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 *Wolbachia* 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 *Wolbachia* 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, wMelPop2 and wMelOctoless, 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 wMelPop. The degree of the Octomom region amplification determines the proliferation rate of wMelPop 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 wMelCS_b as our starting variant because of its genetic proximity to the over-proliferative and virulent wMelPop 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 (wMelCS_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.
We designed the screen to find new mutants of *Wolbachia*, and not host mutants, that lead to the endosymbiotic proliferation. However, EMS will most likely also induce mutations in the host germline, in the nuclear or mitochondrial genomes, that can be transmitted. To minimize the influence of new host nuclear mutations on our screen, we backcrossed the EMS-treated females and their progeny, at every generation, with males from the control isogenic line. To verify that new mutations in the host were not the cause of *Wolbachia* over-proliferation, we replaced the first, second and third chromosomes of *Drosophila* females carrying the over-proliferating *Wolbachia* variants, in line 1A and line 2A, with the chromosomes of the control line, through the use of balancer chromosomes (S4 Fig.). We repeated *Wolbachia* titres quantification and found that the over-proliferative phenotypes were maintained (Fig 1C; Imm, p-value< 0.001 for both).

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

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

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

To test our assembly pipeline we assembled a previously characterized Cluster III wMel *Wolbachia* variant, named wMel [16], which derives from the line used for the original wMel reference genome (GenBank: AE017196.1) [27]. The new wMel genome (GenBank: CP046925.1) was also circular and comparable in size, structure and number of ORFs with previously published wMel genomes [27,28], including the wMel reference genome (S4 Table). We found, however, nine differences (2 SNPs and seven indels) relative to
wMel reference genome, which we confirmed using Sanger sequencing (S5 Table). These results validate our sequencing pipeline.

The only difference between the genome of the over-proliferative *Wolbachia* variant in Line 1A and wMelCS_b was an amplification of the Octomom region (Fig 2A and S1 Text). There were three more copies of this region, giving a genome size difference of 62,814bp. The Octomom region amplification, and lack of other differences, was also confirmed by the mapping of the Illumina sequencing reads of Line 1A on the genome of wMelCS_b (GenBank: CP046924.1) (Fig 2B). These results show that Octomom amplification is the cause of over-proliferation, consistently with previous findings with the variant wMelPop [16,17,29]. Given the nature of the genomic change inducing the over-proliferation we named this variant wMelPop2.

As shown before for wMelPop [17], we observed variation in the Octomom copy number in wMelPop2-carrying flies. For further analyses of the phenotype of these variant we established, through selection (as in [17]), *Drosophila* lines carrying wMelPop2 with specific low (2-3) and high Octomom (8-9) copy number (S5 Fig).

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

wMel (Accession: AE017196.1)

wMelCS_b (Accession: CP046924.1)

wMelPop2 (Accession: CP046922.1)

wMelOctoless (Accession: CP046923.1)

wMel (Accession: CP046925.1)

B

Relative coverage

Control | wMelCS_b

Line 1A | wMelPop2

Line 2A | wMelOctoless

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 wMelCS_b, wMelOctoless and wMelPop2 were aligned using Mauve v2.4.0 to identify mutations causing over-proliferation. The three-fold amplification of Octomom in Line 1A (wMelPop2) and its deletion in Line 2A (wMelOctoless) were the only mutations identified when compared to wMelCS_b. For illustrative purposes, we used MultiGeneBlast v1.1.13 ([http://multigeneblast.sourceforge.net/](http://multigeneblast.sourceforge.net/)) to find, in Line 1A, 2A and a newly assembled wMel genome, homologous proteins to those of wMel 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 *wMelCS_b* genome (GenBank: CP046924.1). We confirmed the amplification or deletion of Octomom as the only difference between over-proliferating *Wolbachia* and *wMelCS_b*.

A second line isolated together with wMelOctoless, line 2B, also lost the Octomom region (S7 Fig). And this was the only observed difference with *wMelCS_b* when mapping the Illumina reads on *wMelCS_b* (S7 Fig). No differences were observed when the Illumina reads were mapped to the wMelOctoless genome (GenBank: CP046923.1). Since wMelOctoless 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-
proliferative line, isolated in a different batch of treatment, line 3A (S3 and S7 Fig).

Mapping the Illumina sequence reads of this line identifies the loss of Octomom as the
only difference with wMelCS_b, and identifies no difference to wMelOctoless. We named
this line wMelOctoless2. These results further confirm that loss of the Octomom region
leads to an over-proliferative phenotype in *Wolbachia*.

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

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

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

In order to characterize better the phenotypes of the new *Wolbachia* variants
wMelOctoless and wMelPop2 we analysed their growth, together with wMelCS_b and
wMelPop, in adult males kept at 18°C, 25°C, and 29°C (Fig 3 and S8 Fig). These flies were
all reared at 25°C and placed at the different temperatures when 0-1 days old. At this initial
point, at adult eclosion, there are differences in titres between lines carrying different copy
numbers of Octomom genomic region (S9 Fig, *p* < 0.028 for all comparisons). Flies with
*Wolbachia* with one copy of Octomom, wMelCS_b, have the lowest relative titre of
*Wolbachia*. Flies with variants with low amplification of the Octomom region have
approximately twice the titres of *Wolbachia*, while flies with variants with high copy number
of this region have three times more *Wolbachia* than wMelCS_b. Flies with wMelOctoless
have the highest titres, approximately 4-fold higher than flies carrying wMelCS_b. Therefor, the deletion or amplification of the Octomom region impact *Wolbachia* titres in
adults at eclosion.
A Relative Wolbachia titer at 18°C

B Relative Wolbachia titer at 25°C

C Relative Wolbachia titer at 29°C

Fig 3. The amplification or deletion of Octomom increase Wolbachia proliferation rate.

Wolbachia proliferation dynamics at 18°C (A), 25°C (B) and 29°C (C). D. melanogaster males used in these experiments developed at 25°C, were collected on the day of eclosion and aged to specific time-point at a given temperature (18°C, 25°C or 29°C). 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 wMelCS_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
variants at different temperatures, from approximately one day to seventeen days. A model with all the data shows a complex interaction between growth rate, *Wolbachia* variant and temperature (imm, \( p < 0.001 \)).

<table>
<thead>
<tr>
<th><em>Wolbachia</em> variants</th>
<th>Adults</th>
<th></th>
<th></th>
<th>Larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18°C</td>
<td>25°C</td>
<td>29°C</td>
<td>25°C</td>
</tr>
<tr>
<td></td>
<td>Doubling time (days) (±SE)</td>
<td>Doubling time (days) (±SE)</td>
<td>Doubling time (days) (±SE)</td>
<td>Doubling time (days) (±SE)</td>
</tr>
<tr>
<td><strong>wMelCS_b</strong></td>
<td>16.79 (14.80-19.39)</td>
<td>13.90 (11.49-17.59)</td>
<td>4.07 (3.67-4.56)</td>
<td>0.68 (0.60-0.79)</td>
</tr>
<tr>
<td><strong>wMelOctoless</strong></td>
<td>9.41 (8.75-10.18)</td>
<td>10.46 (9.03-12.43)</td>
<td>3.37 (3.09-3.71)</td>
<td>0.48 (0.44-0.54)</td>
</tr>
<tr>
<td><strong>wMelPop2 (3 copies)</strong></td>
<td>17.68 (15.49-20.59)</td>
<td>3.50 (3.23-3.80)</td>
<td>1.28 (1.20-1.38)</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>wMelPop2 (8-9 copies)</strong></td>
<td>11.86 (10.83-13.10)</td>
<td>2.26 (1.96-2.66)</td>
<td>1.07 (0.98-1.17)</td>
<td>0.57 (0.52-0.65)</td>
</tr>
<tr>
<td><strong>wMelPop (2-3 copies)</strong></td>
<td>15.44 (13.74-17.61)</td>
<td>5.30 (4.86-5.83)</td>
<td>1.38 (1.29-1.49)</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>wMelPop (8-9 copies)</strong></td>
<td>13.82 (12.43-15.56)</td>
<td>2.79 (2.35-3.44)</td>
<td>0.88 (0.82-0.95)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

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

A direct comparison between *wMelOctoless* with *wMelCS_b* shows that this new variant replicates faster than *wMelCS_b* (imm, \( p < 0.001 \)), although it is a relatively small difference at all temperatures (in the full model with all variants, however, the growth of *wMelOctoless* and *wMelCS_b* is only statistically different at 18°C, Table 1). Both strains interact equally with temperature. Their growth rate does not significantly change between 18°C and 25°C (\( p = 0.94 \)), but increases at 29°C (\( p < 0.001 \)).

We also compared the effect of Octomom copy number in a model taking genotype (*wMelCS_b*, for the control and new variants generated here, or *wMelPop*) and Octomom copy number (zero, one, low and high) as factors. At 18°C the amplification of the Octomom copy number does not lead to a statistically significant increase of growth rate, compared with *wMelCS_b* (imm, \( p > 0.125 \) for both low and high Octomom copy number compared with control). At this temperature only the deletion of Octomom leads to a significantly higher growth, as seen above. However, variants with Octomom amplification
grow more at 25°C than at 18°C, and grow more at 29°C than at 25°C (p < 0.001 for all comparisons). At both 25°C and 29°C, variants with low Octomom copy numbers have a higher growth rate than the variants with one copy or deletion of Octomom region (p < 0.001 for all comparisons). At these temperatures the variants with high Octomom copy numbers grow faster than all the other variants (p < 0.003 for all comparisons). These results confirm that the degree of amplification of the Octomom region controls the intensity of the over-proliferation of these variants, as shown before [17]. The data also demonstrate a strong interaction between temperature and the increased proliferation of variants with amplification of the Octomom region. The effect of the amplification is not significant at 18°C and becomes increasingly stronger with higher temperature. On the other hand, loss of Octomom leads to a smaller effect in growth, but similar at all temperatures. Therefore, although both genomic mutations lead to an increase in Wolbachia titres they have different impacts in the growth rates and their interaction with temperature.

The statistical model taking into account the wMelCs_b or wMelPop genotype, which differ only in two SNPs (see above), indicated a significant difference in growth between them, at 25°C (p < 0.001). This could indicate that these two SNPs also influence growth of Wolbachia. However, this could also be due to the fact that the copy number of the Octomom region was not equally controlled in wMelPop and wMelPop2 lines during these experiments. wMelPop2 low copy number line had 2-3 copies of Octomom, while the wMelPop line had 3 copies. To test if wMelPop and wMelPop2 indeed vary in proliferation rate, we repeated this experiment with a more tightly controlled Octomom copy number in these two lines, at 25°C (S10 Fig A-D). Both wMelPop and wMelPop2 carrying 3 copies of Octomom grow faster than wMelCS_b (Imm, p < 0.001 for both) and there is no difference in growth between them (p = 0.32). This indicates that the genetic differences between these lines do not affect their growth and that they are equally influenced by Octomom copy number.

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

We also analysed the growth of wMelCS_b, wMelOoctoless and wMelPop2 (8-9 Octomom copies) during host development. *D. melanogaster* develops very fast from egg to adult, in only 10 days at 25°C. We predicted that Wolbachia would grow much faster during this period than during adult life, in order to grow from the small population, present in the egg, 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, (ln, 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 (ln, 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).
<table>
<thead>
<tr>
<th>Genome copy number (± SE)</th>
<th>Wolbachia variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>wMelCS_b</td>
<td>wMelOctoless</td>
</tr>
<tr>
<td>Embryos</td>
<td>4,100 (±1,200)</td>
</tr>
<tr>
<td>Adults</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>619,000 (±179,000)</td>
</tr>
<tr>
<td>Females</td>
<td>841,000 (±245,000)</td>
</tr>
</tbody>
</table>

**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). wMelCS_b has an estimated doubling time of approximately 16h, wMelPop2 of 14h, and wMelOctoless 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 wMelCS_b, wMelOctoless, and wMelPop2 (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 wMelCS_b and wMelPop2, 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 wMelPop and wMelPop2 (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 wMelPop has been associated with a shortening of the host lifespan [16,22]. We, therefore, tested if these new over-proliferative variants also shorten...
the lifespan of their host, at different temperatures, in males (Fig 5, S12 Fig). We also performed this assay in females at 25°C, with similar results to males at 25°C (S12 Fig). There was a significant interaction between survival, Wolbachia variant, and temperature (Cox proportional hazard model with mixed effects (CHR), p < 0.001). All lines, including the Wolbachia-free line have a shorter lifespan at 25°C than 18°C, and even shorter at 29°C (p < 0.001 for all these comparisons). wMelCS_b did not affect the host lifespan at any temperature (p > 0.16 for all comparisons with the line without Wolbachia). wMelOctoless strongly reduces host lifespan at all tested temperatures (p < 0.001, each comparison with wMelCS_b) (Fig 5, S12 Fig). This deleterious effect is stronger at 18°C, where wMelOctoless is the tested variant with the highest impact on lifespan, although very similar and not statistically different from wMelPop2 with high Octomom copy number (p < 0.001, for all comparisons with other lines, p = 1 when compared with wMelPop2 with high Octomom copy number). At 25°C the effect of wMelOctoless on host lifespan is weaker (p = 0.001), and similar at 25°C and 29°C (p = 0.95). These results demonstrate that the new over-proliferative wMelOctoless also induces a cost to the host in terms of lifespan and the effect interacts with temperature, being stronger at lower temperature.
Fig 5. MelOctoless and MelPop2 are pathogenic.
Life span of males with different Wolbachia variants at 18°C (A), 25°C (B), and 29°C (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 represent 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 18°C (E), 25°C (F), and 29°C (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.

wMelPop2, as wMelPop, 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 wMelCS_b). This effect is much stronger at 25°C than at 18°C (p < 0.001 for contrasts between both lines wMelCS_b), and similar at 25°C and 29°C (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). wMelPop2 and wMelPop 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 25°C than 18°C, and stronger at 29°C than 25°C (p < 0.05 for these comparisons). In fact, they are not pathogenic at 18°C and an effect on host lifespan is only observed at 25°C and 29°C. 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.

wMelPop2 and wMelPop, 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 wMelPop2 and wMelPop lines with tightly controlled Octomom copy number (S10 Fig). At 25°C lines with both wMelPop and wMelPop2 with 3 copies of the Octomom region had a shorter lifespan than the line with wMelCS_b (p < 0.001), and no difference between them (p = 0.29). These results show that wMelPop2 and wMelPop 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 wMelCS_b and flies carrying either wMelOctoless or wMelPop2 (carrying 3 or 8-9 copies of Octomom) and followed the survival of their female progeny at 29°C. 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 wMelPop 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 18°C. 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). wMelCS_b was the least protective variant, while wMelOctoless the one that provided the highest protection. In general the over-proliferative Wolbachia variants confer stronger protection to DCV than wMelCS_b, although this difference is not always significant (Fig 6C, p < 0.001 for all comparisons, except for wMelPop (2-3 copies), p = 0.10).
Fig 6. wMelOctoless and wMelPop2 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 (10^3 TCID50/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 titers 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.
We also tested the correlation between the antiviral protection and *Wolbachia* proliferation. We found no correlation at either 18°C (*p* = 0.21), the temperature where the flies were kept after infection, or 25°C (*p* = 0.35), the temperature in which flies developed and were kept until being infected with DCV (Fig S14). We also tested the correlation between the antiviral strength of *Wolbachia*-induced anti-viral protection with *Wolbachia* titres in 0-1 day-old flies, as a proxy for *Wolbachia* titre at the day of infection, which is 3-5 day-old flies. We found a significant correlation between these parameters (Fig 6F, *p* = 0.024, |r| = 0.87). Overall, the new over-proliferative variants give more protection to viruses than wMelCS_b, and the strength of this protection is correlated with *Wolbachia* levels at the time of infection.

**Discussion**

We developed a new forward genetic screen and identified new *Wolbachia* over-proliferative variants. We characterized in detail two of these new mutants, wMelPop2 and wMelOctoless, and identified the genetic bases of their over-proliferation. wMelPop2 had an amplification of the Octomom region, which has been previously shown to lead to over-proliferation in the wMelPop variant [16,17]. wMelOctoless, on the other hand, had a deletion of this same Octomom region. These results further confirm and develop the complex role of this genomic region in the control of *Wolbachia* proliferation. An extensive phenotypic characterization of two of these lines showed both *Wolbachia* variants to shorten the host lifespan, as well as to increase antiviral protection. Moreover, we show that *Wolbachia* proliferation rate in *D. melanogaster* depends on the interaction between Octomom copy number, the host developmental stage, and temperature. Our analysis also suggests that the life shortening and antiviral protection phenotypes of *Wolbachia* are dependent on the related, but different, properties of the endosymbiont, rate of proliferation and titres at time of infection, respectively.

**An unbiased approach for genetically intractable symbionts**

A difficulty in studying obligate intracellular bacteria is that they are often genetically intractable, mostly because of their dependency on the host cells to survive and difficulty in isolating and growing them clonally. We decided to try to directly mutagenize and screen *Wolbachia* in the host. The main difficulty of this approach is how to identify a new mutant and link it with a phenotype in the *Wolbachia* population present within a host. We
calculated here that newly emerged female adults, with wMelCS_b, carry approximately 840,000 Wolbachia genome copies, probably corresponding to the same number of Wolbachia cells [30]. Since EMS induces random mutations, we expected mosaicism in the Wolbachia population at the individual fly level. Each new mutant, when generated, would be a unique cell within these 840,000 other cells. Since the Wolbachia phenotypes are normally measured at the individual host levels (e.g. Wolbachia titres, antiviral protection), the properties of individual or small numbers of mutant Wolbachia could be diluted and unmeasurable.

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

**Genetic bases of Wolbachia over-proliferation**

After discarding the possibility that mutations in the host nuclear or mitochondrial genomes were the cause of Wolbachia over-proliferation, we performed de novo assembly of the ancestral, wMelCS_b, and the new mutant variants, wMelPop2 and wMelOctoless. The assemblies generated complete full chromosomes of these Wolbachia. wMel and wMelPop were also assembled. This allowed us to identify single nucleotide differences and structural differences between these genomes [33]. To validate our pipeline we compared our wMel genome assembly to the reference wMel genome. We identified only seven indels and two SNPs, which we confirmed to be present in our line, by Sanger sequencing. Our assembly results also allowed us to confirm two previously identified SNPs between wMelCS_b (but also wMelOctoless and wMelPop2) and wMelPop. Additionally, our assembly provides a significant improvement over the previous wMelPop genome [26].

The only differences between the new over-proliferative variants and wMelCS_b were structural differences in the Octomom region. wMelPop2 has an amplification of this
region. The assembly confirms that the Octomom region is amplified in tandem [17], and that all copies are located in the *Wolbachia* genome. We had shown before that amplification of the Octomom region and the degree of this amplification determined wMelPop over-proliferative phenotype [16,17]. If the Octomom region copy number in wMelPop reverted to one, the variant became phenotypically identical to wMelCS_b [17]. We now show that if we start from wMelCS_b with one copy of Octomom, an increase in the copy number of this genomic region leads to an over-proliferative phenotype. Moreover, wMelPop and wMelPop2 variants, carrying the exact same copy number of Octomom, have identical phenotypes. These results further confirm the role of amplification of Octomom region in the over-proliferation of *Wolbachia*.

wMelOctoless has a deletion of the entire Octomom region. The deletion leaves behind one of the direct repeats flanking the Octomom region [16], suggesting that excision might be mediated by recombination. The causal link between deletion of this region and over-proliferation is further supported by an independent over-proliferative variant isolated in the screen, wMelOctoless2. This *Wolbachia* also has a deletion of Octomom as the only difference with wMelCS_b. These data show that deletion of the Octomom region leads to an over-proliferative phenotype in *Wolbachia*. Thus, we identified the second known *Wolbachia* mutation with a clear link between genotype and phenotype.

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

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

Both the deletion or the amplification of the Octomom region causing an over-proliferative phenotype seems to be a paradox. The resolution of this paradox and the mechanisms leading to these phenotypes will rely on the functional characterization of genes in the
Octomom region. These genes may be involved in interaction with the host, transcriptional regulation or DNA repair [16].

One possibility is that the amplification of the Octomom region and over-expression of particular genes in this region lead, mechanistically, to the same result as the absence of the genes. There are many examples of over-expression of a gene leading to a dominant negative phenotype. For instance, both over-expression or loss of a protein forming a gradient, abolish the gradient [36]. Also, over-expression of a member of protein complex may lead to loss of stoichiometry and therefore loss of functional complexes [37]. Another possibility is that the phenotypes are mediated by loss and amplification of different genes in the Octomom region. The second hypothesis is supported by the fact that the two mutant variants have similar but not identical phenotypes, and interact differently with temperature. Furthermore, wMelPop and wMelPop2 have a higher rate of proliferation than wMelOctoless at 25°C and 29°C. Therefore, their phenotype is stronger than the phenotype associated with the Octomom region loss-of-function.

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

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

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

We found a complex interaction between temperature and proliferation of the different Wolbachia variants in adults. wMelOctoless proliferates faster than wMelCS_b, to a similar extent, at all temperatures. wMelPop and wMelPop2, however, strongly interact with temperature and proliferate much faster at higher temperatures. We also confirmed here that the Octomom copy number in these variants modulates the proliferation rate [17], and interacts with temperature.

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

The new over-proliferative variants shorten the lifespan of *D. melanogaster*, as wMelPop does. Furthermore, we showed this phenotype to result from the interaction of Wolbachia genotype and temperature. wMelOctoless had a similar life shortening phenotype at all temperatures, although it was stronger at 18°C. wMelPop and wMelPop2 responded strongly to temperature, being much more costly at higher temperatures, as shown before for wMelPop [17,22,41]. Octomom copy number also influenced this interaction. While low copy number variants had no phenotype at 18°C, the high copy number wMelPop and wMelPop2 are pathogenic at this temperature. Therefore these variants can also be pathogenic at 18°C, contrary to previous data [29,41,42]. Interestingly, we find at all
temperatures a significant correlation between the proliferation rate of the *Wolbachia* variants and the life shortening phenotype. The faster the variants proliferate the shorter the host lifespan.

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

In summary, our results show the feasibility of forward genetic screens to study *Wolbachia* biology. Similar strategies may be used in the future to study other aspects of *Wolbachia*-host interactions or the biology of other genetically intractable endosymbionts. The new over-proliferative variant wMelPop2 confirms the causal link between amplification of the Octomom region and increase proliferation. Whereas the new loss-of-function mutant wMelOctoless reveals that this region is also required to control *Wolbachia* proliferation.

**Materials and Methods**

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

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

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

The bacterial community associated with the fly stocks was homogenized as in Pais *et al.* [44], with minor modifications. Briefly, we collected eggs for 6 hours in fresh agar plates supplemented with live yeast and sterilized the eggs surface by consecutive washes on
2.1% sodium hypochlorite (NaOCl) solution (10 minutes), 70% ethanol (5 minutes) and sterile water (5 minutes). Next, we transferred axenic eggs to sterile fly food supplemented with 40µL of 1:1 overnight culture of *Acetobacter* OTU 2753 and *Lactobacillus* OTU 1865 [44]. We confirmed the presence of these bacterial species by squashing five females aged 3–6 days in sterile 1x PBS, plating 30µL of the lysate in mannitol plates, incubate them at 25°C for 72h, and identify bacteria by colony morphology.

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

To select for flies carrying wMelPop and wMelPop2 with a desired Octomom copy number, we proceeded as in Chrostek and Teixeira [17], with minor modifications. Briefly, we allowed 5–20 virgin females to cross with 2–3 *Wolbachia*-uninfected males of the Drosdel w^1118^ isogenic background in individual vials, and lay eggs for 3-4 days. Females were then collected in individual tubes for DNA extraction and Octomom copy number determination by qPCR.

**Determination of time for Wolbachia titres recovery**

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

**Forward genetic screen**

We attempted to mutagenize *Wolbachia in vivo* by feeding its host with the mutagen EMS. DrosDel w^1118^ isogenic flies carrying wMelCS_b were first raised in standard fly food or fly food supplemented with tetracycline (from 1.5625 to 12.5µg/ml). Virgin females were collected, starved for 6h, and then fed on a 1% sucrose solution with EMS concentrations ranging from 10 to 8,000mM. Control flies fed on sucrose solution only. A dye was added to the feeding solution to confirm intake and feeding proceeded for 13h (overnight).
EMS-fed females (G0), and control females, were mated individually with 2–3 Wolbachia-free Drosdel $w^{1118}$ isogenic males, egg-laying was allowed for 3–4 days, and parents discarded. From the F1 progeny we collected virgin females, mated them individually with 2–3 Wolbachia-free Drosdel $w^{1118}$ isogenic males, and allowed egg laying for 3-4 days. These females were collected when 10 days old, and Wolbachia titres determined by qPCR. We followed the progeny of F1 females showing 50% or higher increase in Wolbachia titres relative to control flies in the same conditions. We have also transferred the progeny of these F1 for three more generations, without selection, and repeated the determination of Wolbachia titres in F4 females. In the same batch of experiments we may have tested more than one F1 or F4 progeny from each G0 female. Hence, over-proliferative Wolbachia variants isolated in the same batch of treated females may be a result of a single event in the G0 female.

**Real time quantitative PCR**

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

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

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

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

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

Protection against Drosophila C Virus

Viral protection assays were blinded (i.e. virus and buffer pricking occurred without previous knowledge of their Wolbachia status). We produced and titrated the virus solution as described in Teixeira et al. [8]. We infected 50 3-5 days old males by dipping insect needles (Austerlitz Insect Pins) into a virus solution (DCV at 10⁵ TCID₅₀/ml in 50mM Tris-HCl, pH 7.5) and pricking flies anaesthetized under CO₂ in the thorax. An equal number of males were pricked with a buffer solution (50mM Tris-HCl, pH 7.5) and served as controls. After pricking, flies were incubated in groups of 10 individuals per vial, and kept at 18°C. Survival was followed as above.

Wolbachia proliferation during development and in adults

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

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

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

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

Genome assembly

Quality reads matching Drosophila genome (BioProject: PRJNA13812) were removed from the datasets through a custom Python script before proceeding with Wolbachia genome assembly. Illumina and Oxford Nanopore reads were mapped to the fly genome using BWA mem v0.7.12-r1039 [48] and minimap2 v2.17-r941 [49] respectively. We used Unicycler v0.4.8-beta [50] assembly pipeline on the remaining reads in order to assemble the Wolbachia genomes. Briefly, Unicycler uses Illumina data to produce a repeats-limited image graph using Spades v3.9.0 [51], which was further refined through Bandage v0.8.1 [52]. Both small short nodes and nodes with no homology with wMel genome (AE017196.1) upon blastn v2.8.1+ [53] search were removed. Next, repeats were resolved
by bridging Spades’ assemblies with Oxford Nanopore long reads. The resulting draft assemblies were polished using Racon v1.3.1 [54] and Pilon v1.23 [55] and rotated so that genomes begin at the dnaA gene.

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

Read mapping and mutation calling

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

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

Statistical analysis

All the statistical comparisons were performed in R v4.0.0 [60]. To compare Wolbachia titres across multiple groups, we used log linear models (LMM) or generalized linear mixed models assuming Gamma errors (GLMM). The effect of EMS on Wolbachia titre was tested using non-linear regression. We estimated the doubling time of Wolbachia variants using the equation \(\log(2)/\beta\), with \(\beta\) being the coefficients of a log linear 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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References


Supporting information

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

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

S2 Fig. EMS decreases female fecundity and *Wolbachia* titre in the next generation in a dose-dependent manner.
The total number of eggs (A) and adults (B) of females treated with varying doses of EMS. The reproductive output of 10 females was determined in the first ten days after EMS treatment by daily transferring females to new vials for egg laying. Females fed on a sucrose solution served as controls. Each dot represents the total number of eggs (A) or adults (B) laid by individual females during ten days. The effect of EMS on the reproductive output of females was estimated using a non-linear model and was highly significant (p < 0.001 for both numbers of eggs and adults per female). (C-D) Wolbachia titres in the F1 progeny of flies treated with varying EMS doses. Wolbachia titre was quantified on individual females (n=5–13), after laying eggs for three days. Wolbachia titres were normalized against the titres of untreated females. Dashed red lines represent the mean value predicted using non-linear models. The effect of EMS on Wolbachia titres in the next generation was highly significant (p < 0.001 for both panels).

S3 Fig. Isolation of over-proliferative Wolbachia variants.

(A-D) Relative Wolbachia titres in a control (wMelCS_b) and EMS-treated Drosophila lines. Lines to set up the next generation was selected as described for Fig 1. Line 2B was isolated in the same batch as for Line 2A (wMelOctoless) and they may be not independent. Likewise, Lines 3A (wMelOctoless2), 3B, and 3C were also isolated in the same batch.

S4 Fig. Generation of isogenic Drosophila lines with wMelPop2 and wMelOctoless.

The first, second and third chromosomes of flies carrying wMelPop2 and wMelOctoless were replaced through the use of balancer chromosomes. Wolbachia infection (and also mitochondria) was kept in the stock by crossing females with Wolbachia with selected males. The mitochondria are only shown in females because of its strictly maternal transmission. All males were free of Wolbachia infection. Dashed lines indicate the genotype selected from the previous cross. Virgin female in the first cross were considered mutant in all chromosomes (*), for illustrative purposes. Question marks (?) represent recombined chromosomes.

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

The relative coverage in a selected genomic region. As detailed for Fig 2B, Illumina paired-end reads were mapped to wMelCS_b (GenBank: CP046924.1) genome, and the number of reads mapping to each position were normalized by dividing to the median coverage across the genome. Coverage information for wMelCS_b, wMelPop2 and wMelOctoless is also given in Fig 2B. We identified the deletion of Octomom as the cause of over-proliferation in lines 2B and line 3A (wMelOctoless2), as no other difference was found when compared to wMelCS_b.

S6 Fig. Confirmation of deletion of Octomom genes in wMelOctoless by qPCR.

The deletion of individual Octomom genes was confirmed using qPCR. The copy number of three genes outside the Octomom region were also determined. Five females carrying wMelCS_b or wMelOctoless were used in the analysis. The copy number of wMelOctoless genes is relative to that of wMelCS_b.
S7 Fig. Selection for stocks with a desired Octomom copy number.

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

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

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

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

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

S10 Fig. wMelPop2 and wMelPop are phenotypically indistinguishable.

(A) WD0513 copy number of wMelPop2 and wMelPop in two experimental replicates. Using WD0513 as a proxy, the Octomom copy number of wMelPop2 and wMelPop was tightly controlled prior to phenotypic comparison. (B) Wolbachia relative titres at 25°C. The progeny of wMelPop2- and wMelPop-infected females carrying three copies of Octomom was used to set up the experiments. Males that developed at 25°C were collected upon eclosion, aged to specific time-points and used to determine Wolbachia titers using qPCR. Wolbachia titers were normalized to that of wMelCS_b-carrying flies collected on the day of eclosion. Proliferation rates of wMelPop2 and wMelPop were not different (p = 0.32). (C) Lifespan of males (solid lines) and females (dashed lines) flies at 25°C. The experiment was performed twice, as described for Fig 3. Males were transferred to new vials every five days, while females every four days. (D) Coefficients of a Cox mixed model, representing the effect of wMelPop2 and wMelPop on the lifespan relative to wMelCS_b-carrying flies. wMelPop2 and wMelPop was equally pathogenic (p = 0.29).

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

(A) Relative copies of WD0513 throughout the fly development. Samples were collected and processed as described in Fig 4. Relative copies of WD0513 was determined using qPCR and are normalized to wMelCS_b. There is no significant change of Octomom copy number in wMelPop2 during fly development (p = 0.88). (B) The relative copies of WD0513 during the adult life at 18°C, 25°C, and 29°C. 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 18°C (A), 25°C (B), and 29°C (C). An experimental replicate of these experiments is given in Fig 5A-C, respectively. (D) Lifespan of females at 25°C in two experimental replicates. Experiments consisted of collecting 50 females upon adult eclosion and following their survival at 25°C 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 29°C. Virgin females carrying wMelCS_b were crossed with males with wMelOctoless or wMelPop2. 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 29°C. 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 18°C (A), 25°C (B), and 29°C (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 18°C (D) and 25°C (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 \textit{w}^{118} isogenic males, allowed to egg-laying for 3–4 days and used in qPCR reations to determine \textit{Wolbachia} titres relative to the progeny of control females.

\textbf{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 \textit{Wolbachia} or mitochondria of \textit{D. melanogaster} Release 6 genome sequence (KJ947872.2:1–14,000) using BWA mem v0.7.12-r1039. \textit{Wolbachia} reference genome used for mapping the different reads are indicated. ND – not determined.

\textbf{S3 Table. Flies with \textit{w}^{MelCS}_b, \textit{w}^{MelOctoless} and \textit{w}^{MelPop2} carry an identical mitochondrial genome.}

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

\textbf{S4 Table. Assembly and annotation statistics.}

\textit{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 \textit{Wolbachia} genome size but the resulting genomes encoded for the same number of transfer, ribosomal and non-coding RNAs.

\textbf{S5 Table. SNPs and indels differences between different \textit{w}^{Mel} genomes.}

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

\textbf{S1 Text. Alignment of a genomic region, including Octomom, in different Wolbachia variants.}

An alignment summary of a genomic region, including Octomom, in \textit{w}^{MelCS}, \textit{w}^{MelPop2} and \textit{w}^{MelOctoless}.

\textbf{S2 Text. Confirmation of a SNP in the mitochondrial genome.}

Sanger sequencing of a PCR amplicon containing a SNP present in \textit{w}^{MelCS}_b, \textit{w}^{MelPop2} and \textit{w}^{MelOctoless}, but absent in \textit{w}^{MelPop} and \textit{w}^{Mel}.
Relative *Wolbachia* titer

![Box plots showing the relative Wolbachia titer at different tetracycline concentrations and fly generations post-treatment.](image-url)
Copy number of individual genes

WD0505
WD0507
WD0508
WD0509
WD0510
WD0511
WD0512
WD0513
WD0514
WD0519
rpoD

wMelCS_b
wMelOctoless
Relative copies of WD0513
Relative Wolbachia titer at the day of eclosion

- wMelCS\textsubscript{b} (1 copy)
- wMelOctoless (0 copies)
- wMelPop\textsubscript{2} (3 copies)
- wMelPop\textsubscript{2} (8-9 copies)
- wMelPop (2-3 copies)
- wMelPop (8-9 copies)
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

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 29°C

wMelCS_b (1 copy)

wMelPop2 (3 copies)

wMelPop2 (8-9 copies)

wMelPop (2-3 copies)

wMelPop (8-9 copies)

Days post eclosion
Figure 1: Survival rates of males and females across different temperatures and Wolbachia strains.

A) Percentage of males alive at 18°C
B) Percentage of males alive at 25°C
C) Percentage of males alive at 29°C

D) Percentage of females alive at 25°C - Replicate 1
E) Percentage of females alive at 25°C - Replicate 2

The graph in E shows the coefficients of the Cox mixed model, with significant differences indicated by letters a, b, c, d, e, and f.
**A** Percentage of females alive at 29°C (Replicate 1)

- **wMelCS_b (1 copy)** × **wMelOctoless (0 copies)**
- **wMelCS_b (1 copy)** × **wMelPop2 (3 copies)**
- **wMelCS_b (1 copy)** × **wMelPop2 (8-9 copies)**

**B** Percentage of females alive at 29°C (Replicate 2)

- **wMelCS_b (1 copy)** × **wMelOctoless (0 copies)**
- **wMelCS_b (1 copy)** × **wMelPop2 (3 copies)**
- **wMelCS_b (1 copy)** × **wMelPop2 (8-9 copies)**

**C** Coefficients of Cox mixed model

- \( \gamma \text{ wMelCS}_b \text{ (1 copy)} \times \sigma \text{ wMelOctoless} \text{ (0 copies)} \)
- \( \gamma \text{ wMelCS}_b \text{ (1 copy)} \times \sigma \text{ wMelPop2} \text{ (3 copies)} \)
- \( \gamma \text{ wMelCS}_b \text{ (1 copy)} \times \sigma \text{ wMelPop2} \text{ (8-9 copies)} \)
- \( \gamma \text{ wMelOctoless} \text{ (0 copies)} \times \sigma \text{ wMelCS}_b \text{ (1 copy)} \)
- \( \gamma \text{ wMelPop2} \text{ (3 copies)} \times \sigma \text{ wMelCS}_b \text{ (1 copy)} \)
- \( \gamma \text{ wMelPop2} \text{ (8-9 copies)} \times \sigma \text{ wMelCS}_b \text{ (1 copy)} \)
A. Life-shortening (18°C) vs. Wolbachia titer at the day of eclosion

B. Life-shortening (25°C) vs. Wolbachia titer at the day of eclosion

C. Life-shortening (29°C) vs. Wolbachia titer at the day of eclosion

D. Anti-viral protection vs. doubling time 18°C

E. Anti-viral protection vs. doubling time 25°C
A Percentage of flies alive after DCV infection

B Percentage of flies alive after buffer pricking

Days post infection

0 5 10 15 20 25 30 35 40

Percentage

100% 75% 50% 25% 0%

Days post infection

0 5 10 15 20 25 30 35 40

Percentage

100% 75% 50% 25% 0%

Wolbachia-free

wMelCS_b (1 copy)

wMelOctoless (0 copies)

wMelPop2 (3 copies)

wMelPop2 (8-9 copies)

wMelPop (2-3 copies)

wMelPop (8-9 copies)