and *w*MelPop (position 920,191: T in *w*MelPop
243 and C in *w*MelPop2; and position 1,005,339: A in *w*MelPop and G in *w*MelPop2 [16]). We
244 also compared the mitogenome of flies carrying *w*MelPop and *w*MelPop2 and found one
245 single substitution (position 10,793: G in wMelPop and A in wMelPop2) (Table S3). We
246 confirmed this SNP using Sanger sequencing (S2 Text).

247 In summary, we were able to identify the genomic changes associated with the new over-
248 proliferative variants and all map to loss or amplification of the Octomom region.

249

250 **Deletion and amplification of the Octomom region differently impact titres and**
251 **growth of *Wolbachia***

252 In order to characterize better the phenotypes of the new *Wolbachia* variants
253 *w*MelOctoless and *w*MelPop2 we analysed their growth, together with *w*MelCS_b and
254 *w*MelPop, in adult males kept at 18ºC, 25ºC, and 29ºC (Fig 3 and S8 Fig). These flies were
255 all reared at 25ºC and placed at the different temperatures when 0-1 days old. At this initial
256 point, at adult eclosion, there are differences in titres between lines carrying different copy
257 numbers of Octomom genomic region (S9 Fig, $p < 0.028$ for all comparisons). Flies with
258 *Wolbachia* with one copy of Octomom, *w*MelCS_b, have the lowest relative titre of
259 *Wolbachia.* Flies with variants with low amplification of the Octomom region have
260 approximately twice the titres of *Wolbachia*, while flies with variants with high copy number
261 of this region have three times more *Wolbachia* than *w*MelCS_b. Flies with *w*MelOctoless
262 have the highest titres, approximately 4-fold higher than flies carrying *w*MelCS_b.
263 Therefore, the deletion or amplification of the Octomom region impact *Wolbachia* titres in
264 adults at eclosion.

265

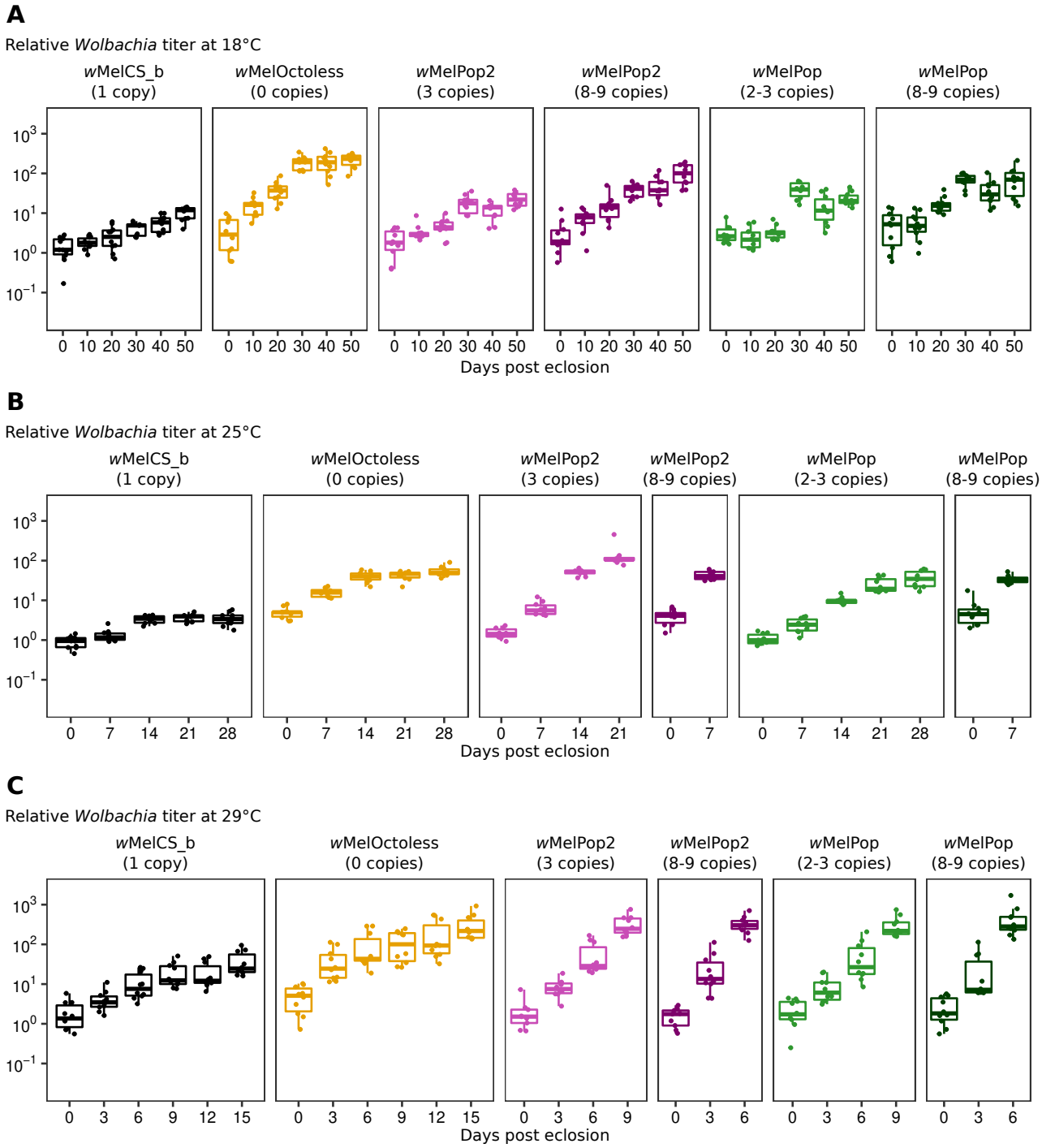

Fig 3. The amplification or deletion of Octomom increase *Wolbachia* proliferation rate. *Wolbachia* proliferation dynamics at 18ºC (A), 25ºC (B) and 29ºC (C). *D. melanogaster* males used in these experiments developed at 25ºC, were collected on the day of eclosion and aged to specific time-point at a given temperature (18ºC, 25ºC or 29ºC). Ten males were collected at each time-point for *Wolbachia* titre measurement using qPCR. *Wolbachia* titres were normalized to that of 0-1 days-old *w*MelCS_b-infected males. A replicate of the experiment is given in S8 Fig. Exponential models were used to estimate Wolbachia doubling time, and a summary of the results is given in Table 1. Both replicates were used for statistical analysis. Each dot represents relative *Wolbachia* titre of a single male.

To analyse growth during adult life we fitted an exponential model to these data, from which we also estimated doubling time of the *Wolbachia* variants, at the different temperatures (Table 1). There is a wide range of doubling times for different *Wolbachia*

279 variants at different temperatures, from approximately one day to seventeen days. A model
280 with all the data shows a complex interaction between growth rate, *Wolbachia* variant and
281 temperature (lmm , $p < 0.001$).

282

	Adults			Larvae
	18ºC	25ºC	29ºC	25ºC
Wolbachia variants	Doubling time (days) (±SE)	Doubling time (days) (±SE)	Doubling time (days) (±SE)	Doubling time (days) (±SE)
*w*MelCS_b	16.79 (14.80-19.39)	13.90 (11.49-17.59)	4.07 (3.67-4.56)	0.68 (0.60-0.79)
*w*MelOctoless	9.41 (8.75-10.18)	10.46 (9.03-12.43)	3.37 (3.09-3.71)	0.48 (0.44-0.54)
*w*MelPop2 (3 copies)	17.68 (15.49-20.59)	3.50 (3.23-3.80)	1.28 (1.20-1.38)	n.d.
*w*MelPop2 (8-9 copies)	11.86 (10.83-13.10)	2.26 (1.96-2.66)	1.07 (0.98-1.17)	0.57 (0.52-0.65)
*w*MelPop (2-3 copies)	15.44 (13.74-17.61)	5.30 (4.86-5.83)	1.38 (1.29-1.49)	n.d.
*w*MelPop (8-9 copies)	13.82 (12.43-15.56)	2.79 (2.35-3.44)	0.88 (0.82-0.95)	n.d.

283

284 Table 1 – Doubling time, in days, of *Wolbachia* variants at different temperatures or developmental stages.
285 N.d. - not determined.

286

287 A direct comparison between *w*MelOctoless with *w*MelCS_b shows that this new variant
288 replicates faster than *w*MelCS_b (lmm, $p < 0.001$), although it is a relatively small
289 difference at all temperatures (in the full model with all variants, however, the growth of
290 *w*MelOctoless and *w*MelCS_b is only statistically different at 18ºC, Table 1). Both strains
291 interact equally with temperature. Their growth rate does not significantly change between
292 18ºC and 25ºC ($p = 0.94$), but increases at 29ºC ($p < 0.001$).

293 We also compared the effect of Octomom copy number in a model taking genotype
294 (wMelCS_b, for the control and new variants generated here, or wMelPop) and Octomom
295 copy number (zero, one, low and high) as factors. At 18ºC the amplification of the
296 Octomom copy number does not lead to a statistically significant increase of growth rate,
297 compared with wMelCS_b (lmm, $p > 0.125$ for both low and high Octomom copy number
298 compared with control). At this temperature only the deletion of Octomom leads to a
299 significantly higher growth, as seen above. However, variants with Octomom amplification

300 grow more at 25ºC than at 18ºC, and grow more at 29ºC than at 25ºC ($p < 0.001$ for all
301 comparisons). At both 25ºC and 29ºC, variants with low Octomom copy numbers have a
302 higher growth rate than the variants with one copy or deletion of Octomom region ($p <$
303 0.001 for all comparisons). At these temperatures the variants with high Octomom copy
304 numbers grow faster than all the other variants ($p < 0.003$ for all comparisons). These
305 results confirm that the degree of amplification of the Octomom region controls the
306 intensity of the over-proliferation of these variants, as shown before [17]. The data also
307 demonstrate a strong interaction between temperature and the increased proliferation of
308 variants with amplification of the Octomom region. The effect of the amplification is not
309 significant at 18ºC and becomes increasingly stronger with higher temperature. On the
310 other hand, loss of Octomom leads to a smaller effect in growth, but similar at all
311 temperatures. Therefore, although both genomic mutations lead to an increase in
312 *Wolbachia* titres they have different impacts in the growth rates and their interaction with
313 temperature.

314 The statistical model taking into account the *w*MelCs_b or *w*MelPop genotype, which differ
315 only in two SNPs (see above), indicated a significant difference in growth between them,
316 at 25ºC ($p < 0.001$). This could indicate that these two SNPs also influence growth of
317 *Wolbachi*a. However, this could also be due to the fact that the copy number of the
318 Octomom region was not equally controlled in *w*MelPop and *w*MelPop2 lines during these
319 experiments. *w*MelPop2 low copy number line had 2-3 copies of Octomom, while the
320 wMelPop line had 3 copies. To test if *w*MelPop and *w*MelPop2 indeed vary in proliferation
321 rate, we repeated this experiment with a more tightly controlled Octomom copy number in
322 these two lines, at 25ºC (S10 Fig A-D). Both *w*MelPop and *w*MelPop2 carrying 3 copies of
323 Octomom grow faster than wMelCS_b (lmm, $p < 0.001$ for both) and there is no difference
324 in growth between them ($p = 0.32$). This indicates that the genetic differences between
325 these lines do not affect their growth and that they are equally influenced by Octomom
326 copy number.

327

328 **Rapid proliferation of *Wolbachia* during the host development**

329 We also analysed the growth of *w*MelCS_b, *w*MelOctoless and *w*MelPop2 (8-9 Octomom
330 copies) during host development. *D. melanogaster* develops very fast from egg to adult, in
331 only 10 days at 25ºC. We predicted that *Wolbachi*a would grow much faster during this
332 period than during adult life, in order to grow from the small population, present in the egg,
333 to the population spread throughout the adult tissues. We, therefore, estimated absolute

numbers of *Wolbachia* genome copies in individuals at the different stages of development, using qPCR and a calibration curve. Assuming one genome per *Wolbachia* cell [30], these number correspond to *Wolbachia* cells. Embryos with 0-2h had between 4,000 and 4,700 *Wolbachia* genome copies on average, with no statistically significant difference between *Wolbachia* variants, (lm, p = 0.89 for the effect of *Wolbachia* variant, Fig 4, Table 2). Interestingly, at the end of the experiment, in newly eclosed adults, and as also observed above, there are significant differences between the three variants (lm, p < 0.009 for all comparisons, Table 2, Fig 4). Also, males carry significantly less *Wolbachia* than females (p = 0.029). Adults carried from approximately 600,000 to 4,500,000 *Wolbachia* genome copies.

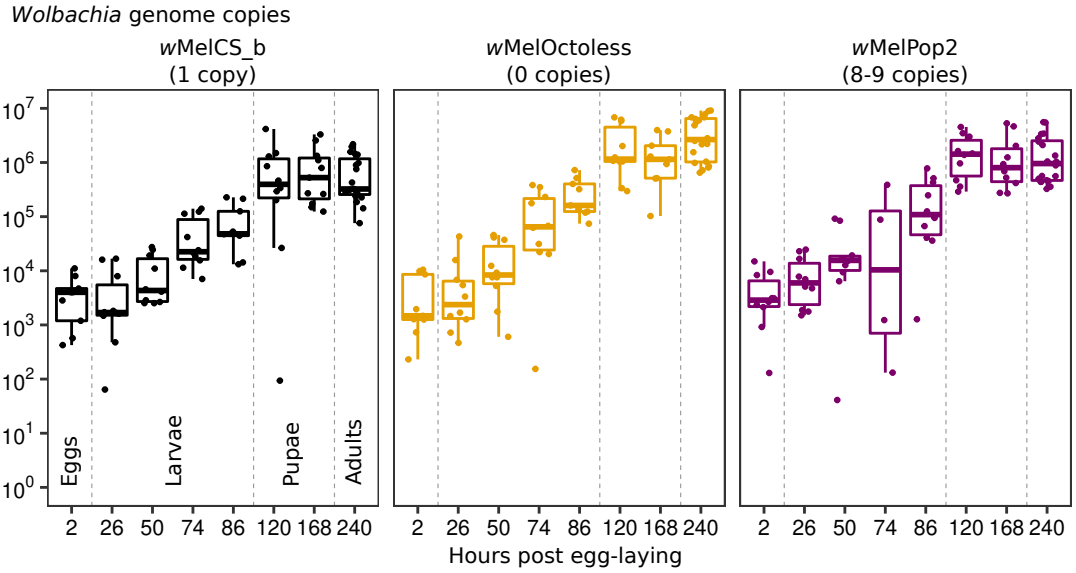

Fig 4. *Wolbachia* proliferates rapidly during larval development.
Wolbachia genome copies per individual throughout *Drosophila* development. Dots represent either data from a pool of 10 individuals (eggs and larvae) or from a single individual (pupae and adults). *Wolbachia* proliferation in the first 120 hours were analysed using a exponential model. A summary is given in Table 1. Vertical dashed lines separate developmental stages (i.e. eggs, larvae, pupae, and adults).

	Genome copy number (± SE)		
	Wolbachia variants		
	*w*MelCS_b	*w*MelOctoless	*w*MelPop2
Embryos	4,100 (±1,200)	4,000 (±1,400)	4,700 (±1,500)
Adults			
Males	619,000 (±179,000)	3,334,000 (±975,000)	918,000 (±591,000)
Females	841,000 (±245,000)	4,528,000 (±928,000)	2,439,000 (±323,000)

Table 2. *Wolbachia* genome copies in embryos and in newly eclosed adult flies. Mean (and standard error) of *Wolbachia* genome copies were estimated using qPCR.

Wolbachia growth seems to be restricted to the period between egg and white prepupae (120h), since there is no significant growth from this stage to adults (lm, $p = 0.44$). From eggs to white prepupae there is rapid exponential growth of all variants (Fig 5, Table 1). *w*MelCS_b has an estimated doubling time of approximately 16h, *w*MelPop2 of 14h, and *w*MelOctoless of 12h. These different doubling times probably explain how *Wolbachia* variants reach different amounts per individual host in adults, starting from the same estimated amount in embryos. However, in this analysis the difference between growth rates is not statistically significant ($p = 0.10$ for interaction between *Wolbachia* variants and growth). The growth rates of these variants are, therefore, very similar during this stage, and much faster than in adults. At the same temperature, we estimated doubling times in adults of *w*MelCS_b, *w*MelOctoless, and *w*MelPop2 (high-copy) to be, approximately, 13.9, 10.5, and 2.3 days, respectively. Therefore, *Wolbachia* growth at different stages of *D. melanogaster* can vary dramatically, and the different variants respond differently to host development stages.

We also asked if *Wolbachia* Octomom copy number changed in *w*MelCS_b and *w*MelPop2, throughout development, as *Wolbachia* is proliferating so fast, and found no evidence of so (lm, $p = 0.88$, S11 Fig). However, as shown before [31], during adult life there was a small increase of Octomom copy number with age, in *w*MelPop and *w*MelPop2 (an increase of 0.032 per day, lmer, $p = 0.009$, S11 Fig).

Wolbachia variants with a deletion or amplification of the Octomom region differently induce a life-shortening phenotype

The over-proliferation of *w*MelPop has been associated with a shortening of the host lifespan [16,22]. We, therefore, tested if these new over-proliferative variants also shorten

380 the lifespan of their host, at different temperatures, in males (Fig 5, S12 Fig). We also
381 performed this assay in females at 25ºC, with similar results to males at 25ºC (S12 Fig).
382 There was a significant interaction between survival, *Wolbachia* variant, and temperature
383 (Cox proportional hazard model with mixed effects (CHR), $p < 0.001$). All lines, including
384 the *Wolbachia*-free line have a shorter lifespan at 25ºC than 18ºC, and even shorter at
385 29ºC ($p < 0.001$ for all these comparisons). *w*MelCS_b did not affect the host lifespan at
386 any temperature ($p > 0.16$ for all comparisons with the line without *Wolbachia*).

387 *w*MelOctoless strongly reduces host lifespan at all tested temperatures ($p < 0.001$, each
388 comparison with *w*MelCS_b) (Fig 5, S12 Fig). This deleterious effect is stronger at 18ºC,
389 where wMelOctoless is the tested variant with the highest impact on lifespan, although
390 very similar and not statistically different from *w*MelPop2 with high Octomom copy number
391 ($p < 0.001$, for all comparisons with other lines, $p = 1$ when compared with *w*MelPop2 with
392 high Octomom copy number). At 25ºC the effect of wMelOctoless on host lifespan is
393 weaker ($p = 0.001$), and similar at 25ºC and 29ºC ($p = 0.95$). These results demonstrate
394 that the new over-proliferative *w*MelOctoless also induces a cost to the host in terms of
395 lifespan and the effect interacts with temperature, being stronger at lower temperature.

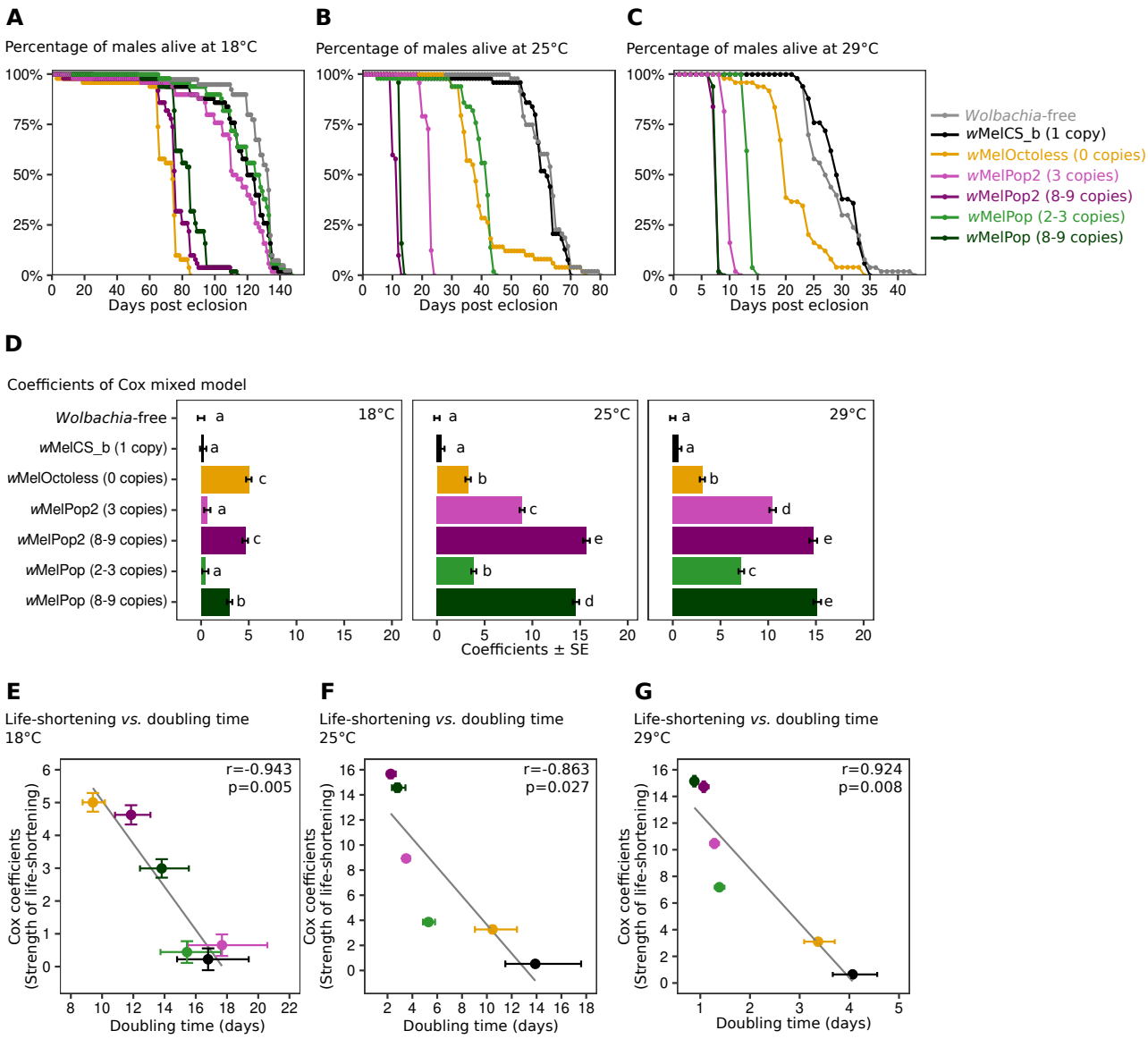

Fig 5. *w*MelOctoless and *w*MelPop2 are pathogenic.
Lifespan of males with different *Wolbachia* variants at 18ºC (A), 25ºC (B), and 29ºC (C). For survival analyses, fifty males were collected on the day of eclosion and kept in groups of 10 per vial until all flies had died. Flies were transferred to new vials every five days. A replicate of these experiments is given in S12A-C Fig. (D) Coefficients of a Cox mixed model, which represent the effect of *Wolbachia* on the lifespan of flies relative to the lifespan of *Wolbachia*-free flies. Both replicate experiments performed at each temperature were pooled for statistical comparisons. Bars represent the standard error of the coefficient, and letters statistical significant groups after *p*-value correction. A significant interaction between *Wolbachia* variant and temperature determined the lifespan ($p < 0.001$). (E-G) The correlation between the strength of life-shortening phenotype and *Wolbachia* doubling time at 18ºC (E), 25ºC (F), and 29ºC (G). The y-axis represents the strength of *Wolbachia* life-shortening phenotype (estimated using Cox mixed models) and the x-axis *Wolbachia* doubling time (in days). The Pearson correlation coefficient (*r*) and its significance (*p*) are given in each panel.

*w*MelPop2, as *w*MelPop, also shortens host lifespan (Fig 5, S12 Fig). The variants containing high copy number of Octomom shorten lifespan at all temperatures ($p < 0.001$, for each comparison with *w*MelCS_b). This effect is much stronger at 25ºC than at 18ºC ($p < 0.001$ for contrasts between both lines *w*MelCS_b), and similar at 25ºC and 29ºC ($p > 0.21$ for these contrasts). At these two higher temperatures the lines carrying the variants

with high copy number of Octomom have the shortest lifespan of all tested lines ($p < 0.001$ for all comparisons). *w*MelPop2 and *w*MelPop with low copy number of Octomom always have a weaker effect than high copy number variants ($p < 0.001$ for all these comparisons), demonstrating the effect of the degree of amplification in this phenotype. As observed with the high copy number variants their effect increases with temperature and is stronger at 25ºC than 18ºC, and stronger at 29ºC than 25ºC ($p < 0.05$ for these comparisons). In fact, they are not pathogenic at 18ºC and an effect on host lifespan is only observed at 25ºC and 29ºC. These data confirm the association of Octomom region amplification with host lifespan shortening, an increase in the severity of the phenotype with increase in the Octomom copy number, and the increase in the severity of the phenotype with temperature.

*w*MelPop2 and *w*MelPop, high and low copy number variants, had significantly different effects in several comparisons at the three tested temperatures (Fig 5). This indicated that there could be differences in this phenotype between these two lines. However, the Octomom copy number was not exactly the same between these lines. Therefore, and as done above in the analysis of proliferation, we compared the lifespan phenotype in *w*MelPop2 and *w*MelPop lines with tightly controlled Octomom copy number (S10 Fig). At 25ºC lines with both *w*MelPop and *w*MelPop2 with 3 copies of the Octomom region had a shorter lifespan than the line with *w*MelCS_b ($p < 0.001$), and no difference between them ($p = 0.29$). These results show that *w*MelPop2 and *w*MelPop have the same phenotype.

To further demonstrate that the life shortening phenotypes were due to the new *Wolbachia* variants, and not an EMS-induced mutation in the host nuclear genome, we performed reciprocal crosses between flies with *w*MelCS_b and flies carrying either *w*MelOctoless or *w*MelPop2 (carrying 3 or 8-9 copies of Octomom) and followed the survival of their female progeny at 29ºC. Females progeny from reciprocal crosses should be identical in the nuclear genome but differ in the *Wolbachia* variant that is maternally transmitted. We observed maternal segregation of the life-shortening phenotype, thus demonstrating that the *Wolbachia* variant carried by the lines is the cause of the phenotype (S13 Fig). The relative strength of the life-shortening phenotype of these female progenies matches what was observed above for the maternal lines. Moreover, all the tested lines that inherited wMelCS_b had a similar lifespan ($p > 0.78$ for all comparisons), indicating no contribution of the host genotype in this set of experiments.

The life shortening phenotype of *w*MelPop has been associated with its over proliferation since its discovery [22]. We tested if these phenotypes were correlated by taking

advantage of the data on proliferation and shortening of lifespan, at different temperatures, we collected from these set of variants. At all temperatures, we found a negative correlation between the strength of the life-shortening phenotype and *Wolbachia* doubling time (Fig 5, $|r| > 0.86$, $p < 0.03$, for all correlations). We found a much weaker or no significant correlation between the strength of the life-shortening phenotype and *Wolbachia* titres in 0-1 day-old adults (S14 Fig). These results show that over-proliferative variants shorten the host lifespan and the strength of this phenotype correlates with their proliferation rates.

Wolbachia **variants with deletion or amplification of the Octomom region provide stronger protection against DCV**

Previous studies established a link between *Wolbachia* titres and the strength of anti-viral protection [16–20]. To test if wMelOctoless and wMelPop also provide a stronger protection against viruses, we infected flies with *Drosophila* C virus (DCV), by pricking, and followed their survival for 40 days at 18ºC. All *Wolbachia* variants tested provided protection against DCV (CHR, $p < 0.001$ for all comparisons with the *Wolbachia* free line, Fig 6A and S15 Fig). Importantly, the survival rate of flies pricked with a buffer solution was not different from each other (Fig 6B, 6D and S15B Fig, $p > 0.05$ for all comparisons). *w*MelCS_b was the least protective variant, while *w*MelOctoless the one that provided the highest protection. In general the over-proliferative *Wolbachi*a variants confer stronger protection to DCV than *w*MelCS_b, although this difference is not always significant (Fig 6C, $p < 0.001$ for all comparisons, except for *w*MelPop (2-3 copies), $p = 0.10$).

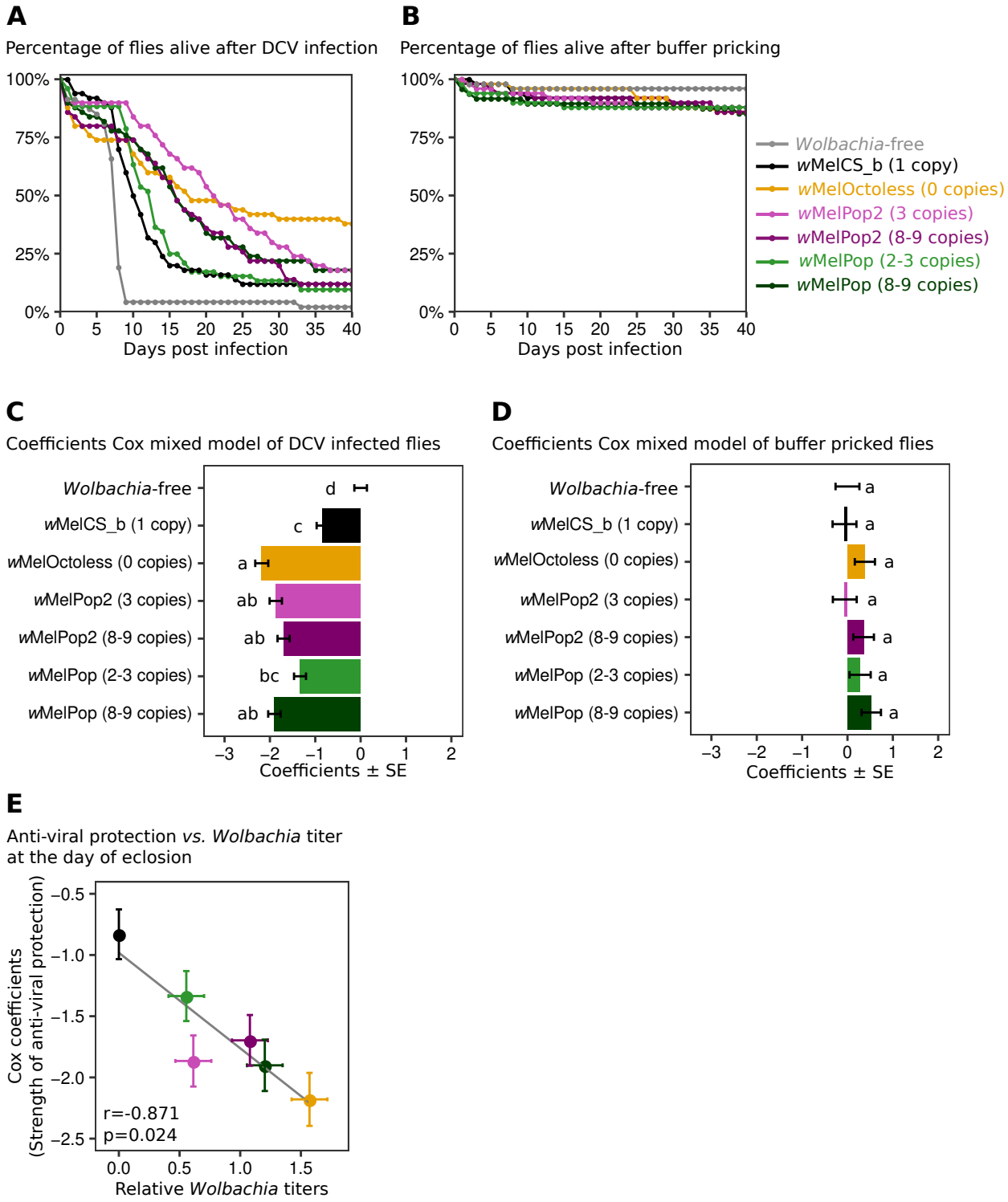

Fig 6. *w*MelOctoless and *w*MelPop2 provide strong protection against DCV.
Survival of males carrying different *Wolbachia* variants after a challenge with DCV (A) or a buffer solution (B). 50 3-5 days-old *Drosophila* males were pricked with DCV (10^9 $TCID_{50}$/ml), or a buffer solution, and survival curves were determined at 18ºC for 40 days. A replicate of the experiment is given in S15 Fig. Both survival curves of DCV-infected (C) or control-pricked flies (D) were analysed using Cox mixed models. The resulting coefficients represent the effect of *Wolbachia* infection on survival relative to the *Wolbachia*-free flies. Bars represent the standard error of the estimate, and the letters the statistical significant groups after *p*-value correction. *Wolbachia* infection improved the survival of DCV-infected flies ($p < 0.001$) but did not change the survival of buffer-pricked flies in this timeframe ($p > 0.5$). (E) Correlation between the strength of anti-viral protection (represented as the coefficients of a Cox mixed model in the y-axis) and *Wolbachia* titres at the day of eclosion, as a proxy for *Wolbachia* titer in the day of infection. The direction and strength of the correlation were estimated using Person correlation coefficient.

486 We also tested the correlation between the antiviral protection and *Wolbachia* proliferation.
487 We found no correlation at either 18ºC (p = 0.21), the temperature where the flies were
488 kept after infection, or 25ºC (p = 0.35), the temperature in which flies developed and were
489 kept until being infected with DCV (Fig S14). We also tested the correlation between the
490 antiviral strength of *Wolbachia*-induced anti-viral protection with *Wolbachia* titres in 0-1
491 day-old flies, as a proxy for *Wolbachia* titre at the day of infection, which is 3-5 day-old
492 flies. We found a significant correlation between these parameters (Fig 6F, p = 0.024, |r| =
493 0.87). Overall, the new over-proliferative variants give more protection to viruses than
494 *w*MelCS_b, and the strength of this protection is correlated with *Wolbachia* levels at the
495 time of infection.

496

497 **Discussion**

498 We developed a new forward genetic screen and identified new *Wolbachia* over-
499 proliferative variants. We characterized in detail two of these new mutants, *w*MelPop2 and
500 *w*MelOctoless, and identified the genetic bases of their over-proliferation. *w*MelPop2 had
501 an amplification of the Octomom region, which has been previously shown to lead to over-
502 proliferation in the wMelPop variant [16,17]. *w*MelOctoless, on the other hand, had a
503 deletion of this same Octomom region. These results further confirm and develop the
504 complex role of this genomic region in the control of *Wolbachia* proliferation. An extensive
505 phenotypic characterization of two of these lines showed both *Wolbachia* variants to
506 shorten the host lifespan, as well as to increase antiviral protection. Moreover, we show
507 that *Wolbachia* proliferation rate in *D. melanogaster* depends on the interaction between
508 Octomom copy number, the host developmental stage, and temperature. Our analysis also
509 suggests that the life shortening and antiviral protection phenotypes of *Wolbachia* are
510 dependent on the related, but different, properties of the endosymbiont, rate of proliferation
511 and titres at time of infection, respectively.

512

513 **An unbiased approach for genetically intractable symbionts**

514 A difficulty in studying obligate intracellular bacteria is that they are often genetically
515 intractable, mostly because of their dependency on the host cells to survive and difficulty in
516 isolating and growing them clonally. We decided to try to directly mutagenize and screen
517 *Wolbachia* in the host. The main difficulty of this approach is how to identify a new mutant
518 and link it with a phenotype in the *Wolbachia* population present within a host. We

519 calculated here that newly emerged female adults, with *w*MelCS_b, carry approximately
520 840,000 *Wolbachia* genome copies, probably corresponding to the same number of
521 *Wolbachia* cells [30]. Since EMS induces random mutations, we expected mosaicism in
522 the *Wolbachia* population at the individual fly level. Each new mutant, when generated,
523 would be a unique cell within these 840,000 other cells. Since the *Wolbachia* phenotypes
524 are normally measured at the individual host levels (e.g. *Wolbachia* titres, antiviral
525 protection), the properties of individual or small numbers of mutant *Wolbachia* could be
526 diluted and unmeasurable.

527 We hypothesized, however, that over-proliferating *Wolbachia* cells could overtake the
528 population and that the resulting higher titres could be detectable. Indeed, fast proliferative
529 *Wolbachia* can be selected at the level of a single host [31]. To increase the probability of
530 isolating rare over-proliferating *Wolbachia* variants, we also relied on the bottleneck
531 imposed in the vertical transmission of *Wolbachia*. We calculated here that single embryos
532 carry approximately 4,000 *Wolbachia* genomes, which is consistent with previous
533 estimates [32]. Moreover, we treated flies with tetracycline to further reduce this
534 population, and increase drift. By screening at the immediate progeny (F1) of EMS-treated
535 females or three generations later (F4) we were able to select new over-proliferative
536 *Wolbachia* mutants.

537

538 **Genetic bases of *Wolbachia* over-proliferation**

539 After discarding the possibility that mutations in the host nuclear or mitochondrial genomes
540 were the cause of *Wolbachia* over-proliferation, we performed *de novo* assembly of the
541 ancestral, *w*MelCS_b, and the new mutant variants, *w*MelPop2 and *w*MelOctoless. The
542 assemblies generated complete full chromosomes of these *Wolbachia*. *w*Mel and
543 *w*MelPop were also assembled. This allowed us to identify single nucleotide differences
544 and structural differences between these genomes [33]. To validate our pipeline we
545 compared our *w*Mel genome assembly to the reference *w*Mel genome. We identified only
546 seven indels and two SNPs, which we confirmed to be present in our line, by Sanger
547 sequencing. Our assembly results also allowed us to confirm two previously identified
548 SNPs between *w*MelCS_b (but also *w*MelOctoless and *w*MelPop2) and *w*MelPop.
549 Additionally, our assembly provides a significant improvement over the previous *w*MelPop
550 genome [26].

551 The only differences between the new over-proliferative variants and *w*MelCS_b were
552 structural differences in the Octomom region. *w*MelPop2 has an amplification of this

553 region. The assembly confirms that the Octomom region is amplified in tandem [17], and
554 that all copies are located in the *Wolbachia* genome. We had shown before that
555 amplification of the Octomom region and the degree of this amplification determined
556 *w*MelPop over-proliferative phenotype [16,17]. If the Octomom region copy number in
557 *w*MelPop reverted to one, the variant became phenotypically identical to *w*MelCS_b [17].
558 We now show that if we start from *w*MelCS_b with one copy of Octomom, an increase in
559 the copy number of this genomic region leads to an over-proliferative phenotype.
560 Moreover, *w*MelPop and *w*MelPop2 variants, carrying the exact same copy number of
561 Octomom, have identical phenotypes. These results further confirm the role of
562 amplification of Octomom region in the over-proliferation of *Wolbachia*.

563 *w*MelOctoless has a deletion of the entire Octomom region. The deletion leaves behind
564 one of the direct repeats flanking the Octomom region [16], suggesting that excision might
565 be mediated by recombination. The causal link between deletion of this region and over-
566 proliferation is further supported by an independent over-proliferative variant isolated in the
567 screen, *w*MelOctoless2. This *Wolbachia* also has a deletion of Octomom as the only
568 difference with *w*MelCS_b. These data show that deletion of the Octomom region leads to
569 an over-proliferative phenotype in *Wolbachia*. Thus, we identified the second known
570 *Wolbachia* mutation with a clear link between genotype and phenotype.

571 The mutations identified in the new variants are deletions and amplifications. We did not
572 detect any new SNPs in these variants. However, we expected EMS to mainly induce
573 single nucleotide mutations. It is possible that the deletion or amplification of the Octomom
574 region in these over-proliferative variants were independent of the EMS treatment. For
575 instance, loss of the Octomom region has been twice reported, in cell culture [26,34],
576 suggesting that it can occur spontaneously. However, chemical mutagens such as EMS
577 can activate DNA damage response and transposable elements [35], and some genes of
578 the Octomom region and its flaking genes are predicted to be potentially involved in
579 transposition and DNA repair [16]. Therefore, we cannot rule out that the EMS treatment
580 induced the mutations in this genomic region.

581

582 **Opposing mutations lead to a similar *Wolbachia* over-proliferative phenotype**

583 Both the deletion or the amplification of the Octomom region causing an over-proliferative
584 phenotype seems to be a paradox. The resolution of this paradox and the mechanisms
585 leading to these phenotypes will rely on the functional characterization of genes in the

586 Octomom region. These genes may be involved in interaction with the host, transcriptional
587 regulation or DNA repair [16].

588 One possibility is that the amplification of the Octomom region and over-expression of
589 particular genes in this region lead, mechanistically, to the same result as the absence of
590 the genes. There are many examples of over-expression of a gene leading to a dominant
591 negative phenotype. For instance, both over-expression or loss of a protein forming a
592 gradient, abolish the gradient [36]. Also, over-expression of a member of protein complex
593 may lead to loss of stoichiometry and therefore loss of functional complexes [37]. Another
594 possibility is that the phenotypes are mediated by loss and amplification of different genes
595 in the Octomom region. The second hypothesis is supported by the fact that the two
596 mutant variants have similar but not identical phenotypes, and interact differently with
597 temperature. Furthermore, *w*MelPop and *w*MelPop2 have a higher rate of proliferation
598 than *w*MelOctoless at 25ºC and 29ºC. Therefore, their phenotype is stronger than the
599 phenotype associated with the Octomom region loss-of-function.

600 Although complex, these results help to explain some of the data from an over-proliferative
601 variant trans-infected in *Aedes aegypti*. The *D. melanogaster* variant *w*MelPop was
602 transinfected into *Aedes albopictus* cells, and then transinfected into *A. aegypti*. In the
603 process of cell culture adaptation the Octomom region, which was amplified in *w*MelPop,
604 was deleted [26]. The *w*MelPop-PGYP variant in *A. aegypti* lacks, therefore, the Octomom
605 region. Nonetheless, this variant over-proliferates and is highly pathogenic in *A. aegypti*
606 [38]. If one assumed the same genetic basis for the pathogenicity of *w*MelPop in *D.*
607 *melanogaster* and *w*MelPop-PGYP in *A. aegypti*, one might conclude that the Octomom
608 region was not related with these phenotypes [26]. However, similarly to
609 wMelPop/wMelPop-PGYP variants our results show that both amplification and deletion of
610 the Octomom region lead to increased *Wolbachia* pathogenicity. Thus, deletion of the
611 Octomom region in *w*MelPop-PGYP is probably why it is also pathogenic.

612 The Octomom region has the properties of a genomic island and it is not part of the
613 *Wolbachia* core genome, since many *Wolbachia* strains lack this region [16,39]. Although
614 the *w*Mel strain can lose this region and be viable in several scenarios (here and [26,34],
615 natural variants of *w*Mel without Octomom are not known [40]. The over-proliferative
616 phenotype, and the associated shortening of host lifespan, may lead to a fitness
617 disadvantage to a host harbouring such mutant. This in turn may lead to loss of these
618 variants from the host population. Therefore, this genomic region, absent in many other

619 strains of *Wolbachia*, and through its integration in the regulation of proliferation, became
620 addictive to the *w*Mel strain.

621

622 ***Wolbachia* proliferation, pathogenicity, and antiviral protection**

623 We found a complex interaction between temperature and proliferation of the different
624 *Wolbachia* variants in adults. *w*MelOctoless proliferates faster than *w*MelCS_b, to a similar
625 extent, at all temperatures. *w*MelPop and *w*MelPop2, however, strongly interact with
626 temperature and proliferate much faster at higher temperatures. We also confirmed here
627 that the Octomom copy number in these variants modulates the proliferation rate [17], and
628 interacts with temperature.

629 Throughout the range of tested temperatures, the different variants have very different
630 proliferation rates. The titres of *w*MelPop2 at 25ºC, for instance, double every 2 days, while
631 wMelCS_b titres only double every 14 days. Interestingly the proliferation rates of
632 *w*MelCS_b, *w*MelOctoless, and *w*MelPop2 during larval development were much faster
633 and very similar between them. During development, at 25ºC, their doubling time varies
634 between 12 to 16 hours. Thus, rapid exponential proliferation of *Wolbachia* occurs
635 simultaneously with the fast growth of the host in the larval stages. These results show
636 that although *w*MelCS_b has a relatively low proliferation rate in adult flies, it is capable of
637 a very fast proliferation. Moreover, these data show that *Wolbachia*, somehow, coordinates
638 its proliferation with host developmental stage. The Octomom region influences this
639 coordination. While in *w*MelCS_b the proliferation rate is severely reduced in the adult
640 stages, in *w*MelOctoless is less reduced, and in wMelPop and wMelPop2 is still kept very
641 high. The over-proliferative phenotypes of these variants, in particular of *w*MelPop and
642 *w*MelPop2, can be interpreted as an inability of the *Wolbachia* variants to properly respond
643 to the host developmental stage or its own population density within the host.

644 The new over-proliferative variants shorten the lifespan of *D. melanogaster*, as *w*MelPop
645 does. Furthermore, we showed this phenotype to result from the interaction of *Wolbachia*
646 genotype and temperature. *w*MelOctoless had a similar life shortening phenotype at all
647 temperatures, although it was stronger at 18ºC. *w*MelPop and *w*MelPop2 responded
648 strongly to temperature, being much more costly at higher temperatures, as shown before
649 for *w*MelPop [17,22,41]. Octomom copy number also influenced this interaction. While low
650 copy number variants had no phenotype at 18ºC, the high copy number *w*MelPop and
651 *w*MelPop2 are pathogenic at this temperature. Therefore these variants can also be
652 pathogenic at 18ºC, contrary to previous data [29,41,42]. Interestingly, we find at all

653 temperatures a significant correlation between the proliferation rate of the *Wolbachia*
654 variants and the life shortening phenotype. The faster the variants proliferate the shorter
655 the host lifespan.

656 These over-proliferative variants also increased antiviral resistance, with *w*MelOctoless
657 conferring the strongest protection. This phenotype correlated poorly with proliferation
658 rates at 25ºC or 18ºC, the temperature before and after infection with DCV, respectively.
659 However, the strength of the antiviral resistance correlated with the titres of *Wolbachia*
660 near the day of infection. Thus, the cost of harbouring *Wolbachia* in terms of lifespan, and
661 the benefit of the antiviral protection, correlate with related but different parameters.
662 Therefore, it may be possible to select for highly protective *Wolbachia* variants without
663 necessarily having a high cost to the host. These would be *Wolbachia* variants with high
664 titres but low proliferation in adults. Such variants would be particularly useful in the use of
665 *Wolbachia*-transinfected mosquito to prevent arboviruses transmission.

666

667 In summary, our results show the feasibility of forward genetic screens to study *Wolbachia*
668 biology. Similar strategies may be used in the future to study other aspects of *Wolbachia*-
669 host interactions or the biology of other genetically intractable endosymbionts. The new
670 over-proliferative variant *w*MelPop2 confirms the causal link between amplification of the
671 Octomom region and increase proliferation. Whereas the new loss-of-function mutant
672 *w*MelOctoless reveals that this region is also required to control *Wolbachia* proliferation.

673

674 **Materials and Methods**

675 **Fly genotypes, infection status, and maintenance**

676 Flies were reared on fly food, supplemented with live yeast, at 25ºC, 70% humidity. Fly
677 food was composed of molasses (37.5g/L), sugar (62.5g/L), cornflour (58.3g/L), yeast
678 extract (16.7g/L), and agar (8.3g/L) in distilled water. The mixture was sterilized by
679 autoclaving and cooled to 45ºC. For each litre of food we added 29.2 mL of a solution with
680 100g of methylparaben and 0.2g of Carbendazim for 1L absolute ethanol.

681 All fly stocks used had the Drosdel w^{1118} isogenic background [16,43].

682 The bacterial community associated with the fly stocks was homogenized as in Pais *et al.*
683 [44], with minor modifications. Briefly, we collected eggs for 6 hours in fresh agar plates
684 supplemented with live yeast and sterilized the eggs surface by consecutive washes on

685 2.1% sodium hypochlorite (NaOCl) solution (10 minutes), 70% ethanol (5 minutes) and
686 sterile water (5 minutes). Next, we transferred axenic eggs to sterile fly food supplemented
687 with 40µL of 1:1 overnight culture of *Acectobater* OTU 2753 and *Lactobacillus* OTU 1865
688 [44]. We confirmed the presence of these bacterial species by squashing five females
689 aged 3–6 days in sterile 1x PBS, plating 30µL of the lysate in mannitol plates, incubate
690 them at 25ºC for 72h, and identify bacteria by colony morphology.

691

692 **Selection of *D. melanogaster* lines carrying *Wolbachia* with specific Octomom copy**
693 **number**

694 To select for flies carrying *w*MelPop and *w*MelPop2 with a desired Octomom copy number,
695 we proceeded as in Chrostek and Teixeira [17], with minor modifications. Briefly, we
696 allowed 5–20 virgin females to cross with 2–3 *Wolbachia*-uninfected males of the Drosdel
697 w^{1118} isogenic background in individual vials, and lay eggs for 3-4 days. Females were
698 then collected in individual tubes for DNA extraction and Octomom copy number
699 determination by qPCR.

700

701 **Determination of time for *Wolbachia* titres recovery**

702 Flies with *w*MelCS_b developed in fly food supplemented with tetracycline at the
703 concentrations 1.5625µg/ml, 3.125µg/ml, 6.25µg/ml, 12.5µg/ml, 25µg/ml, and 50µg/ml.
704 Three isofemale lines were established from each dose. In the F1, we randomly selected
705 four virgin females for egg-laying and *Wolbachia* titre measurement using qPCR. We set
706 up the next four generations using the progeny of a female with the median *Wolbachia*
707 titres.

708

709 **Forward genetic screen**

710 We attempted to mutagenize *Wolbachia in vivo* by feeding its host with the mutagen EMS.
711 DrosDel w^{1118} isogenic flies carrying *w*MelCS_b were first raised in standard fly food or fly
712 food supplemented with tetracycline (from 1.5625 to 12.5µg/ml). Virgin females were
713 collected, starved for 6h, and then fed on a 1% sucrose solution with EMS concentrations
714 ranging from 10 to 8,000mM. Control flies fed on sucrose solution only. A dye was added
715 to the feeding solution to confirm intake and feeding proceeded for 13h (overnight).

716 EMS-fed females (G0), and control females, were mated individually with 2–3 *Wolbachia*-
717 free Drosdel w^{1118} isogenic males, egg-laying was allowed for 3–4 days, and parents
718 discarded. From the F1 progeny we collected virgin females, mated them individually with
719 2–3 *Wolbachia*-free Drosdel w^{1118} isogenic males, and allowed egg laying for 3-4 days.
720 These females were collected when 10 days old, and *Wolbachia* titres determined by
721 qPCR. We followed the progeny of F1 females showing 50% or higher increase in
722 *Wolbachia* titres relative to control flies in the same conditions. We have also transferred
723 the progeny of these F1 for three more generations, without selection, and repeated the
724 determination of *Wolbachia* titres in F4 females. In the same batch of experiments we may
725 have tested more than one F1 or F4 progeny from each G0 female. Hence, over-
726 proliferative *Wolbachia* variants isolated in the same batch of treated females may be a
727 result of a single event in the G0 female.

728

729 **Real time quantitative PCR**

730 DNA extraction for qPCR was performed as described before [17].

731 The qPCR reactions were performed in the QuantStudioTM 7 Flex (Applied Biosystems).
732 The reaction mix and PCR program used were described before [16]. The specificity of the
733 amplicons were confirmed by analysing the melting curve profile.

734 Relative levels of the target genes was determined using the Pfaffl method [45]. To
735 quantify relative *Wolbachia* titres were performed using *Drosophila RpL32* gene as
736 calibrator, and *Wolbachia wsp* as the target gene. To determine the copy number of the
737 Octomom region, *Wolbachia wsp* gene was used as the calibrator and *WD0513* used as
738 the target gene. For determination of copy number of other Octomom region genes, or
739 control genes, *wsp* was also used as a calibrator.

740 For absolute quantification of *Wolbachia* genome copies the full-length *Wolbachia wsp*
741 gene was cloned into a pMT/V5 *Drosophila* expression vector (Invitrogen). The plasmid
742 was amplified in *Escherichia coli* strain DH5-α, purified using midiprep (QIAGEN) and its
743 concentration determined using Qubit® 2.0 (Thermo Fisher Scientific). Standard curves of
744 1:10 serial dilutions were run to calibrate the assay each time.

745

746 **Determination of lifespans**

747 For each replicate a total of 50 males or 50 females were collected on the day of eclosion.
748 Flies (10 per vials) were then incubated at 18ºC, 25ºC or 29ºC, being transferred to new
749 fresh vials every four (females) or five (males) days. The number of dead flies was
750 recorded daily until all the flies had died. Censored observations (i.e. flies lost or trapped in
751 the vial plug) were recorded and taken into account during data analysis.

752

753 **Protection against *Drosophila* C Virus**

754 Viral protection assays were blinded (i.e. virus and buffer pricking occurred without
755 previous knowledge of their *Wolbachia* status). We produced and titrated the virus solution
756 as described in Teixeira *et al.* [8]. We infected 50 3-5 days old males by dipping insect
757 needles (Austerlitz Insect Pins) into a virus solution (DCV at 10^9 $TCID_{50}$/ml in 50mM Tris-
758 HCl, pH 7.5) and pricking flies anaesthetized under CO2 in the thorax. An equal number of
759 males were pricked with a buffer solution (50mM Tris-HCl, pH 7.5) and served as controls.
760 After pricking, flies were incubated in groups of 10 individuals per vial, and kept at 18ºC.
761 Survival was followed as above.

762

763 ***Wolbachia* proliferation during development and in adults**

764 To determine *Wolbachia* growth during development flies carrying *w*MelCS_b,
765 *w*MelOctoless, and wMelPop2 laid eggs for 2 hours in apple juice agar plates
766 supplemented with live yeast. Eggs were transferred to fly food-containing bottles and
767 allowed to develop at 25ºC. For *Wolbachia* titre assessment we sampled eggs (2 hours),
768 L1 larvae (24 hours post-egg-laying), newly moulted L2 larvae (48 hours), L3 larvae (72
769 and 84 hours), white prepupae pupae (120 hours), P8 staged pupae (168 hours), and
770 newly eclosed adult males and females (240 hours). Ten samples per time point were
771 analysed. Samples included ten individuals each for eggs and larvae and one individual
772 each for pupae and adults. Except for adults who were collected within 24 hours post-
773 emergence, all samples were collected within two hours interval.

774 For assessment of titres dynamics in adult flies, newly eclosed males, raised at 25ºC, were
775 incubated at 18ºC, 25ºC, and 29ºC. Flies were collected every three (29ºC), seven (25ºC)
776 or ten days (18ºC) for *Wolbachia* titre measurement. Ten individuals were processed for
777 each time point, and the experiment was performed twice. Each sample consisted of a
778 single fly.

779

780 ***Wolbachia* genomes sequencing and quality control**

781 For *Wolbachia* genomic sequencing (Illumina and Oxford Nanopore) we enriched the
782 sample for *Wolbachia* cells before DNA purification. To this end, approximately 500 10-
783 days old flies were squashed for 5 minutes in 10ml Schneider's Insect Medium (Thermo
784 Fisher Scientific) using glass beads. Next, we pelleted host debris by centrifugation at
785 1,000g for 5 min and filtered the supernatant solution thought a 5µm pore. Bacterial cells
786 were pelleted by centrifugation at 13,000rpm for 15 minutes, and DNA was extracted. All
787 centrifugations were carried out at 4ºC. DNA was extracted with a phenol-chloroform
788 isolation protocol and resuspended in 10mM Tris-HCl (pH 8).

789 Sequencing was performed at the Genomics Facility at the Instituto Gulbenkian de
790 Ciência, (Portugal). Both Illumina and Oxford Nanopore sequencing was done on genomic
791 DNA extracted from the same biological material. Illumina 300bp paired-end libraries were
792 prepared using the Pico Nextera kit according to the manufacturer's instructions and
793 sequenced by the MiSeq sequencing. Data quality was assessed via FastQC v.0.11.5 [46]
794 and reads were trimmed using Trimmomatic v.0.36 [47]. Genomic samples for Oxford
795 Nanopore sequencing were processed with minimal shearing to maximize the size of the
796 libraries. After ligation (kit SQK-LSK108), libraries were sequenced in MinION Mk1b
797 portable sequencing device using SpotON flow cell (R9.4.1). The status of the sequencing
798 pores was monitored using MinKNOW (v2.0.1). Sequencing lasted for up to 48 hours.
799 Albacore (v2.3.1) and Porechop (v0.2.2) were used for base-calling and read trimming,
800 respectively.

801

802 **Genome assembly**

803 Quality reads matching *Drosophila* genome (BioProject: PRJNA13812) were removed
804 from the datasets through a custom Python script before proceeding with *Wolbachia*
805 genome assembly. Illumina and Oxford Nanopore reads were mapped to the fly genome
806 using BWA mem v0.7.12-r1039 [48] and minimap2 v2.17-r941 [49] respectively. We used
807 Unicycler v0.4.8-beta [50] assembly pipeline on the remaining reads in order to assemble
808 the *Wolbachia* genomes. Briefly, Unicycler uses Illumina data to produce a repeats-limited
809 image graph using Spades v3.9.0 [51], which was further refined through Bandage v0.8.1
810 [52]. Both small short nodes and nodes with no homology with *w*Mel genome
811 (AE017196.1) upon blastn v2.8.1+ [53] search were removed. Next, repeats were resolved

812 by bridging Spades' assemblies with Oxford Nanopore long reads. The resulting draft
813 assemblies were polished using Racon v1.3.1 [54] and Pilon v1.23 [55] and rotated so that
814 genomes begin at the *dna*A gene.

815 We further refined our genome assemblies using a two-stepped pipeline. First, we mapped
816 Illumina reads to the corresponded draft genomes to identify mismatches, which were later
817 corrected via Sanger sequencing. Next, we improved genome assemblies by aligning
818 them using Mauve v2.4.0 [56], and the predicted differences were also confirmed by
819 Sanger sequencing. Only two predicted SNPs which are unique to *w*MelPop were real.

820

821 **Read mapping and mutation calling**

822 We identified mutations relative to a genome following a previously published pipeline [57].
823 It consisted of mapping quality checked reads to a reference genome using BWA mem
824 v0.7.12-r1039 algorithm [48] and saving the output as Sequence Alignment/Map file format
825 (SAM). After conversion to the Binary Alignment/Map format (BAM), the file was sorted,
826 duplicates removed and indexed using SAMtools v0.1.19 [58]. Next, we generate mpileup
827 files, also using SAMtools (option '-d 1,000,000'), after which was converted to Variant Call
828 Format (VCF) files using BCFtools v1.9-209. We visually confirmed all inferred mutations
829 in IGV v2.4.2 [59]. We did not consider mutations associated with homopolymer regions or
830 in regions with low coverage (<10X). The set of mutations were compared between
831 *Wolbachia* variants using custom Python and R scripts.

832 To compare the set of mutations in the mitochondria of flies infected with different
833 *Wolbachia* variant, we mapped Illumina reads to the *D. melanogaster* Release 6 genome
834 sequence (KJ947872.2:1–14,000) and proceed as previously. Mutations following the
835 criteria previously described were also compared by using custom scripts.

836

837 **Statistical analysis**

838 All the statistical comparisons were performed in R v4.0.0 [60].

839 To compare *Wolbachia* titres across multiple groups, we used log linear models (LMM) or
840 generalized linear mixed models assuming Gamma errors (GLMM). The effect of EMS on
841 *Wolbachia* titre was tested using non-linear regression. We estimated the doubling time of
842 *Wolbachia* variants using the equation $\log(2)/\beta$, with β being the coefficients of a log linear
843 model.

The lifespan datasets and survival curves after challenge with DCV were analysed with mixed effect Cox models [61].

The significance of correlations were tested using Pearson's correlation coefficient.

In multiple comparisons were necessary, the p-values were adjusted as proposed by Holm [62]. When multivariate techniques were applied, all the relevant covariates were included in the model, and the final model was selected as proposed by Burnham & Anderson [63].

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875 **References**

876 1. Moran NA, McCutcheon JP, Nakabachi A. Genomics and Evolution of Heritable
877 Bacterial Symbionts. Annual review of genetics. 2008;42: 165–190.
878 doi:10.1146/annurev.genet.41.110306.130119

879 2. Jaenike J. Coupled population dynamics of endosymbionts within and between hosts.
880 Oikos. 2009;118: 353–362.

881 3. López-Madrigal S, Duarte EH. Titer regulation in arthropod-Wolbachia symbioses.
882 FEMS microbiology letters. 2019;366: 118–9. doi:10.1093/femsle/fnz232

883 4. Zug R, Hammerstein P. Still a host of hosts for wolbachia: analysis of recent data
884 suggests that 40% of terrestrial arthropod species are infected. PLoS ONE. 2012;7:
885 e38544. doi:10.1371/journal.pone.0038544

886 5. Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate
887 biology. Nature reviews Microbiology. 2008;6: 741–751. doi:10.1038/nrmicro1969

888 6. Hosokawa T, Koga R, Kikuchi Y, Meng X-Y, Fukatsu T. Wolbachia as a bacteriocyte-
889 associated nutritional mutualist. Proceedings of the National Academy of Sciences of
890 the United States of America. 2010;107: 769–774. doi:10.1073/pnas.0911476107

891 7. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. Wolbachia and virus protection in
892 insects - Supp. Science (New York, NY). 2008;322: 702. doi:10.1126/science.1162418

893 8. Teixeira L, Ferreira A, Ashburner M. The bacterial symbiont Wolbachia induces
894 resistance to RNA viral infections in Drosophila melanogaster. PLoS biology. 2008;6:
895 e1000002. doi:10.1371/journal.pbio.1000002

896 9. Ross PA, Turelli M, Hoffmann AA. Evolutionary Ecology of Wolbachia Releases for
897 Disease Control. Annual review of genetics. 2019;53: 93–116. doi:10.1146/annurev-
898 genet-112618-043609

899 10. Aliota MT, Walker EC, Yepes AU, Velez ID, Christensen BM, Osorio JE. The w Mel
900 Strain of Wolbachia Reduces Transmission of Chikungunya Virus in Aedes aegypti.
901 PLoS Neglected Tropical Diseases. 2016;10: e0004677.
902 doi:10.1371/journal.pntd.0004677

903 11. Dutra HLC, Rocha MN, Dias FBS, Mansur SB, Caragata EP, Moreira LA. Wolbachia
904 Blocks Currently Circulating Zika Virus Isolates in Brazilian Aedes aegypti
905 Mosquitoes. Cell host & microbe. 2016. doi:10.1016/j.chom.2016.04.021

906 12. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A
907 Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and
908 Plasmodium. Cell. 2009;139: 1268–1278. doi:10.1016/j.cell.2009.11.042

909 13. van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, Day A, et al.
910 Impact of Wolbachia on Infection with Chikungunya and Yellow Fever Viruses in the
911 Mosquito Vector Aedes aegypti. PLoS Neglected Tropical Diseases. 2012;6: e1892.
912 doi:10.1371/journal.pntd.0001892

913 14. Nazni WA, Hoffmann AA, NoorAfizah A, Cheong YL, Mancini MV, Golding N, et al.
914 Establishment of Wolbachia Strain wAlbB in Malaysian Populations of Aedes aegypti
915 for Dengue Control. CURBIO. 2019;0. doi:10.1016/j.cub.2019.11.007

916 15. O'Neill SL, Ryan PA, Turley AP, Wilson G, Retzki K, Iturbe-Ormaetxe I, et al. Scaled
917 deployment of *Wolbachia* to protect the community from *Aedes* transmitted
918 arboviruses. Gates Open Research. 2018;2: 36. doi:10.12688/gatesopenres.12844.1

919 16. Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, Jiggins FM, et al.
920 Wolbachia Variants Induce Differential Protection to Viruses in Drosophila
921 melanogaster: A Phenotypic and Phylogenomic Analysis. PLoS genetics. 2013;9:
922 e1003896. doi:10.1371/journal.pgen.1003896

923 17. Chrostek E, Teixeira L. Mutualism breakdown by amplification of Wolbachia genes.
924 PLOS Biol. 2015;13: e1002065. doi:10.1371/journal.pbio.1002065

925 18. Lu P, Bian G, Pan X, Xi Z. Wolbachia Induces Density-Dependent Inhibition to
926 Dengue Virus in Mosquito Cells. PLoS Neglected Tropical Diseases. 2012;6: e1754.
927 doi:10.1371/journal.pntd.0001754

928 19. Osborne SE, Leong YS, O'Neill SL, Johnson KN. Variation in antiviral protection
929 mediated by different Wolbachia strains in Drosophila simulans. PLoS pathogens.
930 2009;5: e1000656. doi:10.1371/journal.ppat.1000656

931 20. Osborne SE, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL, Johnson KN. Antiviral
932 protection and the importance of Wolbachia density and tissue tropism in Drosophila
933 simulans. Applied and environmental microbiology. 2012;78: 6922–6929.
934 doi:10.1128/AEM.01727-12

935 21. McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang Y-F, et al. Stable
936 Introduction of a Life-Shortening Wolbachia Infection into the Mosquito Aedes aegypti
937 - Supp Material. Science (New York, NY). 2009;323: 141–144.
938 doi:10.1126/science.1165326

939 22. Min KT, Benzer S. Wolbachia, normally a symbiont of Drosophila, can be virulent,
940 causing degeneration and early death. Proceedings of the National Academy of
941 Sciences of the United States of America. 1997;94: 10792–10796.

942 23. Ant TH, Sinkins SP. A Wolbachia triple-strain infection generates self-incompatibility in
943 Aedes albopictus and transmission instability in Aedes aegypti. Parasites & Vectors.
944 2018;11: 1–7.

945 24. McClure EE, Chávez ASO, Shaw DK, Carlyon JA, Ganta RR, Noh SM, et al.
946 Engineering of obligate intracellular bacteria: progress, challenges and paradigms.
947 Nature reviews Microbiology. 2017;984: 249. doi:10.1038/nrmicro.2017.59

948 25. Ashburner M, Golic KG, Hawley RS. Drosophila : a laboratory handbook. Cold Spring
949 Harbor, N.Y. : Cold Spring Harbor Laboratory Press; 2005. Available:
950 http://lccn.loc.gov/2004019474

951 26. Woolfit M, Iturbe-Ormaetxe I, Brownlie JC, Walker T, Riegler M, Seleznev A, et al.
952 Genomic evolution of the pathogenic Wolbachia strain, wMelPop. Genome biology
953 and evolution. 2013;5: 2189–2204. doi:10.1093/gbe/evt169

27. Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, et al. Phylogenomics of the reproductive parasite Wolbachia pipientis wMel: a streamlined genome overrun by mobile genetic elements. PLoS biology. 2004;2: E69. doi:10.1371/journal.pbio.0020069

28. Basting PJ, Bergman CM. Complete Genome Assemblies for Three Variants of the Wolbachia Endosymbiont of Drosophila melanogaster. Microbiol Res Announc. 2019;8: 741.

29. Chrostek E, Teixeira L. Comment on Rohrscheib et al. 2016 “Intensity of mutualism breakdown is determined by temperature not amplification of Wolbachia genes.” PLoS pathogens. 2017;13: e1006540. doi:10.1371/journal.ppat.1006540

30. McGarry HF, Egerton GL, Taylor MJ. Population dynamics of Wolbachia bacterial endosymbionts in Brugia malayi. Molecular and Biochemical Parasitology. 2004;135: 57–67. doi:10.1016/j.molbiopara.2004.01.006

31. Chrostek E, Teixeira L. Within host selection for faster replicating bacterial symbionts. PLoS ONE. 2018;13: e0191530. doi:10.1371/journal.pone.0191530

32. Christensen S, Camacho M, Sharmin Z, Momtaz AJMZ, Perez L, Navarro G, et al. Quantitative methods for assessing local and bodywide contributions to Wolbachia titer in maternal germline cells of Drosophila. BMC Microbiol. 2019;19: 1–17. doi:10.1186/s12866-019-1579-3

33. Alkan C, Coe BP, Eichler EE. Genome structural variation discovery and genotyping. Nature reviews Genetics. 2011;12: 363–376.

34. Grobler Y, Yun CY, Kahler DJ, Bergman CM, Lee H, Oliver B, et al. Whole genome screen reveals a novel relationship between Wolbachia levels and Drosophila host translation. PLoS pathogens. 2018;14: e1007445. doi:10.1371/journal.ppat.1007445

35. Burr B, Burr FA. Activation of Silent Transposable Elements. Plant Transposable Elements. Boston, MA: Springer US; 1988. pp. 317–323.

36. Duchek P, Rørth P. Guidance of Cell Migration by EGF Receptor Signaling During Drosophila Oogenesis. Science (New York, NY). 2001;291: 131–133. doi:10.1126/science.291.5501.131

37. Prelich G. Gene overexpression: uses, mechanisms, and interpretation. Genetics. 2012;190: 841–854. doi:10.1534/genetics.111.136911

38. McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang Y-F, et al. Stable Introduction of a Life-Shortening Wolbachia Infection into the Mosquito Aedes aegypti. Science (New York, NY). 2009;323: 141–144. doi:10.1126/science.1165326

39. Iturbe-Ormaetxe I, Burke GR, Riegler M, O’Neill SL. Distribution, expression, and motif variability of ankyrin domain genes in Wolbachia pipientis. Journal of bacteriology. 2005;187: 5136–5145. doi:10.1128/JB.187.15.5136-5145.2005

40. Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, Jiggins FM, et al. Population Genomics of the Wolbachia Endosymbiont in Drosophila melanogaster. PLoS genetics. 2012;8: e1003129. doi:10.1371/journal.pgen.1003129

41. Reynolds KT, Thomson LJ, Hoffmann AA. The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent Wolbachia strain popcorn in Drosophila melanogaster. Genetics. 2003;164: 1027–1034.

42. Rohrscheib CE, Frentiu FD, Horn E, Ritchie FK, van Swinderen B, Weible MW, et al. Intensity of Mutualism Breakdown Is Determined by Temperature Not Amplification of Wolbachia Genes. PLoS pathogens. 2016;12: e1005888. doi:10.1371/journal.ppat.1005888

43. Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, et al. The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. Genetics. 2004;167: 797–813. doi:10.1534/genetics.104.026658

44. Pais IS, Valente RS, Sporniak M, Teixeira L. Drosophila melanogaster establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. PLOS Biol. 2018;16: e2005710. doi:10.1371/journal.pbio.2005710

45. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research. 2001;29: e45.

46. Andrews, Simon. FastQC: A quality control tool for high throughput sequence data. 2010. Available: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

47. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics (Oxford, England). 2014;30: 2114–2120. doi:10.1093/bioinformatics/btu170

48. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics (Oxford, England). 2009;25: 1754–1760. doi:10.1093/bioinformatics/btp324

49. Li H. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics (Oxford, England). 2018;34: 3094–3100.

50. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. bioRxiv. 2016; 096412. doi:10.1101/096412

51. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. Journal of Computational Biology. 2012;19: 455–477. doi:10.1089/cmb.2012.0021

52. Wick RR, Schultz MB, Zobel J, Holt KE. Bandage: interactive visualization of de novogenome assemblies: Bioinformatics (Oxford, England). 2015;31: 3350–3352.

53. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990;215: 403–410.

54. Vaser R, Sovic I, Nagarajan N, Sikic M. Fast and accurate de novo genome assembly from long uncorrected reads. Genome research. 2017;27: 737–746.

1032 55. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: An
1033 Integrated Tool for Comprehensive Microbial Variant Detection and Genome
1034 Assembly Improvement. PLoS ONE. 2014;9: e112963.

1035 56. Darling ACE, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved
1036 genomic sequence with rearrangements. Genome research. 2004;14: 1394–1403.

1037 57. Li H. A statistical framework for SNP calling, mutation discovery, association mapping
1038 and population genetical parameter estimation from sequencing data. Bioinformatics
1039 (Oxford, England). 2011;27: 2987–2993.

1040 58. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence
1041 Alignment/Map format and SAMtools. Bioinformatics (Oxford, England). 2009;25:
1042 2078–2079. doi:10.1093/bioinformatics/btp352

1043 59. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al.
1044 Integrative genomics viewer. Nature biotechnology. 2011;29: 24–26.

1045 60. Team RC. R: A language and environment for statistical computing. R Foundation for
1046 Statistical Computing, Vienna, Austria; 2012. Available: URL http://www.R-project.org/

1047 61. Therneau TM. coxme: Mixed Effects Cox Models. 2020. Available: https://CRAN.R-
1048 project.org/package=coxme

1049 62. Holm S. A simple sequentially rejective multiple test procedure. Scandinavian Journal
1050 of Statistics. 1979;6: 65–70.

1051 63. Burnham KP, Anderson DR. Model selection and multimodel inference : a practical
1052 information-theoretic approach. New York : Springer; 2002.

1053

1054

1055 ## Supporting information

1056 **S1 Fig. *Wolbachia* recovers from severe titre reduction within 3-4 fly generations.**

1057 Relative *Wolbachia* titres of the progeny of tetracycline-treated flies. *w*MelCS_b-carrying females laid eggs in
1058 food containing varying doses of tetracycline. The progeny of three females were used to set up the
1059 experiment. At the first generation, four females were randomly selected for egg-laying in antibiotic-free fly
1060 food and *Wolbachia* titre was measured using qPCR. Titres of untreated females were used to normalize the
1061 qPCR results. The progeny of a female with the median titre was used to set up the next generation.
1062 *Wolbachia* titre in the F1 was significantly determined by the concentration of the antibiotic ($p < 0.001$ for all
1063 doses compared with control at generation 1), but recovered to normal within four fly generations ($p > 0.05$
1064 for all doses compared with control at generation 4).

1065

1066 **S2 Fig. EMS decreases female fecundity and *Wolbachia* titre in the next generation in a dose-**
1067 **dependent manner.**

1068 The total number of eggs (A) and adults (B) of females treated with varying doses of EMS. The reproductive
1069 output of 10 females was determined in the first ten days after EMS treatment by daily transferring females to
1070 new vials for egg laying. Females fed on a sucrose solution served as controls. Each dot represents the total
1071 number of eggs (A) or adults (B) laid by individual females during ten days. The effect of EMS on the
1072 reproductive output of females was estimated using a non-linear model and was highly significant ($p < 0.001$
1073 for both numbers of eggs and adults per female). (C-D) *Wolbachia* titres in the F1 progeny of flies treated
1074 with varying EMS doses. *Wolbachia* titre was quantified on individual females (n=5–13), after laying eggs for
1075 three days. *Wolbachia* titres were normalized against the titres of untreated females. Dashed red lines
1076 represent the mean value predicted using non-linear models. The effect of EMS on *Wolbachia* titres in the
1077 next generation was highly significant ($p < 0.001$ for both panels).

1078

1079 **S3 Fig. Isolation of over-proliferative *Wolbachia* variants.**

1080 (A-D) Relative *Wolbachia* titres in a control (*w*MelCS_b) and EMS-treated *Drosophila* lines. Lines to set up
1081 the next generation was selected as described for Fig 1. Line 2B was isolated in the same batch as for Line
1082 2A (wMelOctoless) and they may be not independent. Likewise, Lines 3A (*w*MelOctoless2), 3B, and 3C were
1083 also isolated in the same batch.

1084

1085 **S4 Fig. Generation of isogenic *Drosophila* lines with *w*MelPop2 and *w*MelOctoless.**

1086 The first, second and third chromosomes of flies carrying *w*MelPop2 and *w*MelOctoless were replaced
1087 through the use of balancer chromosomes. *Wolbachia* infection (and also mitochondria) was kept in the
1088 stock by crossing females with *Wolbachia* with selected males. The mitochondria are only shown in females
1089 because of its strictly maternal transmission. All males were free of *Wolbachia* infection. Dashed lines
1090 indicate the genotype selected from the previous cross. Virgin female in the first cross were considered
1091 mutant in all chromosomes (*), for illustrative purposes. Question marks (?) represent recombined
1092 chromosomes.

1093

1094 **S5 Fig. Identification the genetic bases of *Wolbachia* over-proliferating variants.**

1095 The relative coverage in a selected genomic region. As detailed for Fig 2B, Illumina paired-end reads were
1096 mapped to *w*MelCS_b (GenBank: CP046924.1) genome, and the number of reads mapping to each position
1097 were normalized by dividing to the median coverage across the genome. Coverage information for
1098 *w*MelCS_b, *w*MelPop2 and *w*MelOctoless is also given in Fig 2B. We identified the deletion of Octomom as
1099 the cause of over-proliferation in lines 2B and line 3A (*w*MelOctoless2), as no other difference was found
1100 when compared to *w*MelCS_b.

1101

1102 **S6 Fig. Confirmation of deletion of Octomom genes in *w*MelOctoless by qPCR.**

1103 The deletion of individual Octomom genes was confirmed using qPCR. The copy number of three genes
1104 outside the Octomom region were also determined. Five females carrying *w*MelCS_b or *w*MelOctoless were
1105 used in the analysis. The copy number of *w*MelOctoless genes is relative to that of *w*MelCS_b.

1106

1107 **S7 Fig. Selection for stocks with a desired Octomom copy number.**

1108 The relative copy number of genomic WD0513 in *Wolbachia*-carrying stocks throughout 30 fly generations.
1109 Each generation, 5-20 females were randomly collected for egg-laying for 3-4 days and used to determine
1110 the relative copy number of WD0513 as a proxy for the Octomom copy number. The progeny of a single
1111 female was used to set up the next generation. qPCR results were normalized to that of *w*MelCS_b, which
1112 has a single copy of Octomom per genome.

1113

1114 **S8 Fig. Proliferation dynamics of over-proliferative *Wolbachia* variants at different temperatures.**

1115 *Wolbachia* proliferation dynamics at 18ºC (A), 25ºC (B) and 29ºC (C). *Wolbachia* titres were determined and
1116 analysed as described for Fig 3.

1117

1118 **S9 Fig. Octomom copy number determines *Wolbachia* titres on the day of eclosion.**

1119 Relative *Wolbachia* titres on the day of adults eclosion. Males developed at 25ºC were collected within 24
1120 hours after eclosion for *Wolbachia* titre measurement using qPCR. Data used in this figure were given in Fig
1121 3 and S8 Fig (time point 0). Letters represent significant groups after *p*-value correction.

1122

1123 **S10 Fig. *w*MelPop2 and *w*MelPop are phenotypically indistinguishable.**

1124 (A) WD0513 copy number of *w*MelPop2 and *w*MelPop in two experimental replicates. Using WD0513 as a
1125 proxy, the Octomom copy number of *w*MelPop2 and *w*MelPop was tightly controlled prior to phenotypic
1126 comparison. (B) *Wolbachia* relative titres at 25ºC. The progeny of *w*MelPop2- and *w*MelPop-infected females
1127 carrying three copies of Octomom was used to set up the experiments. Males that developed at 25ºC were
1128 collected upon eclosion, aged to specific time-points and used to determine *Wolbachia* titers using qPCR.
1129 *Wolbachia* titers were normalized to that of *w*MelCS_b-carrying flies collected on the day of eclosion.
1130 Proliferation rates of *w*MelPop2 and *w*MelPop were not different (p = 0.32). (C) Lifespan of males (solid
1131 lines) and females (dashed lines) flies at 25ºC. The experiment was performed twice, as described for Fig 3.
1132 Males were transferred to new vials every five days, while females every four days. (D). Coefficients of a Cox
1133 mixed model, representing the effect of *w*MelPop2 and *w*MelPop on the lifespan relative to *w*MelCS_b-
1134 carrying flies. *w*MelPop2 and *w*MelPop was equally pathogenic (p = 0.29).

1135

1136 **S11 Fig. Octomom copy number dynamics throughout *Drosophila* development and during the adult**
1137 **life.**

1138 (A) Relative copies of WD0513 throughout the fly development. Samples were collected and processed as
1139 described in Fig 4. Relative copies of WD0513 was determined using qPCR and are normalized to
1140 *w*MelCS_b. There is no significant change of Octomom copy number in wMelPop2 during fly development
1141 (p = 0.88). (B) The relative copies of WD0513 during the adult life at 18ºC, 25ºC, and 29ºC. Relative copies

of WD0513 was determined and normalized as previously described. There was a small increase of Octomom copy number with adult age (p = 0.009).

S12 Fig. New over-proliferative Wolbachia variants are pathogenic.

Lifespan of males with different *Wolbachia* variants at 18ºC (A), 25ºC (B), and 29ºC (C). An experimental replicate of these experiments is given in Fig 5A-C, respectively. (D) Lifespan of females at 25ºC in two experimental replicates. Experiments consisted of collecting 50 females upon adult eclosion and following their survival at 25ºC until all flies had died. Females were kept on groups of 10 per vial and transferred to new vials every four days. (E) Coefficients of a Cox mixed model, representing the effect of *Wolbachia* variants on the survival of *Drosophila* females. Bars represent the standard error of the estimates, and letters statistically significant groups.

S13 Fig. *Wolbachia* variants and not the differences in the host genetic background are the cause of the life shortening phenotype.

(A-B) Lifespan of females at 29ºC. Virgin females carrying *w*MelCS_b were crossed with males with *w*MelOctoless or *w*MelPop2. Reciprocal crosses were also performed. The survival of resulting female progeny, which have the same genetic background but differ in *Wolbachia* infection, were assayed at 29ºC. For both experimental replicates, we determined the lifespan of 50 female progeny from each cross. Females were maintained in groups of 10 per vial and transferred to new vial every four days. (C) Coefficients of a Cox mixed model, representing the effect of *Wolbachia* variant on the survival of females. Significance of each group was accessed after *p*-value correction for multiple comparisons, and are represented by letters.

S14 Fig. Correlation between the strength of life-shortening phenotype or anti-viral protection with *Wolbachia* titre and proliferation.

(A-C) Correlation between the strength of life-shortening phenotype at 18ºC (A), 25ºC (B), and 29ºC (C) and *Wolbachia* titres at the day of eclosion. (D-E) Correlation between the strength of *Wolbachia* anti-viral protection and *Wolbachia* doubling times at 18ºC (D) and 25ºC (E). The direction and significant of all correlations was estimated using Person correlation coefficient and are given in each panel.

S15 Fig. Over-proliferative *Wolbachia* variants confer strong protection against DCV.

Survival of males males after a DCV (A) or buffer (B) challenge. An experimental replicate is given in Fig 6. The experiment was set up and analysed as described in Fig 6.

S1 Table. Number of progeny of EMS-treated females screened for over-proliferating *Wolbachia* variants.

Progenies of EMS-treated females were collected as virgin, mated with 2-3 *Wolbachia*-free Drosdel w^{1118} isogenic males, allowed to egg-laying for 3–4 days and used in qPCR reations to determine *Wolbachia* titres relative to the progeny of control females.

S2 Table. Coverage statistics of the sequencing project.

Coverage statistics (median, the first and third quantiles) of Illumina reads across different genomes mapped to either *Wolbachia* or mitochondria of *D. melanogaster* Release 6 genome sequence (KJ947872.2:1–14,000) using BWA mem v0.7.12-r1039. *Wolbachia* reference genome used for mapping the different reads are indicated. ND – not determined.

S3 Table. Flies with *w*MelCS_b, *w*MelOctoless and *w*MelPop2 carry an identical mitochondrial genome.

Illumina reads of fly stocks varying on *Wolbachia* variants were mapped to the *D. melanogaster* Release 6 genome sequence (KJ947872.2:1–14,000) using BWA mem v0.7.12-r1039. The mitogenome of flies infected with *w*MelCS_b, *w*MelOctoless and *w*MelPop2 was identical. We found a SNP unique to flies infected with *w*MelCS-like variants (G→A on position 10,793) but absent in flies infected with *w*MelPop or *w*Mel. We confirmed the substitution using Sanger sequencing (S2 Text).

S4 Table. Assembly and annotation statistics.

Wolbachia genomes were assembled using the Unicycler v0.4.8-beta pipeline and annotated using NCBI Prokaryotic Genome Annotation Pipeline v4.10. We found variant in *Wolbachia* genome size but the resulting genomes encoded for the same number of transfer, ribosomal and non-coding RNAs.

S5 Table. SNPs and indels differences between different *w*Mel genomes.

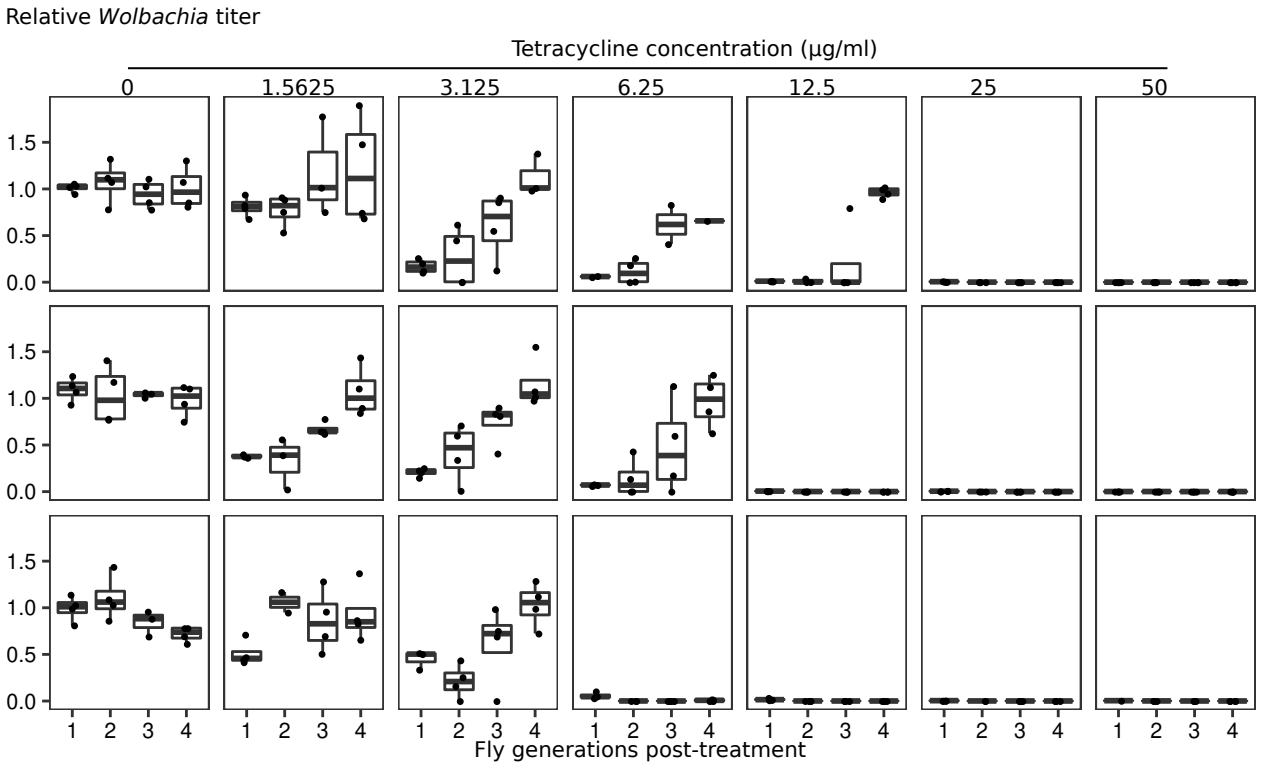
The genome of a newly assembled Cluster III *Wolbachia* variant, called *w*Mel, was compared to *w*Mel reference genome. Nine differences were identified and validated using Sanger sequencing.

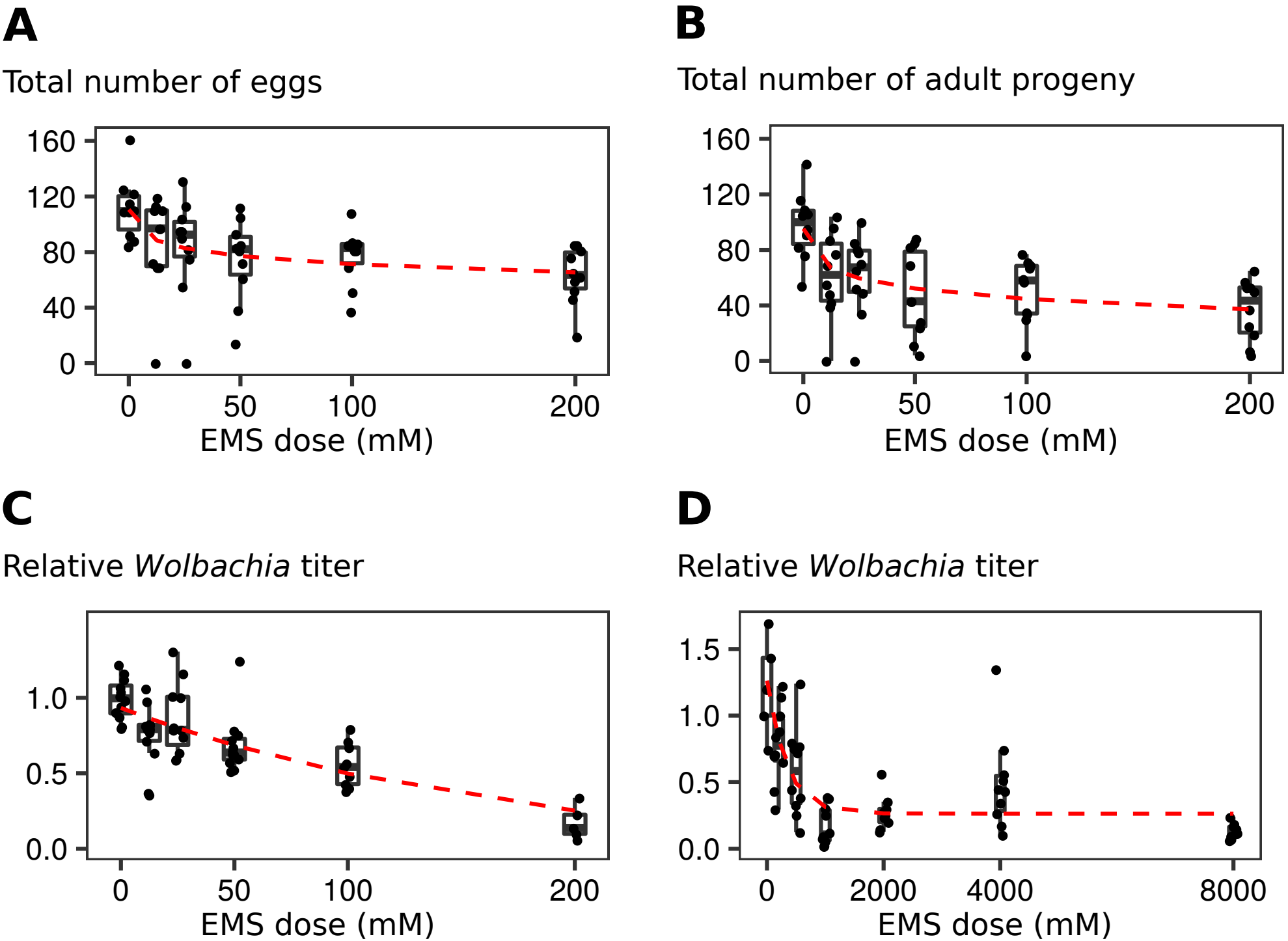
S1 Text. Alignment of a genomic region, including Octomom, in different Wolbachia variants.

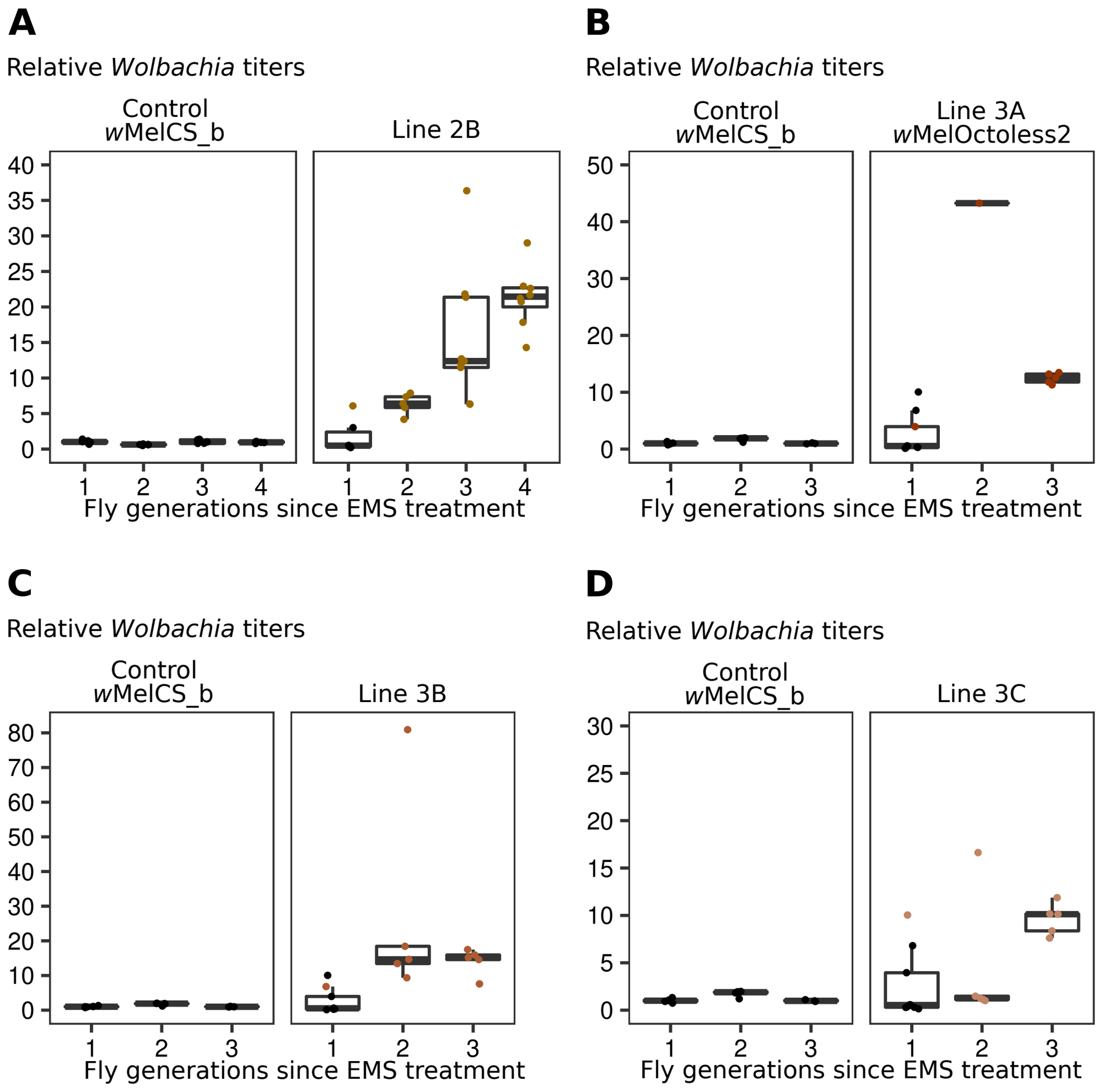
An alignment summary of a genomic region, including Octomom, in *w*MelCS, *w*MelPop2 and *w*MelOctoless.

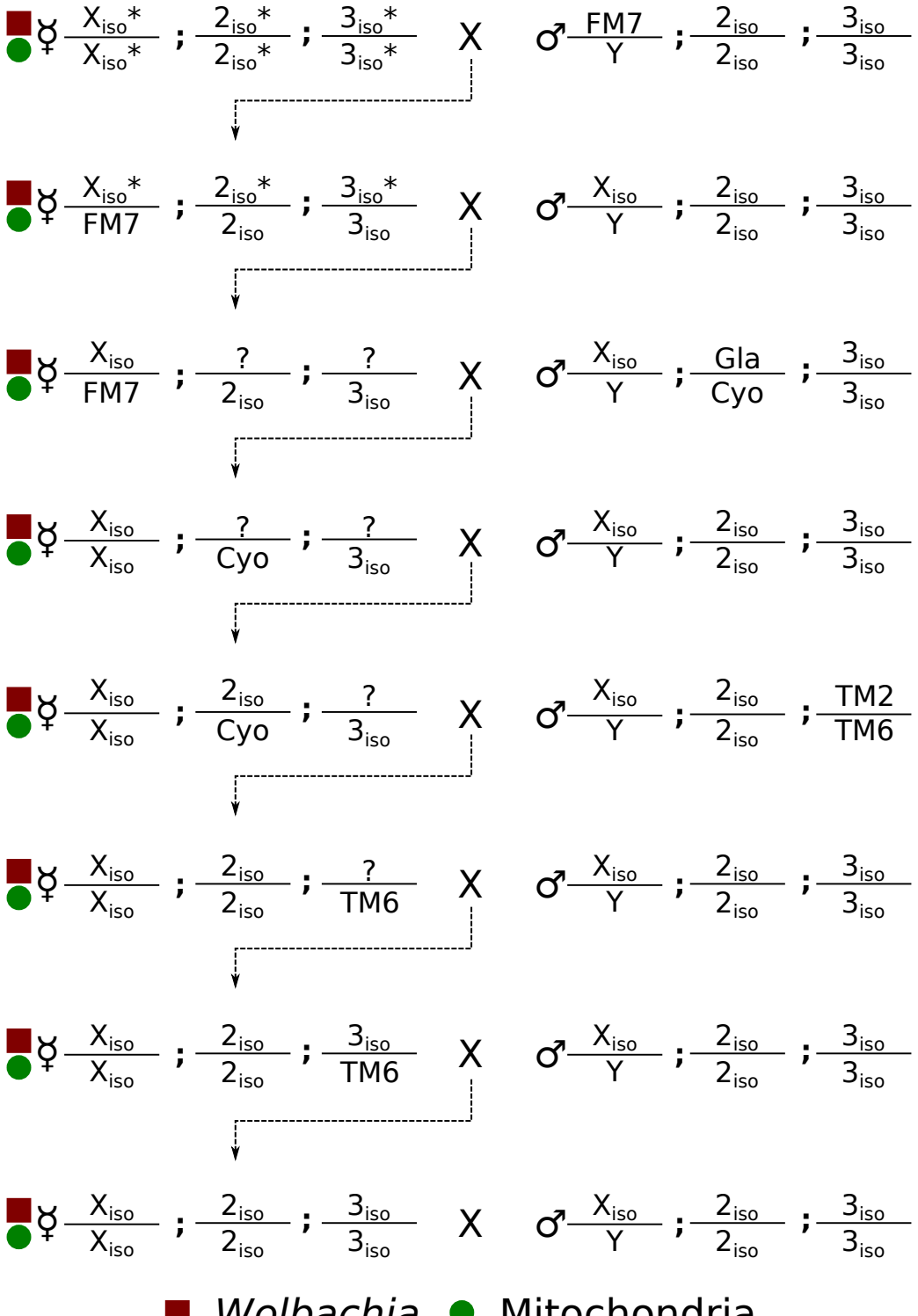
S2 Text. Confirmation of a SNP in the mitochondrial genome.

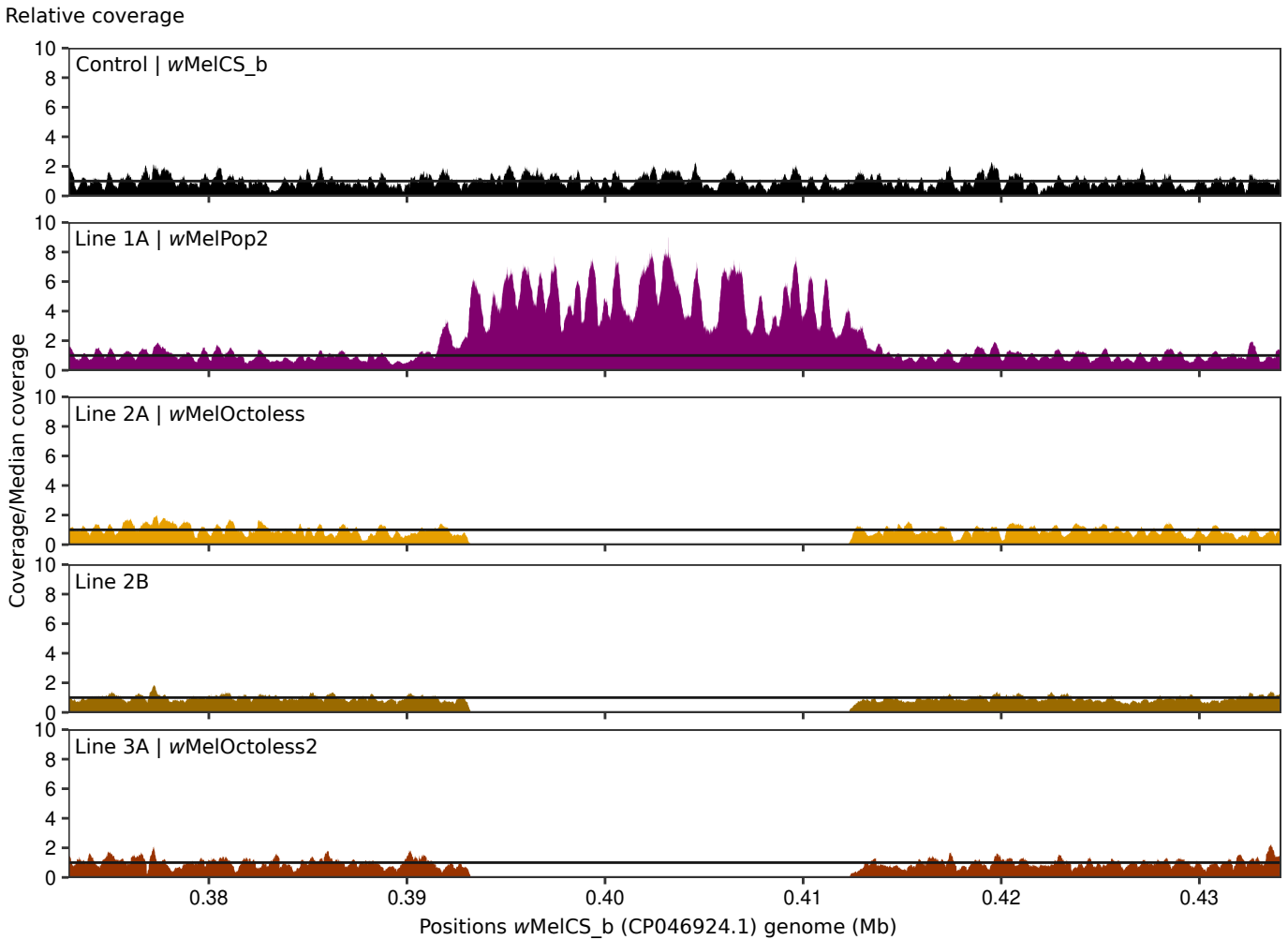
Sanger sequencing of a PCR amplicon containing a SNP present in *w*MelCS_b, *w*MelPop2 and *w*MelOctoless, but absent in *w*MelPop and *w*Mel.


Relative *Wolbachia* titer
Tetracycline concentration (µg/ml)
0
1.5625
3.125
6.25
12.5
25
50
1.5
1.0
0.5
0.0
1
2
3
4
Fly generations post-treatment

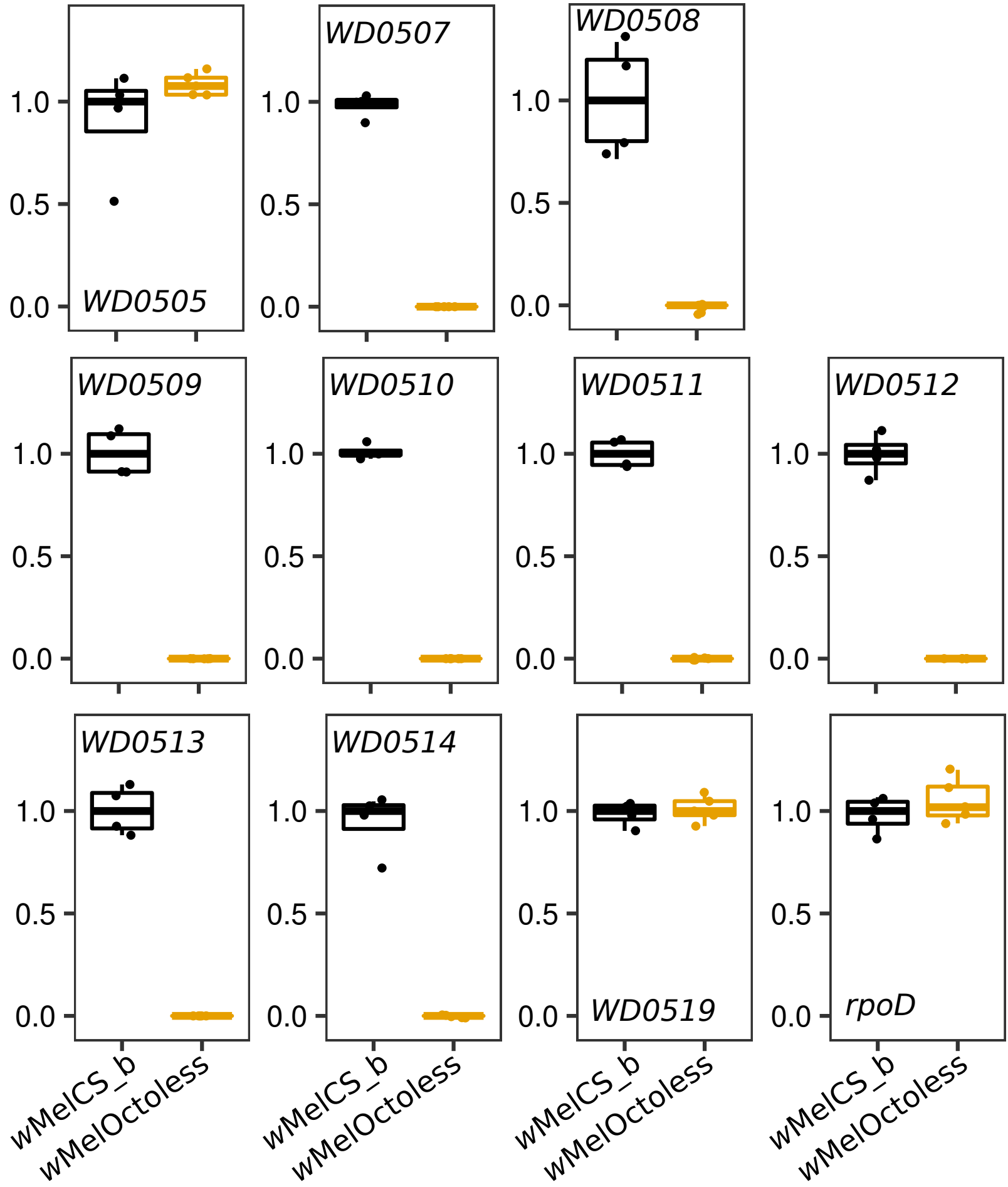

A
Total number of eggs
160
120
80
40
0
0
50
100
200
EMS dose (mM)
B
Total number of adult progeny
160
120
80
40
0
0
50
100
200
EMS dose (mM)
C
Relative *Wolbachia* titer
1.0
0.5
0.0
0
50
100
200
EMS dose (mM)
D
Relative *Wolbachia* titer
1.5
1.0
0.5
0.0
0
2000
4000
8000
EMS dose (mM)

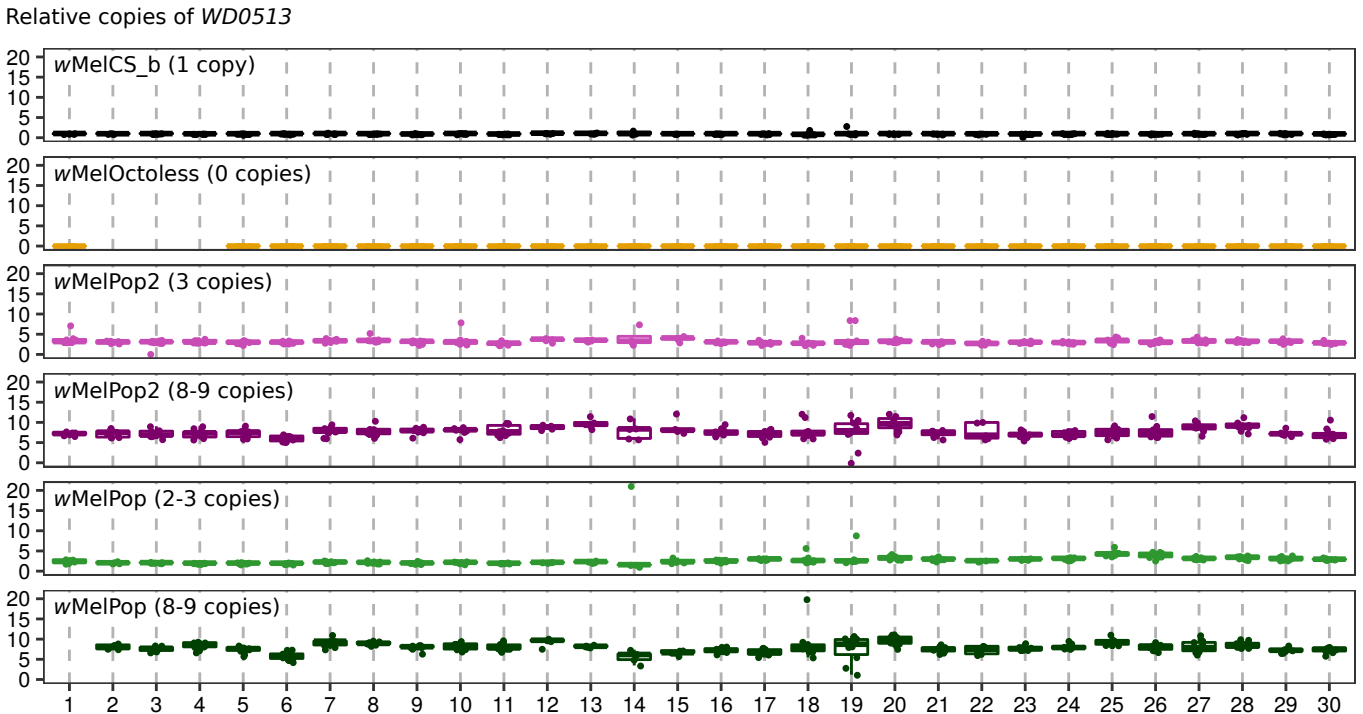

A
Relative Wolbachia titers
Control
wMelCS_b
Line 2B
40
35
30
25
20
15
10
5
0
1
2
3
4
Fly generations since EMS treatment
B
Relative Wolbachia titers
Control
wMelCS_b
Line 3A
wMelOctoless2
50
40
30
20
10
0
1
2
3
Fly generations since EMS treatment
C
Relative Wolbachia titers
Control
wMelCS_b
Line 3B
80
70
60
50
40
30
20
10
0
1
2
3
Fly generations since EMS treatment
D
Relative Wolbachia titers
Control
wMelCS_b
Line 3C
30
25
20
15
10
5
0
1
2
3
Fly generations since EMS treatment

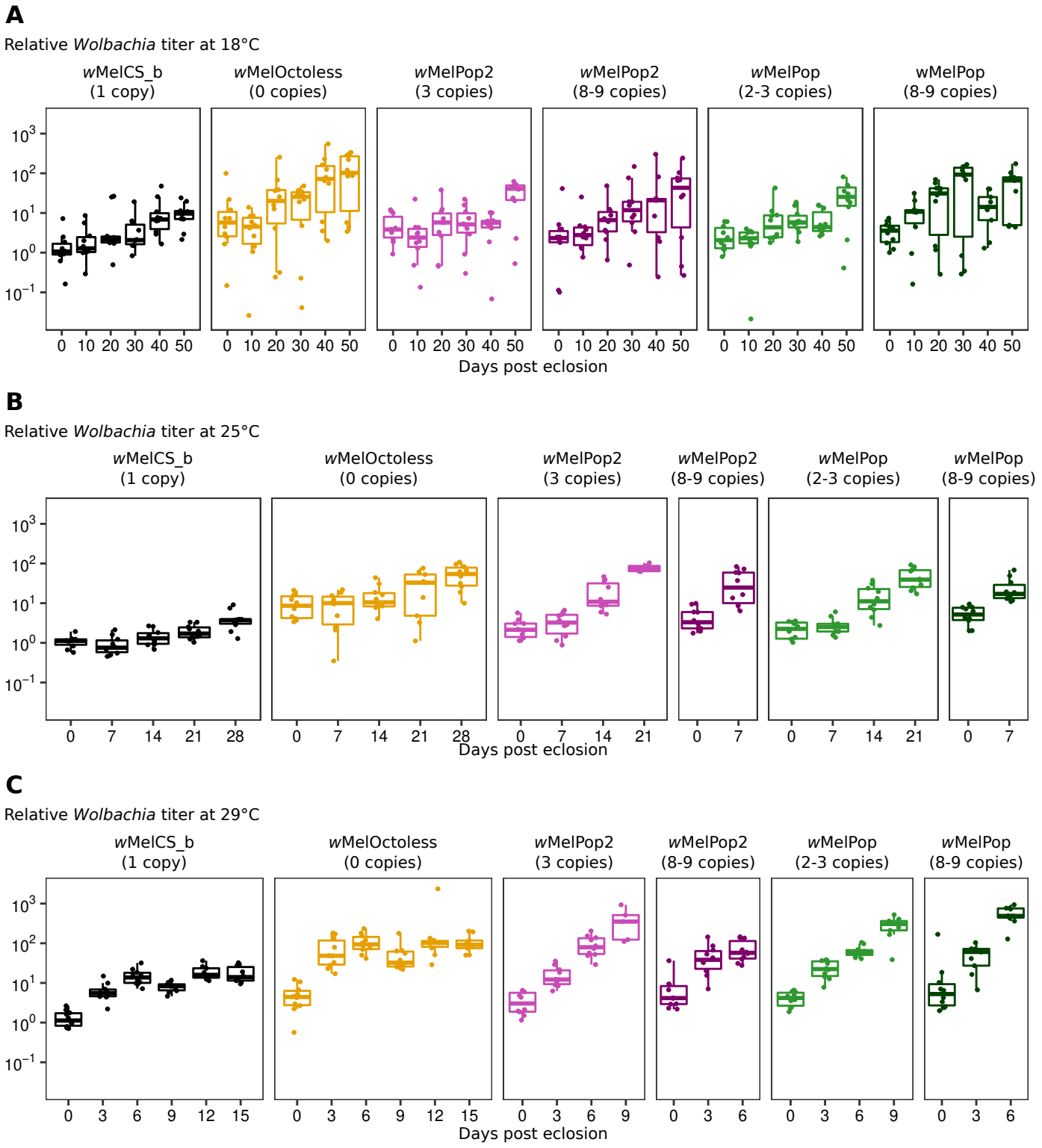

♀ X_iso* / X_iso* ; 2_iso* / 2_iso* ; 3_iso* / 3_iso* X ♂ FM7 / Y ; 2_iso / 2_iso ; 3_iso / 3_iso
♀ X_iso* / FM7 ; 2_iso* / 2_iso ; 3_iso* / 3_iso X ♂ X_iso / Y ; 2_iso / 2_iso ; 3_iso / 3_iso
♀ X_iso / FM7 ; ? / 2_iso ; ? / 3_iso X ♂ X_iso / Y ; Gla / Cyo ; 3_iso / 3_iso
♀ X_iso / X_iso ; ? / Cyo ; ? / 3_iso X ♂ X_iso / Y ; 2_iso / 2_iso ; 3_iso / 3_iso
♀ X_iso / X_iso ; 2_iso / Cyo ; ? / 3_iso X ♂ X_iso / Y ; 2_iso / 2_iso ; TM2 / TM6
♀ X_iso / X_iso ; 2_iso / 2_iso ; ? / TM6 X ♂ X_iso / Y ; 2_iso / 2_iso ; 3_iso / 3_iso
♀ X_iso / X_iso ; 2_iso / 2_iso ; 3_iso / TM6 X ♂ X_iso / Y ; 2_iso / 2_iso ; 3_iso / 3_iso
♀ X_iso / X_iso ; 2_iso / 2_iso ; 3_iso / 3_iso X ♂ X_iso / Y ; 2_iso / 2_iso ; 3_iso / 3_iso
Wolbachia
Mitochondria

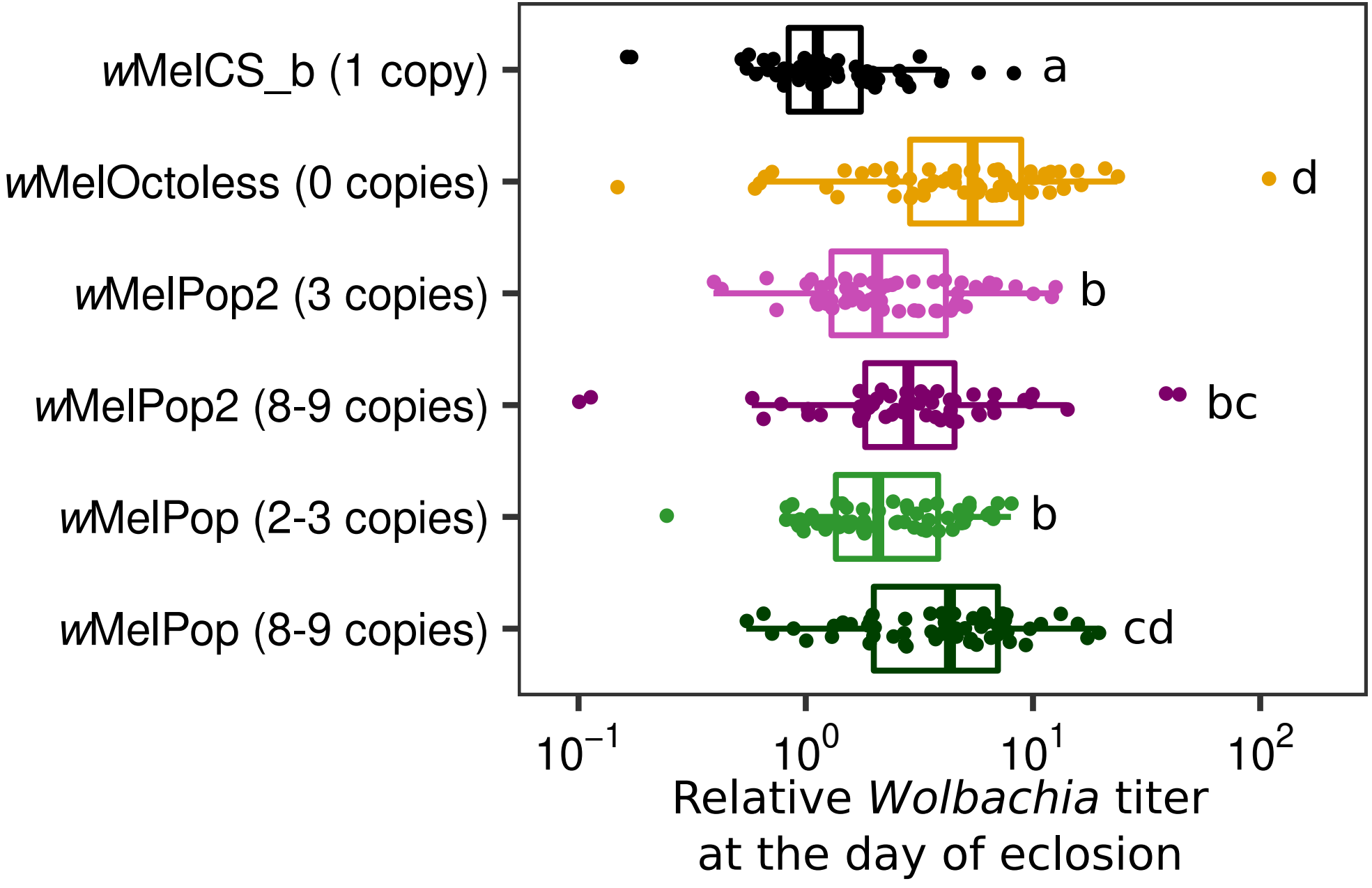

Relative coverage
Control | wMelCS_b
Line 1A | wMelPop2
Line 2A | wMelOctoless
Line 2B
Line 3A | wMelOctoless2
Coverage/Median coverage
0
2
4
6
8
10
0.38
0.39
0.40
0.41
0.42
0.43
Positions wMelCS_b (CP046924.1) genome (Mb)

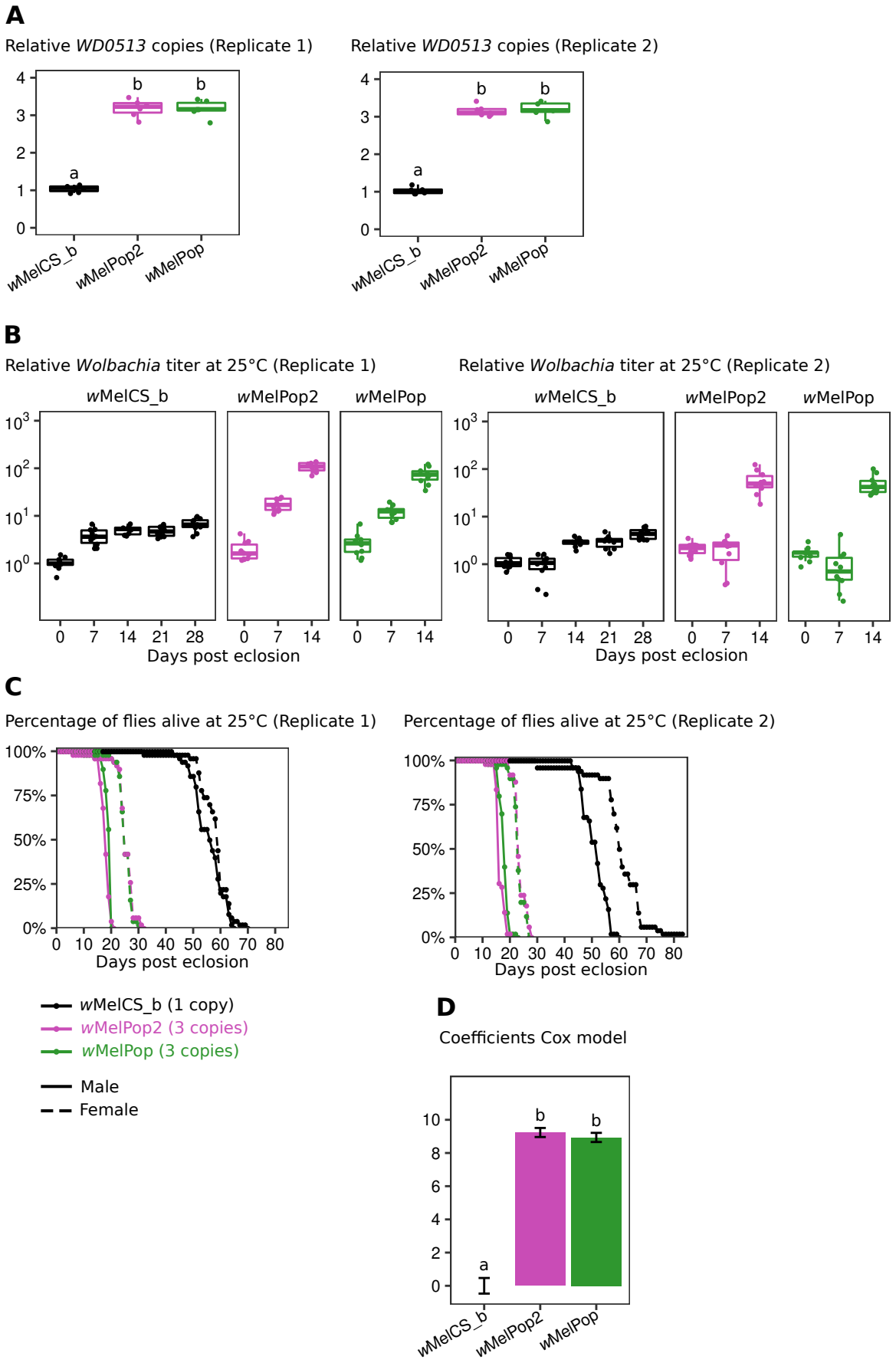
Copy number of individual genes

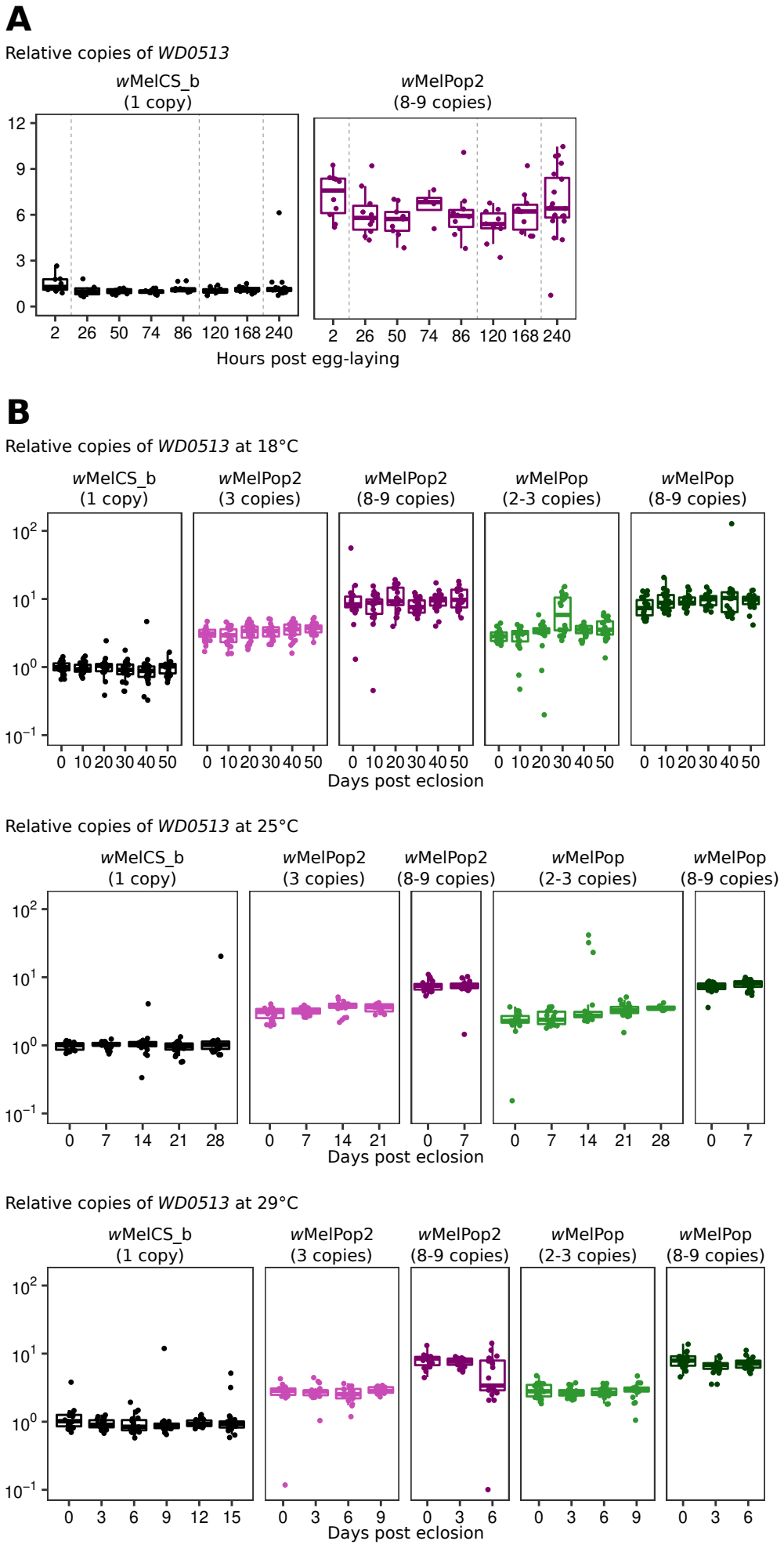


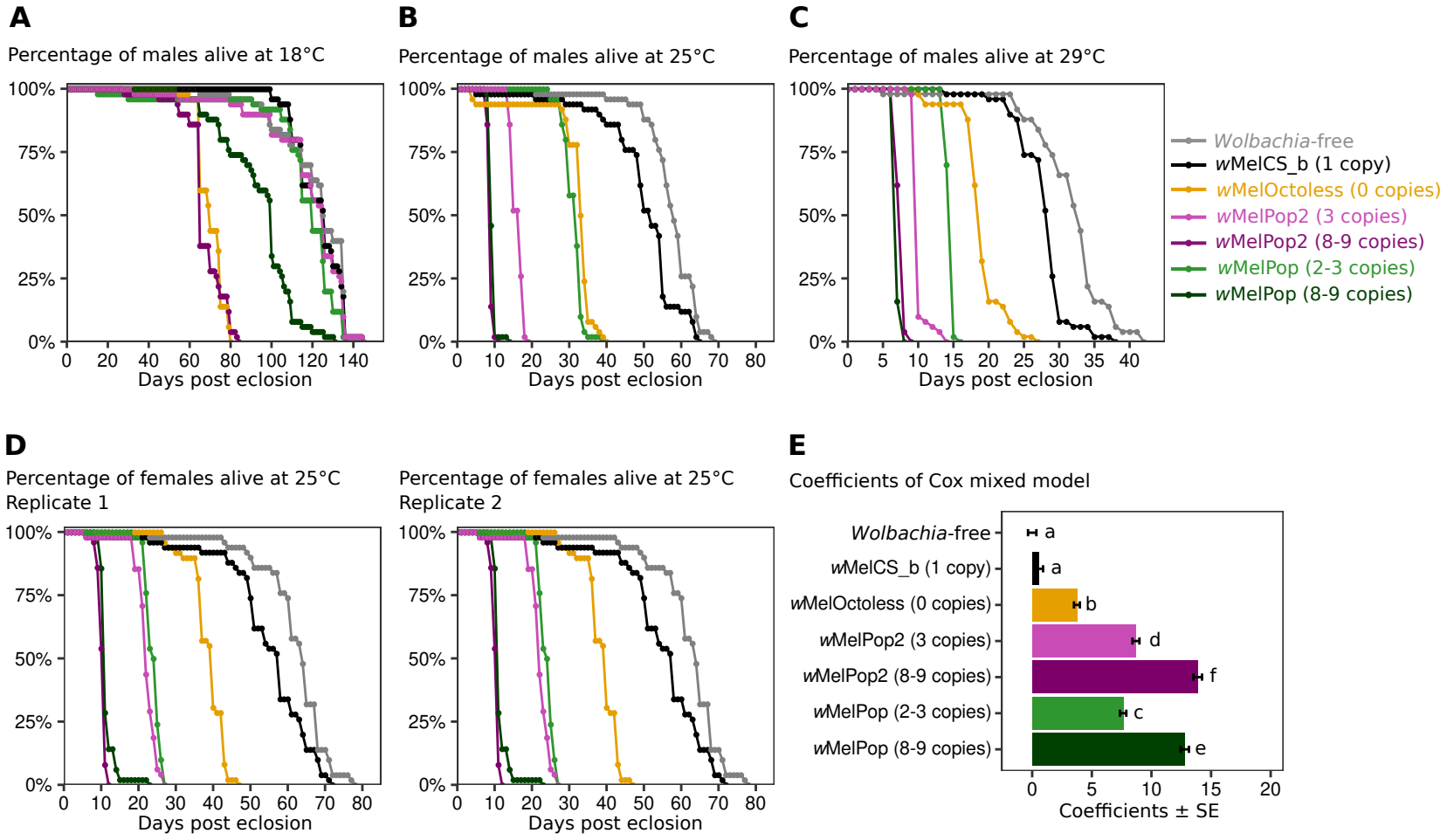

Relative copies of *WD0513*
*w*MelCS_b (1 copy)
*w*MelOctoless (0 copies)
*w*MelPop2 (3 copies)
*w*MelPop2 (8-9 copies)
*w*MelPop (2-3 copies)
*w*MelPop (8-9 copies)
0
5
10
15
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30


A
Relative Wolbachia titer at 18°C
wMelCS_b (1 copy)
wMelOctoless (0 copies)
wMelPop2 (3 copies)
wMelPop2 (8-9 copies)
wMelPop (2-3 copies)
wMelPop (8-9 copies)
Days post eclosion
B
Relative Wolbachia titer at 25°C
wMelCS_b (1 copy)
wMelOctoless (0 copies)
wMelPop2 (3 copies)
wMelPop2 (8-9 copies)
wMelPop (2-3 copies)
wMelPop (8-9 copies)
Days post eclosion
C
Relative Wolbachia titer at 29°C
wMelCS_b (1 copy)
wMelOctoless (0 copies)
wMelPop2 (3 copies)
wMelPop2 (8-9 copies)
wMelPop (2-3 copies)
wMelPop (8-9 copies)
Days post eclosion


wMelCS_b (1 copy)
wMelOctoless (0 copies)
wMelPop2 (3 copies)
wMelPop2 (8-9 copies)
wMelPop (2-3 copies)
wMelPop (8-9 copies)
a
d
b
bc
b
cd
10^{-1}
10^{0}
10^{1}
10^{2}
Relative *Wolbachia* titer
at the day of eclosion

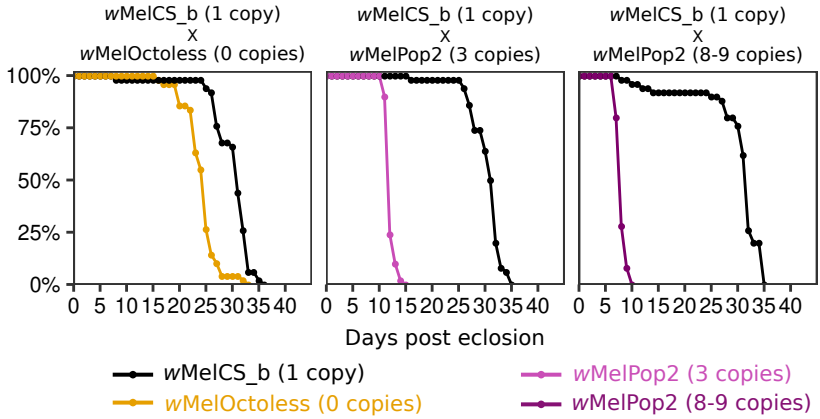

A
Relative WD0513 copies (Replicate 1)
Relative WD0513 copies (Replicate 2)
a
b
b
wMelCS_b
wMelPop2
wMelPop
B
Relative Wolbachia titer at 25°C (Replicate 1)
Relative Wolbachia titer at 25°C (Replicate 2)
wMelCS_b
wMelPop2
wMelPop
Days post eclosion
C
Percentage of flies alive at 25°C (Replicate 1)
Percentage of flies alive at 25°C (Replicate 2)
Days post eclosion
wMelCS_b (1 copy)
wMelPop2 (3 copies)
wMelPop (3 copies)
Male
Female
D
Coefficients Cox model


A
Relative copies of WD0513
wMelCS_b (1 copy)
wMelPop2 (8-9 copies)
Hours post egg-laying
B
Relative copies of WD0513 at 18°C
wMelCS_b (1 copy)
wMelPop2 (3 copies)
wMelPop2 (8-9 copies)
wMelPop (2-3 copies)
wMelPop (8-9 copies)
Days post eclosion
Relative copies of WD0513 at 25°C
Days post eclosion
Relative copies of WD0513 at 29°C
Days post eclosion


A
Percentage of males alive at 18°C
B
Percentage of males alive at 25°C
C
Percentage of males alive at 29°C
100%
75%
50%
25%
0%
Days post eclosion
Wolbachia-free
wMelCS_b (1 copy)
wMelOctoless (0 copies)
wMelPop2 (3 copies)
wMelPop2 (8-9 copies)
wMelPop (2-3 copies)
wMelPop (8-9 copies)
D
Percentage of females alive at 25°C
Replicate 1
Percentage of females alive at 25°C
Replicate 2
E
Coefficients of Cox mixed model
Wolbachia-free
wMelCS_b (1 copy)
wMelOctoless (0 copies)
wMelPop2 (3 copies)
wMelPop2 (8-9 copies)
wMelPop (2-3 copies)
wMelPop (8-9 copies)
a
a
b
d
f
c
e
Coefficients ± SE

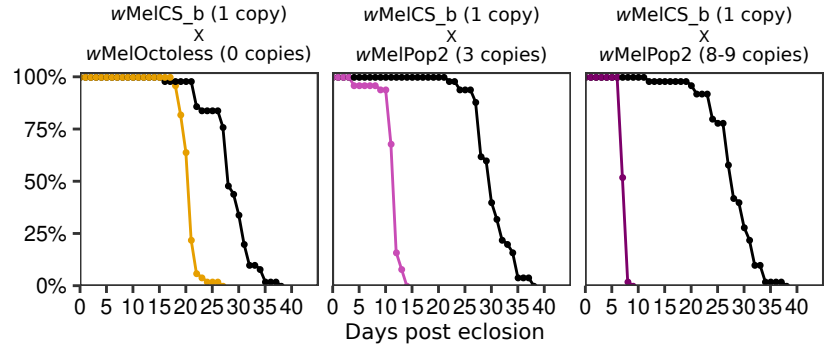
A

Percentage of females alive at 29ºC (Replicate 1)



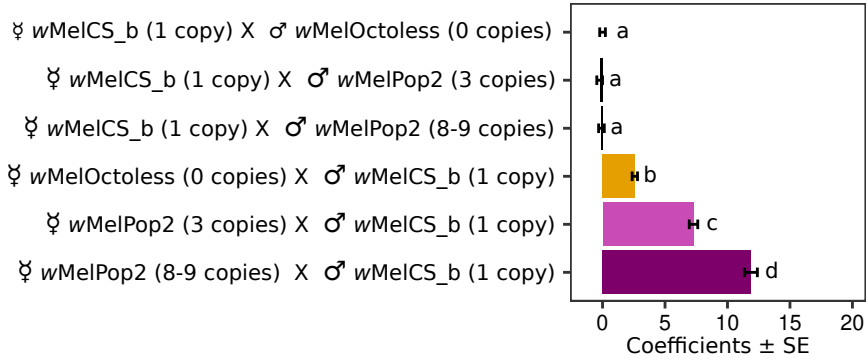
B

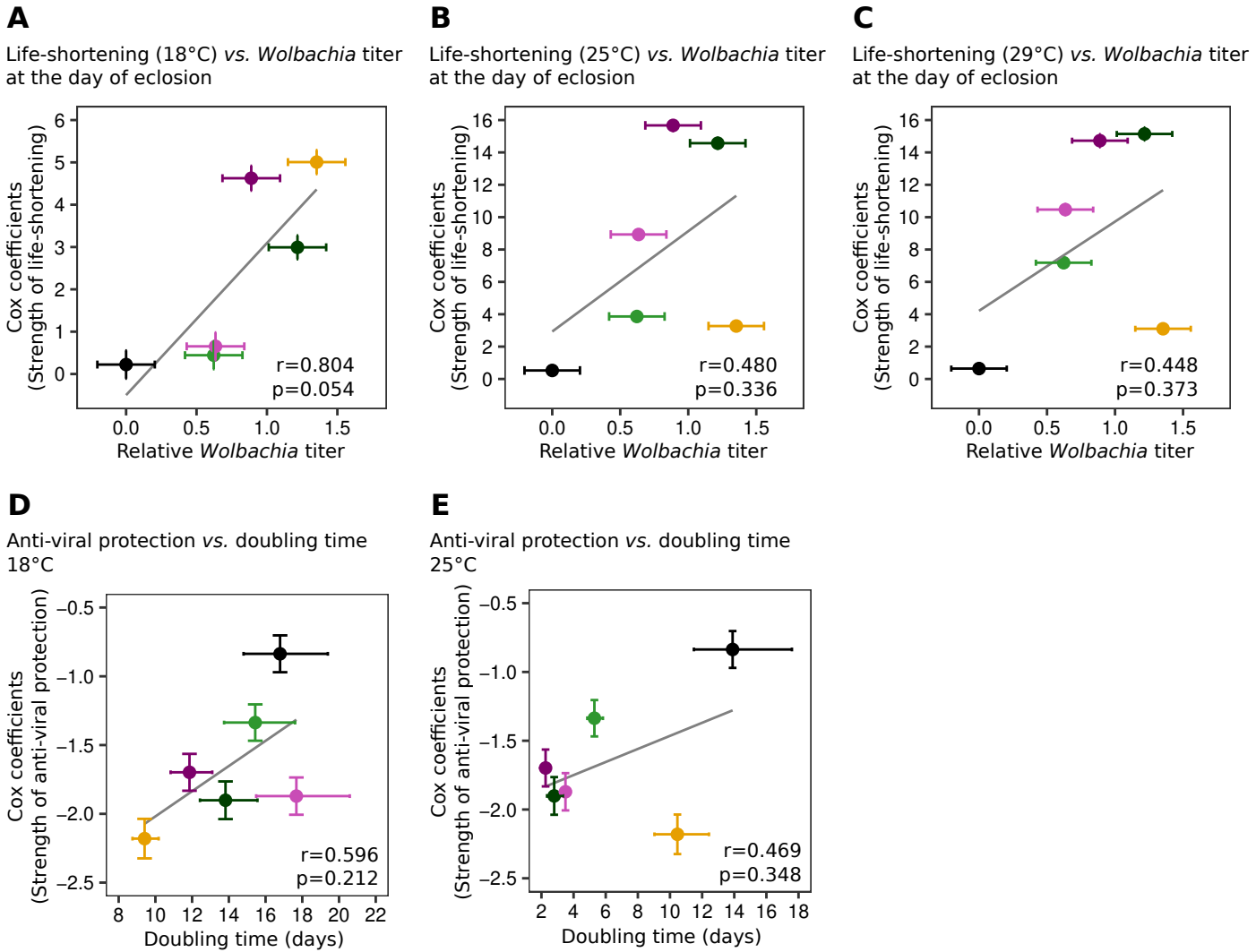
Percentage of females alive at 29ºC (Replicate 2)

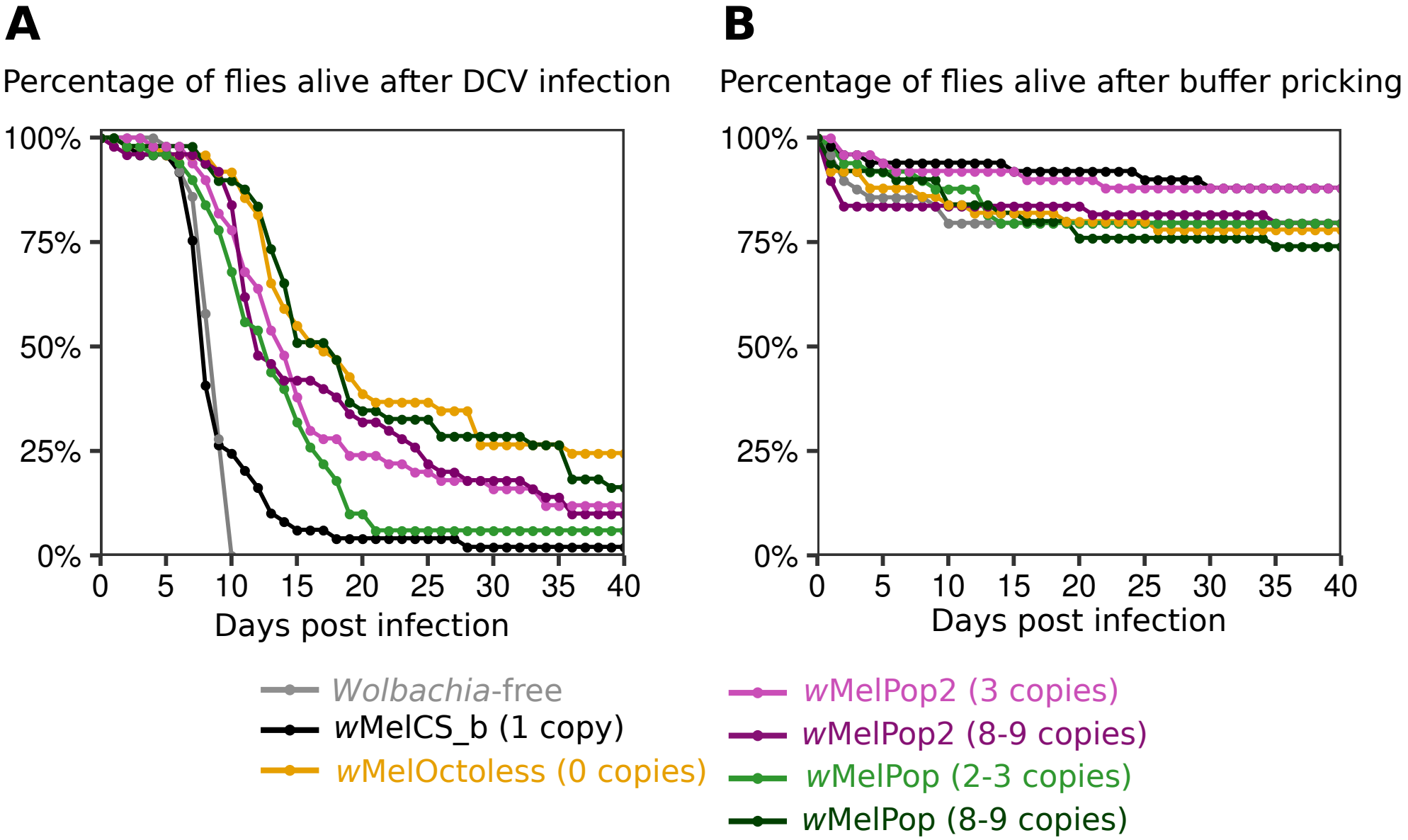


C

Coefficients of Cox mixed model




A
Life-shortening (18°C) vs. Wolbachia titer at the day of eclosion
Cox coefficients (Strength of life-shortening)
Relative Wolbachia titer
r=0.804
p=0.054
B
Life-shortening (25°C) vs. Wolbachia titer at the day of eclosion
Cox coefficients (Strength of life-shortening)
Relative Wolbachia titer
r=0.480
p=0.336
C
Life-shortening (29°C) vs. Wolbachia titer at the day of eclosion
Cox coefficients (Strength of life-shortening)
Relative Wolbachia titer
r=0.448
p=0.373
D
Anti-viral protection vs. doubling time 18°C
Cox coefficients (Strength of anti-viral protection)
Doubling time (days)
r=0.596
p=0.212
E
Anti-viral protection vs. doubling time 25°C
Cox coefficients (Strength of anti-viral protection)
Doubling time (days)
r=0.469
p=0.348


A
Percentage of flies alive after DCV infection
100%
75%
50%
25%
0%
0
5
10
15
20
25
30
35
40
Days post infection
B
Percentage of flies alive after buffer pricking
100%
75%
50%
25%
0%
0
5
10
15
20
25
30
35
40
Days post infection
Wolbachia-free
wMelCS_b (1 copy)
wMelOctoless (0 copies)
wMelPop2 (3 copies)
wMelPop2 (8-9 copies)
wMelPop (2-3 copies)
wMelPop (8-9 copies)