PLOS | BIOLOGY

RESEARCH ARTICLE

Mutualism Breakdown by Amplification of *Wolbachia* Genes

Ewa Chrostek*, Luis Teixeira*

Instituto Gulbenkian de Ciência, Oeiras, Portugal

* e.chrostek@gmail.com (EC); lteixeira@igc.gulbenkian.pt (LT)

Abstract

Most insect species are associated with vertically transmitted endosymbionts. Because of the mode of transmission, the fitness of these symbionts is dependent on the fitness of the hosts. Therefore, these endosymbionts need to control their proliferation in order to minimize their cost for the host. The genetic bases and mechanisms of this regulation remain largely undetermined. The maternally inherited bacteria of the genus *Wolbachia* are the most common endosymbionts of insects, providing some of them with fitness benefits. In *Drosophila melanogaster*, *Wolbachia w*MelPop is a unique virulent variant that proliferates massively in the hosts and shortens their lifespan. The genetic bases of *w*MelPop virulence are unknown, and their identification would allow a better understanding of how *Wolbachia* levels are regulated. Here we show that amplification of a region containing eight *Wolbachia* genes, called Octomom, is responsible for *w*MelPop virulence. Using *Drosophila* lines selected for carrying *Wolbachia* with different Octomom copy numbers, we demonstrate that the number of Octomom copies determines *Wolbachia* titers and the strength of the lethal phenotype. Octomom amplification is unstable, and reversion of copy number to one reverts all the phenotypes. Our results provide a link between genotype and phenotype in *Wolbachia* and identify a genomic region regulating *Wolbachia* proliferation. We also prove that these bacteria can evolve rapidly. Rapid evolution by changes in gene copy number may be common in endosymbionts with a high number of mobile elements and other repeated regions. Understanding *w*MelPop pathogenicity and variability also allows researchers to better control and predict the outcome of releasing mosquitoes transinfected with this variant to block human vector-borne diseases. Our results show that transition from a mutualist to a pathogen may occur because of a single genomic change in the endosymbiont. This implies that there must be constant selection on endosymbionts to control their densities.

Citation: Chrostek E, Teixeira L (2015) Mutualism Breakdown by Amplification of *Wolbachia* Genes. PLoS Biol 13(2): e1002065. doi:10.1371/journal.pbio.1002065

Academic Editor: Harmit S. Malik, Fred Hutchinson Cancer Research Center, UNITED STATES

Received: August 12, 2014

Accepted: January 5, 2015

Published: February 10, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Fundação para a Ciência e Tecnologia (http://www.fct.pt) grant PTDC/SAU-MII/105655/2008 and the Wellcome Trust (http://www.wellcome.ac.uk) grant 094664/Z/10/Z. EC is supported by the PhD fellowship from Fundação para a Ciência e Tecnologia SFRH/BD/51625/2011. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Author Summary

Insects frequently carry intracellular bacteria that are passed from generation to generation through their eggs. These intracellular symbionts can be beneficial or parasitic, but because of their mode of transmission, they are always dependent on the reproduction of their carriers. They therefore have to control their own growth in order to minimize

Abbreviations: DCV, *Drosophila* C virus; qPCR, quantitative PCR.

deleterious effects on the host. Bacteria of the genus *Wolbachia* are the most common maternally transmitted intracellular bacteria in insects. Most *Wolbachia* variants that are naturally associated with the fruit fly *Drosophila melanogaster* are benign to their hosts and provide them with protection against viruses. However, *w*MelPop is a virulent *Wolbachia* variant that over-replicates massively and shortens the lifespan of its fruit fly host. Here we show that amplification of a *Wolbachia* genomic region containing eight genes—called Octomom—is responsible for the pathogenic effects of *w*MelPop. Our results provide a link between genotype and phenotype in *Wolbachia* and show that virulence in symbionts can be simply caused by increases in gene copy number. These results also indicate that gene copy number variation may be a common mechanism underlying rapid evolution of intracellular symbionts.

Introduction

Vertically transmitted bacterial endosymbionts are widespread in arthropods, particularly in insects [1]. Many endosymbionts are mutualists and confer a fitness advantage to the host. The benefits may range from metabolic provisioning to protection against pathogens [2]. Other symbionts act as parasites and manipulate host reproductive biology in order to increase the relative fitness of their carriers [3]. In both cases, the density of endosymbionts within hosts is a crucial factor determining their prevalence in host populations [4,5].

Symbiont densities are determined by host and symbiont genetic diversity and environment [4,6–10]. These densities are under selection at the level of the host and the symbiont. Interestingly, there are conflicting selective forces at the level of the symbiont. Higher symbiont densities are associated with higher transmission fidelity and stronger phenotypes induced in the host [4,5,8,11–17]. Theoretically, this should lead to a selection for higher densities. On the other hand, high symbiont levels may have a negative impact on host fitness [8,9,17–19]. Since vertical transmission leads to dependence of the symbiont on the fitness of the host, it is advantageous for endosymbionts to limit their densities and consequently minimize the cost to their hosts. Thus, a key question in the field of host–microbe interactions is how symbionts regulate their replication and resulting densities to achieve an equilibrium between these opposing selective forces.

Wolbachia is conceivably the most prevalent bacterial endosymbiont of insects [20,21], and its interactions with hosts have been studied extensively. *Wolbachia* is maternally transmitted and exhibits a range of phenotypes. These include cytoplasmic incompatibility and other reproductive manipulations that potentiate *Wolbachia* spread in host populations [22]. Some *Wolbachia* strains have also been shown to be metabolic mutualists [23] or to protect insects from viral infections [24–27]. The *Wolbachia* strain infecting *Drosophila melanogaster*, *w*Mel, exerts only a weak cytoplasmic incompatibility in laboratory conditions [28], and this reproductive manipulation seems not to be expressed in field conditions [29]. Since cytoplasmic incompatibility cannot explain *Wolbachia* prevalence in *D. melanogaster* populations [28,30], it was suggested that *w*Mel exerts positive fitness effects on its hosts [29]. More recently, it was shown that *w*Mel provides *Drosophila* with strong resistance to systemic and oral infection with the natural pathogen *Drosophila* C virus (DCV) [24,25,31]. This protection extends to RNA viruses of different families [24,25,27,32], indicating that *w*Mel protects against a wide range of RNA viruses. Some of the fastest evolving genes in *D. melanogaster* are involved in antiviral RNA interference and are under strong positive selection [33]. Therefore, viruses seem to be a strong selective force in *D. melanogaster*. Moreover, several viruses, including DCV,

have been isolated from natural populations of *D. melanogaster* [34–36]. Although there are no data regarding *Wolbachia* antiviral protection in natural populations, the *D. melanogaster*–*w*Mel–DCV interaction fulfills many of the criteria for defensive mutualism [37]. Therefore, antiviral protection may be the cause of *w*Mel maintenance in *D. melanogaster* natural populations.

Natural *w*Mel variants have a small effect on host longevity, yet they provide a strong antiviral protection [8]. This protection is positively correlated with *Wolbachia* density: the higher the titers of *Wolbachia*, the higher the antiviral protection [8,17,18,26,38–40]. On the other hand, high endosymbiont densities can have a cost in the absence of viral infection, and *Wolbachia* variants conferring strong protection often shorten the lifespan of the flies [8,18]. There is thus a fine balance between density, benefit, and cost to the host.

The *w*Mel variant *w*MelPop breaks this balance and is clearly pathogenic: it over-proliferates and dramatically shortens the lifespan of infected flies [8,19,41,42]. *w*MelPop is, hence, an exceptional vertically transmitted symbiont. Its uniqueness was immediately recognized as providing a tool to better understand regulation of vertically transmitted symbionts and the biology of *Wolbachia* [43].

Understanding the cause of the *w*MelPop phenotype and regulation of *Wolbachia* densities is also important from an applied perspective. Several *Wolbachia* strains, including *w*MelPop, have been transinfected into mosquito vectors of human diseases, where they can interfere with arboviruses or other pathogens [44–52]. The purpose of this research is to release *Wolbachia*-carrying mosquitoes refractory to human pathogens into natural populations and prevent infections in humans. *Aedes aegypti* mosquitoes carrying *Wolbachia* are more resistant to dengue virus and are already being tested in the field [44,50,53–55]. Different variants of *w*Mel transinfected into *A. aegypti* show a trade-off between host fitness and resistance to dengue virus. A *w*MelPop-derived strain gives higher resistance to dengue but has a high fitness cost, which may prevent it from stably infecting natural mosquito populations [56,57]. On the other hand, a *w*Mel-derived strain confers lower protection to dengue virus but is able to stably invade *A. aegypti* populations [50,53–55]. Ideally, a further understanding of the system would allow researchers to use *Wolbachia* strains with a better ratio of antiviral protection to cost. Moreover, since *w*MelPop has been transinfected into mosquitoes [44–48], understanding the pathogenicity of this *Wolbachia* variant is crucial for predicting *w*MelPop dynamics in the released mosquito populations.

Finding the genetic basis of *w*MelPop pathogenicity is essential to understanding its phenotype. Difficulty in the functional analysis of *Wolbachia* lies in its refractoriness to genetic manipulation. Nonetheless, genomic analyses have provided insight into the cause of *w*MelPop pathogenicity. The first genomic map of *w*MelPop was published in 2003 [58], while the full genome of the similar *w*Mel was published in 2004 [59]. Analyses of polymorphic genomic markers and whole genome assemblies have shown that *w*MelPop is closely related to *w*MelCS variants [8,60–62]. We have recently identified genetic differences between *w*MelPop and the closely related non-pathogenic *w*MelCS_b [8]. The *w*MelPop genome contains an amplification of a ~21-kb region, named Octomom, that includes eight *Wolbachia* genes (*WD0507* to *WD0514*) flanked by direct repeats. This amplification in *w*MelPop was also described by Woolfit and colleagues [62]. Apart from this amplification, we found only one synonymous SNP unique to *w*MelPop (position 943,443, G>A) [8]. Therefore, we hypothesized that Octomom region amplification underlies *w*MelPop virulence. Gene amplification has previously been reported to change the pathogenicity of other bacteria and viruses [63–67].

Here we show strong evidence that, in support of our original hypothesis, Octomom region amplification is the cause of the *w*MelPop phenotypes of over-replication and pathogenicity.

Results

Currently, *Wolbachia* cannot be genetically manipulated, which hinders functional studies on *Wolbachia* genes. However, bacterial amplified DNA sequences have been described before as unstable [64], leading us to test the hypothesis that natural variation in Octomom copy number exists and causes distinct phenotypes. To detect Octomom copy number variation, we tested several single *Drosophila* females for the copy number of the Octomom gene *WD0513* in their *Wolbachia* bacteria (Fig. 1A). The copy number of *WD0513* was determined by quantitative PCR on genomic DNA samples from single flies carrying *Wolbachia*, using *Wolbachia wsp* (*Wolbachia* surface protein) as a reference gene. *w*MelCS_b samples were used as reference samples for one *WD0513* copy, based on the coverage analysis of our previous *Wolbachia* sequencing data [8]. We analyzed two fly stocks infected with *w*MelPop: w^{1118}, derived from the original stock in the Benzer lab [19], and a DrosDel isogenic w^{1118} (*iso*) stock into which we introgressed *w*MelPop from the w^{1118} stock [8]. All *w*MelPop samples analyzed had at least a duplication of the Octomom region, with high variation in *WD0513* copy number between individual females, ranging from two to ten copies (Fig. 1A). This copy number corresponds to the average *WD0513* copy number in the *Wolbachia* of each individual female (thus, differences in Octomom copy number between *Wolbachia* cells within each female may exist).

To check whether the *Wolbachia* Octomom region is amplified as a unit, we tested *WD0507* and *WD0513* copy number simultaneously in individual flies. The copy numbers of the two genes are the same in each fly (Figs. 1B and S1), suggesting integrity of the Octomom region. A common mechanism of gene amplification in bacteria leads to tandem duplications and the formation of new junctions between units [64]. We detected the presence of this new predicted *WD0514–WD0507* junction by PCR and Sanger sequencing (Fig. 1C; S1 Text). These data show that Octomom copy number is highly variable and that the amplification is consistent with a tandem duplication.

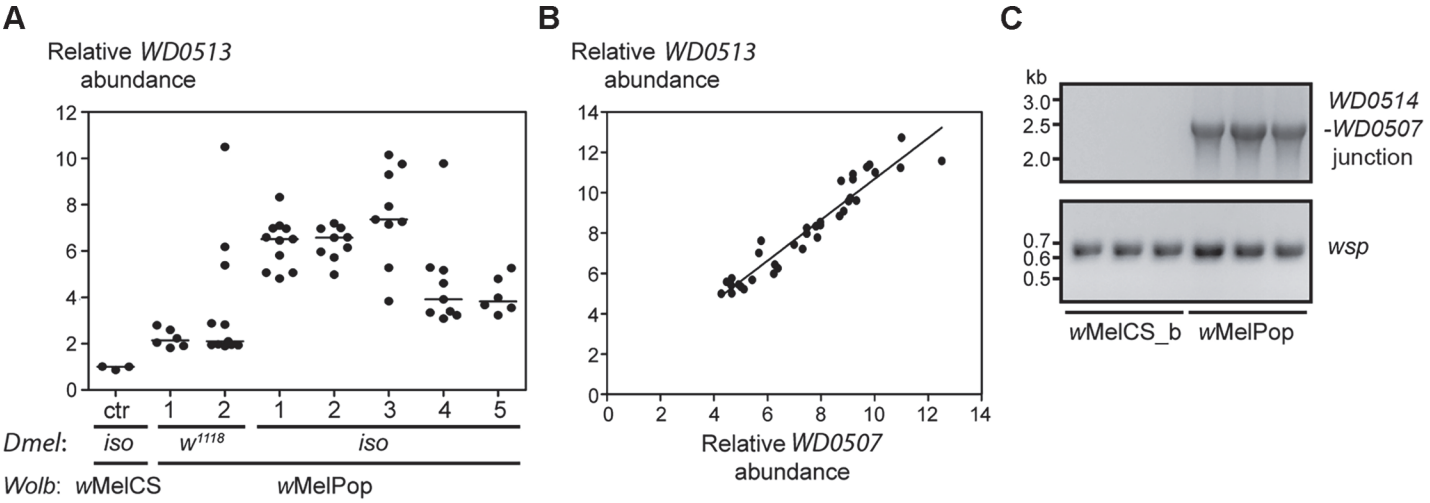

Fig 1. Individual *w*MelPop flies differ in Octomom copy numbers. (A) *WD0513* copy number variability in single females from two *w*MelPop stocks with w^{1118} and *iso* genetic backgrounds, relative to *wsp*. We tested two replicates of w^{1118} stock and five replicates of *iso* stock. *w*MelCS_b *iso* flies were used for copy number normalization (control [ctr]). Lines are medians of the replicates. Supporting data can be found in S1 Data. (B) Relation between *WD0507* and *WD0513* abundance in single *w*MelPop females. Each dot represents a female, and the regression line is shown. The estimates for the fitted regression line are slope = 1.036 ± 0.041, intercept = 0.182 ± 0.204, R^2 = 0.92. Supporting data can be found in S2 Data. (C) PCR of the predicted *WD0514–WD0507* junction in *w*MelPop flies. *w*MelCS_b was used as a negative control. Three samples of each *Wolbachia* variant were used. PCR for *wsp* gene was used as a DNA quality control.

doi:10.1371/journal.pbio.1002065.g001

To test Octomom amplification's effect on *w*MelPop virulence, we established *Drosophila* lines carrying *Wolbachia* with different Octomom copy numbers. Individual females with the highest and the lowest Octomom copy number were selected throughout several generations in both w^{1118} and *iso* backgrounds (Figs. 2 and S2). Octomom copy number is heritable: *Drosophila* mothers carrying high-copy *Wolbachia* produce mostly offspring with high-copy *Wolbachia*, while the inverse is observed for mothers with low-copy *Wolbachia*. In the course of selection for low Octomom copy number in the w^{1118} background, we recovered a *Drosophila* line carrying *w*MelPop with only a single copy of Octomom (Fig. 2C). This single-copy Octomom line had also lost the *WD0514–WD0507* junction detected in *w*MelPop with multiple Octomom copies (S3 Fig.). Therefore, from generation six onwards, we maintained three selection regimes: high, two, and one Octomom copy number. The *w*MelPop unique synonymous SNP is present in all three selection lines, including the line carrying *Wolbachia* with a single Octomom copy (S4 Fig.).

Taking advantage of the different selection lines, we compared the phenotypes of flies with *w*MelPop with different Octomom copy numbers. We predicted that the higher the copy number, the more severe the pathogenic phenotype, and that the one-copy Octomom line would be phenotypically identical to *w*MelCS_b. To perform these assays, we used the progeny of females individually tested for Octomom copy number. As *Wolbachia w*MelCS_b was associated with the *iso* fly genetic background and the one-copy Octomom line appeared only in the w^{1118} background, we used hybrids between *iso* and w^{1118} to directly compare the two (S1 and S2 Tables). All female hybrids resulting from these crosses have the same host genetic background

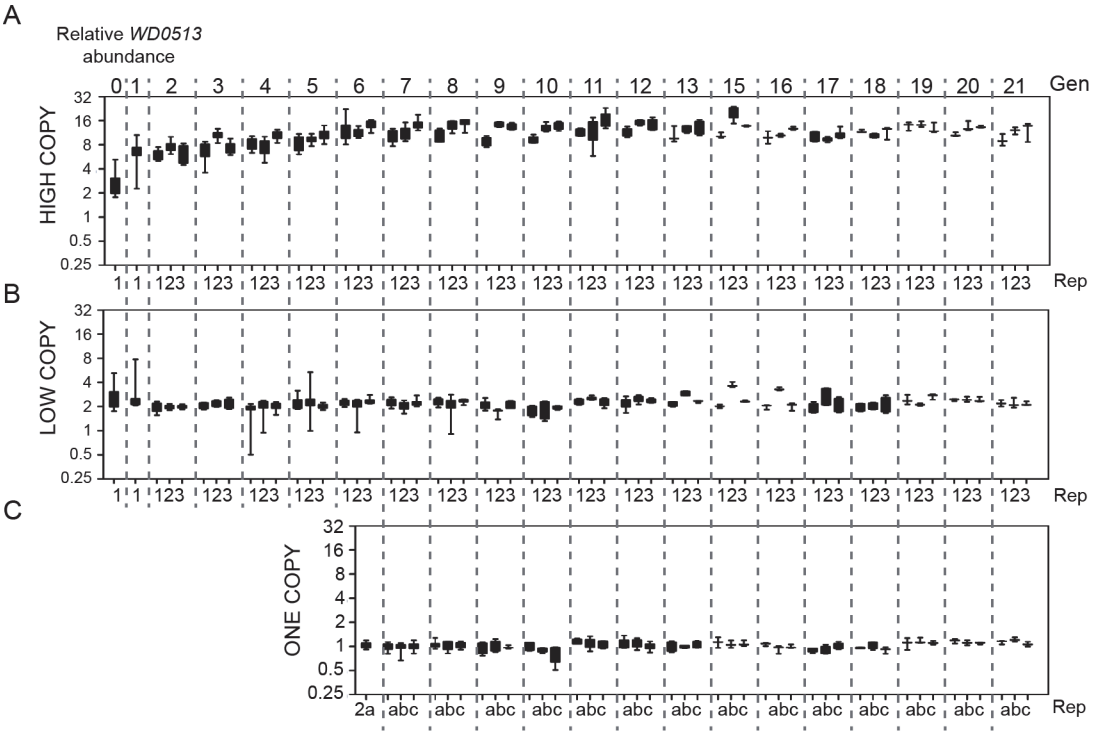

Fig 2. Octomom copy number is heritable and can be selected. Selection for high (A), low (B), and one (C) *WD0513* copy number in *w*MelPop in w^{1118} flies. Selection was started with females coming from one vial (Generation zero). The female with the highest (A) and lowest (B) *WD0513* abundance was always the founder of the next generation. At generation two, both selection regimes were split into three replicate lines. At generation six, we derived a one-copy line from the low-copy selection line two that was subsequently split into three lines kept independently (C). From that point on, the low-copy regime was maintained at two Octomom copies. The boxes extend from the 25th to 75th percentile, and the whiskers include all values. Dashed lines separate generations. Supporting data can be found in S3 Data. Gen, generation; Rep, replicate.

doi:10.1371/journal.pbio.1002065.g002

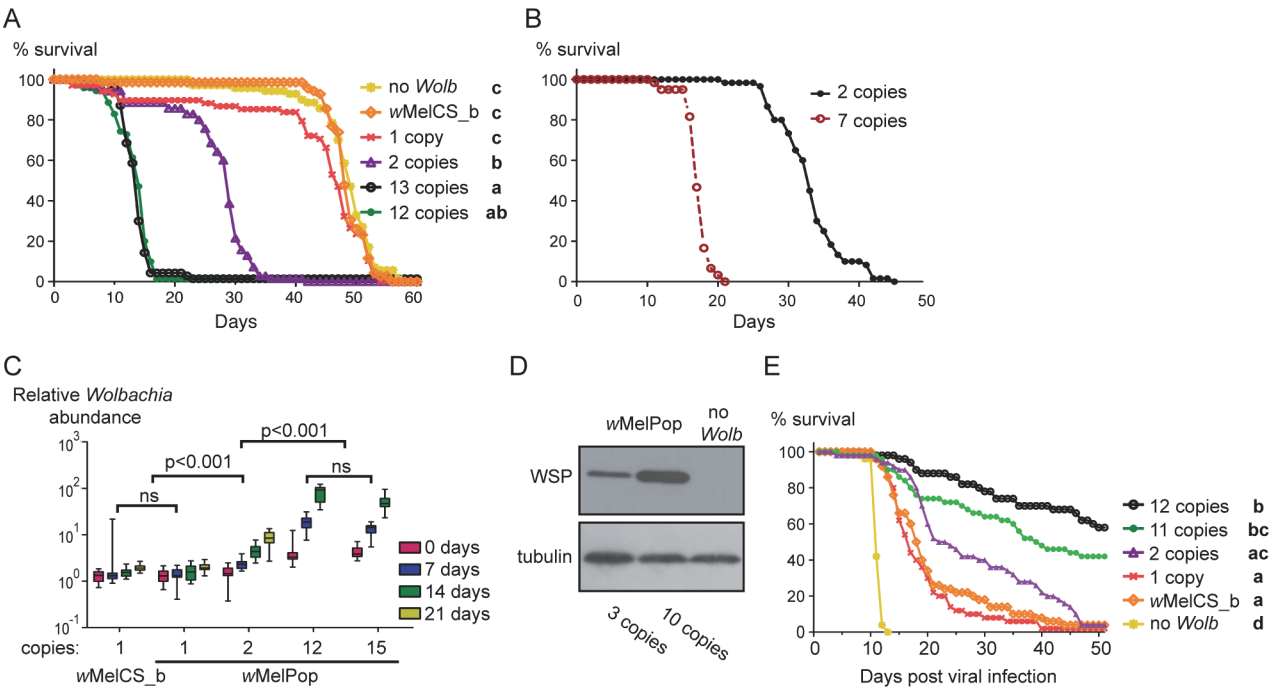

Fig 3. Octomom copy number determines *w*MelPop phenotypes. (A) Lifespan of female flies with different *w*MelPop Octomom copy numbers, flies with *w*MelCS_b, and *Wolbachia*-free controls at 29°C. Seventy females per line were analyzed; flies are the progeny from crosses between *iso* and w^{1118} lines. Bold letters on the right indicate groups with significantly different survival curves by Tukey's test of all pairwise comparisons of Cox hazard ratios. Supporting data can be found in S4 Data. (B) Lifespan of female flies from the forward selection *iso* low-copy line two (two Octomom copies) and the matched reverse selection line (seven copies) at 25°C. Mixed effects Cox model fit, $p < 0.001$. Supporting data can be found in S5 Data. (C) Time-course analysis of *Wolbachia* densities in female flies with different *w*MelPop Octomom copy numbers, starting at eclosion (day zero). Each bar represents *wsp* genomic levels in 16–20 single females (progeny from crosses between *iso* and w^{1118} lines). The boxes extend from the 25th to 75th percentile, and the whiskers include all values. Statistical analysis was performed using a log-linear model, and the *p*-values refer to comparisons of slopes. Supporting data can be found in S6 Data. ns, non-significant. (D) Western blot with anti-WSP antibody of pools of ten 10-d-old *iso* female flies with three or ten Octomom copies. *Drosophila* tubulin was used as a loading control. (E) Survival of female flies with different *w*MelPop Octomom copy numbers upon viral infection at 18°C. Fifty females per line were analyzed; flies are the progeny from crosses between *iso* and w^{1118} lines. Bold letters on the right indicate groups with significantly different survival curves by Tukey's test of all pairwise comparisons of Cox hazard ratios. Supporting data can be found in S7 Data.

doi:10.1371/journal.pbio.1002065.g003

(heterozygous between *iso* and w^{1118}) and differ in the *Wolbachia* inherited from the mother. Two high-copy Octomom lines, one in each genetic background, were used to control for potential host-genotype-specific maternal effects. Survival data demonstrate that differences in Octomom copy number lead to differences in host longevity: the more Octomom copies, the earlier the flies die (Figs. 3A and S5A–G). The line with one Octomom copy derived from *w*MelPop is indistinguishable from *w*MelCS_b and *Wolbachia*-free control (Figs. 3A and S5E–G). Even a single duplication of this region is enough to significantly shorten the host lifespan (median time to death is reduced by 39%) (Figs. 3A and S5E–G). The lifespan of flies from the two high-copy Octomom lines is further reduced, and there is no difference between these two lines (Figs. 3A and S5E–G). To further test the dependence of the phenotype on Octomom copy number, we reversed the direction of the selection in selected *iso* lines (choosing females with *w*MelPop with the highest Octomom copy number from the low-copy lines and with the lowest copy number from the high-copy lines, from generation 17 onwards) (S6A Fig.), simultaneously maintaining the forward selection regime as controls (S2 Fig.). Comparison of the lifespans of females from the forward and reverse selections confirmed that *Wolbachia* Octomom copy number determines *w*MelPop pathogenicity (Figs. 3B and S6B–D). Overall, Octomom copy number negatively correlates with longevity (S7 Fig.), and by manipulating copy number we can control *Wolbachia* virulence.

We next asked whether *Wolbachia* growth is associated with Octomom copy number. We tested *Wolbachia* levels in flies carrying *Wolbachia* with different Octomom copy numbers over time by real-time quantitative PCR (qPCR) (Fig. 3C). The higher the Octomom copy number, the higher the density of *Wolbachia*. The levels are different at eclosion, and the growth of *Wolbachia* is faster in flies with higher Octomom copy number. Both high-copy lines have the same *Wolbachia* growth rate, which is higher than the *Wolbachia* growth rate of the two-copy line. This growth rate, in turn, is higher than that of one-copy *w*MelPop and *w*MelCS_b, which have the same *Wolbachia* growth rate (Fig. 3C). We confirmed this effect of Octomom copy number on *Wolbachia* densities by comparing *Wolbachia* WSP protein abundance between flies harboring *w*MelPop with three versus ten Octomom copies (Fig. 3D). Flies carrying *Wolbachia* with ten Octomom copies had more WSP protein than flies harboring *Wolbachia* with three copies.

The density of *Wolbachia* is known to be related with *Wolbachia*-conferred antiviral protection, and *w*MelPop provides very strong protection [8,17,18,26,38–40]. This protective effect is best analyzed when flies are kept at 18°C, the temperature at which *w*MelPop is not pathogenic [41]. In flies that are raised from egg to adult at 25°C, *Wolbachia* levels at the time of infection are still related to Octomom copy number (see Fig. 3C). The survival of virus-infected flies confirmed that the higher the Octomom copy number, the stronger the antiviral protection (Figs. 3E and S5H). As with pathogenicity and growth rate, *w*MelPop with one Octomom copy is phenotypically identical to *w*MelCS_b in terms of antiviral protection.

We showed that Octomom copy number can change rapidly under direct selection (Figs. 2 and S2). Next we questioned whether Octomom copy number would be stable if this selection were relaxed. We observed that releasing our lines from copy number selection and maintaining them at 25°C in crowded vials for five generations caused a decrease in copy number in three out of four lines tested (S8 Fig.). The only line where the copy number did not change over the five generations started with two Octomom copies. Also, examination of another *w*MelPop stock did not show the expected life-shortening phenotype and, accordingly, Octomom amplification (S9 Fig.). Presumably, Octomom copy number reverted to one copy, and the phenotype was lost in this stock. All these results demonstrate that *w*MelPop *Wolbachia* is genetically and, consequently, phenotypically unstable.

Octomom amplification could promote *w*MelPop virulence in several ways, including via local or overall gene expression deregulation. The most parsimonious explanation, however, is that Octomom genes are overexpressed and that this causes the phenotype. Thus, we checked the expression of Octomom genes, immediately adjacent genes, and genes distant from the region by reverse transcription real-time qPCR. All Octomom genes, except *WD0514*, had a statistically significant higher expression in *w*MelPop than in *w*MelCS_b, but immediately adjacent genes did not (S10 Fig.). Moreover, analysis of one Octomom gene (*WD0511*) showed that expression level was dependent on *w*MelPop Octomom copy number (Fig. 4).

Discussion

Here we identify the genetic basis of *Wolbachia w*MelPop virulence. By selecting for *Wolbachia* with different Octomom copy numbers, we show a functional link between copy number and *w*MelPop phenotypes. The more copies of Octomom, the higher the densities of *Wolbachia*, and the faster the hosts die, but the stronger the antiviral protection. The evidence we provide is stronger than a simple correlation because we are controlling Octomom copy number and determining its effect. Furthermore, all *w*MelPop phenotypes are reverted in the line selected for one Octomom copy, establishing that Octomom copy number drives these phenotypes. There is evidence that *Wolbachia* levels determine the strength of the *Wolbachia*-associated

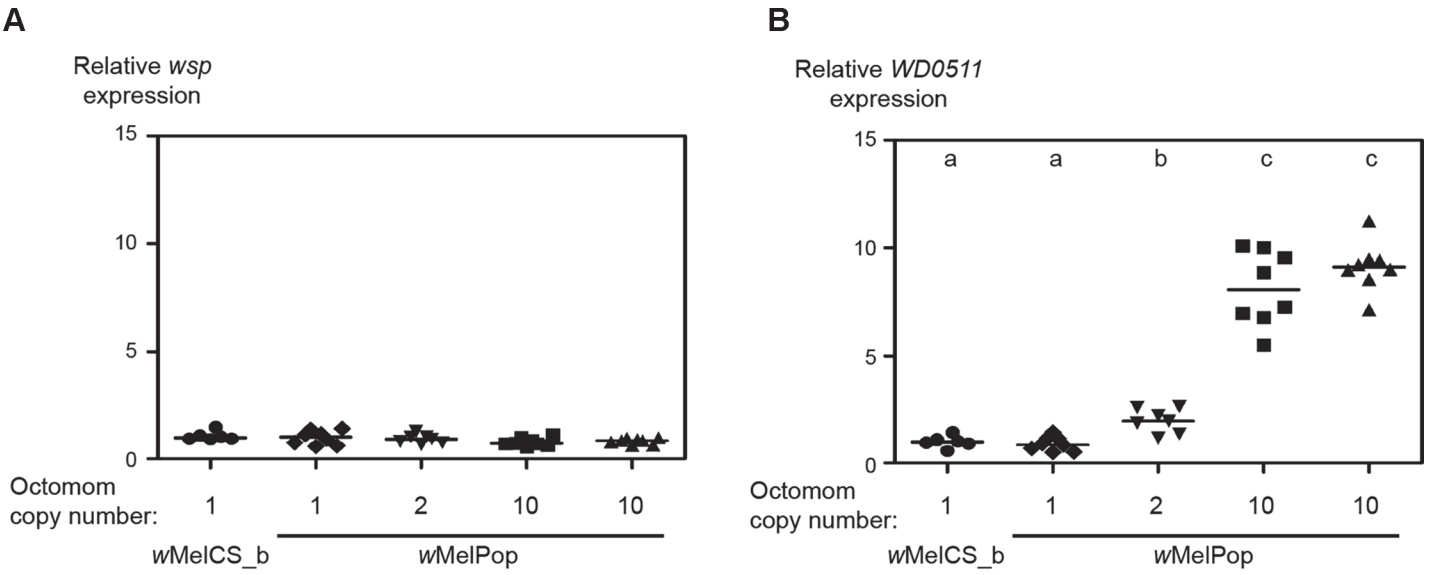

Fig 4. Octomom copy number determines expression of Octomom gene *WD0511*. Expression of *wsp* (outside the Octomom region) (A) and *WD0511* (within the Octomom region) (B) in flies carrying *w*MelPop with different Octomom copy numbers or carrying *w*MelCS_b. *WD0511* is differentially expressed between the lines (Tukey's test on linear model, $p < 0.002$), except between *w*MelCS_b and one-copy Octomom *w*MelPop and between the two high-copy Octomom *w*MelPop lines. Letters indicate groups with significantly different expression levels by Tukey's test of all pairwise comparisons of the linear model fit. There is no significant difference in the expression of *wsp* between any of the lines. All flies are hybrids between *iso* and w^{1118} genetic background. Hybrids represented by circles and triangles are derived from *w*MelCS_b or *w*MelPop *iso* mothers, while hybrids represented by diamonds, inverted triangles, and squares are derived from *w*MelPop w^{1118} mothers. Relative expression for each gene is calculated using *gmk* as a reference gene and is relative to that of the *w*MelCS_b samples. RNA was extracted from eight samples of ten 3- to 6-d-old *iso* males, and real-time qPCR was performed on cDNA with specific primers. Lines are medians of the replicates. Supporting data can be found in S8 Data.

doi:10.1371/journal.pbio.1002065.g004

phenotypes [8,17,18,26,38–40]. Therefore, the different replication capacities of *w*MelPop variants with distinct Octomom copy numbers are the likely cause of the differences in the other phenotypes.

Woolfit and colleagues also identified Octomom amplification in the *D. melanogaster* *w*MelPop genome and a deletion of the Octomom region in a mosquito-adapted *w*MelPop variant, *w*MelPop-PGYP [62]. As *w*MelPop-PGYP retained a strong life-shortening effect in *A. aegypti*, while an *A. aegypti*–adapted *w*Mel variant was benign, the authors dismissed Octomom as responsible for the high virulence of *w*MelPop also in *D. melanogaster*. We argue that the difference between *w*MelPop-PGYP and *w*Mel phenotypes in mosquitoes may be due to other genetic changes accumulated during their adaptation to a new host, some already described for *w*MelPop-PGYP [62]. This phenotypic difference may also exist because *w*Mel and *w*MelPop belong to the two different monophyletic groups of *Wolbachia* from *D. melanogaster*: *w*Mel group and *w*MelCS group [8,68–70]. *w*MelCS-like variants replicate faster than *w*Mel-like variants and sometimes shorten the lifespan of their natural host [8], and this difference may be exacerbated in mosquitoes. Relatedly, some *Wolbachia* bacteria transinfected into a new host species induce new pathogenic phenotypes [18,71–73].

Amplification of Octomom is in agreement with common gene amplification by nonequal recombination in bacteria [64]: (i) Octomom is flanked by direct repeats (see [8,62]), (ii) it seems to amplify as a unit, since different Octomom genes are equally amplified in the same fly (Figs. 1B and S1A), (iii) we confirmed the predicted novel joint point (Figs. 1C and S3; S1 Text), and (iv) the amplification is unstable.

The degree of Octomom amplification, and the associated strength of the phenotypes, can rapidly change and is fully reversible. This shows that *Wolbachia* can evolve rapidly, and adds

to the understanding of genome evolution of endosymbionts. Many endosymbionts have evolutionarily dynamic genomes [1,74]. Genomes of *Wolbachia* and other endosymbionts (including *Hamiltonella defensa*, *Serratia symbiotica*, *Sarocladium oryzae* principal endosymbiont [SOPE], and *Portiera*) are rich in mobile elements, prophages or phage-derived regions, and other repetitive DNA sequences [1,59,74–79]. These DNA elements may mobilize, amplify, or reduce in numbers, leading to genomic changes, but they can also mediate recombination and other genomic rearrangements. Comparative genomics of some closely related endosymbionts show extensive genomic rearrangements [75,76,78,80–85]. The same repetitive DNA elements may serve as a basis for gene amplification, as observed for Octomom. Consequently, gene copy number variation may be a common feature in these endosymbionts and may promote fast but reversible evolutionary changes. Accordingly, gene amplifications in other *w*Mel variants [8], other *Wolbachia* strains [80,81], and the whitefly endosymbiont *Portiera* [78] have previously been reported, although without any associated phenotypes.

Genotype–phenotype links are very rarely established in endosymbionts, as many of them cannot be cultured *in vitro*. Previous examples include a point mutation in *Buchnera aphidicola* that affects thermal tolerance provided to the aphid *Acyrthosiphon pisum* [86] and the loss of a prophage in *Hamiltonella defensa*, abrogating induced protection to parasitoids in the same aphid [87]. The involvement of Octomom genes in *Wolbachia* virulence provides a unique point of entry into understanding *Wolbachia*–host interactions at the molecular level. As Octomom genes are overexpressed and may cause the phenotype, functional analysis of Octomom-encoded proteins is required to better understand the *Wolbachia*–host interaction. The Octomom region is part of the *Wolbachia* accessory genome since it is not present in all *Wolbachia* strains and shows signs of horizontal gene transfer [8,88–91]. There are genes putatively encoding mobile elements in the flanking region (*WD0506* and *WD0515*, in the direct repeats) and inside Octomom (*WD0511*). Because of its structure and associated phenotype, the Octomom region resembles bacterial pathogenicity islands [92,93]. However, the pathogenicity seems to be expressed only when the region is amplified. The functions of Octomom genes are unknown and can only be speculated about based on the sequence of predicted proteins. Proteins encoded by three genes (*WD0512*, *WD0513*, and *WD0514*) have eukaryotic protein domains or homologs in arthropods (mosquitoes and *Daphnia*) and therefore may be effector proteins that interact with the host [8,88–91]. When highly expressed, these proteins could suppress host control over the symbiont. Other genes (*WD0506*–*WD0511* and *WD0515*) encode proteins that may be involved in transposition, DNA replication and repair, or transcriptional regulation [8]. Overexpression of these proteins may increase *Wolbachia*'s replication rate. It is crucial to determine which of these genes are involved in the regulation of *Wolbachia* density and which structural characteristics of the Octomom region are important.

Octomom copy number instability may confound past and future analyses of *w*MelPop phenotypes. For instance, Octomom copy number variation may have contributed to changes in *w*MelPop pathogenicity over time or associated with different host species or host genetic backgrounds [41,42,94]. This instability has to be taken into consideration in future applications of *w*MelPop-transinfected mosquitoes to prevent transmission of human pathogens. In the dengue vector *A. aegypti* transinfected with *w*MelPop-PGYP, currently being tested in the field [44,95], Octomom copy number instability is not a factor since this region is deleted [62]. However, *w*MelPop is also being transfected to other vectors of human diseases, such as the malaria-transmitting *Anopheles gambiae* [96] and the dengue and chikungunya vector *Aedes albopictus* [97].

We have shown variation in *Wolbachia* Octomom copy number between individual hosts within a population and across time. Genetic heterogeneity within individual hosts has been previously shown at the nucleotide level in *w*Cer1 and *w*Cer2 [98]. The instability of Octomom

copy number suggests that there is also a high level of heterogeneity between *Wolbachia* bacteria within individual insects. Analysis of the dynamics and consequences of heterogeneity in gene copy numbers in somatic or germline tissues may be important to understand host–endosymbiont interactions.

Vertically transmitted endosymbionts are subjected to different levels of selection. An increase in replication may confer a fitness advantage to the bacteria in intra-host competition but a disadvantage at the inter-host level, as it can have a high cost to the host and reduce symbiont transmission. A *Drosophila* line harboring *w*MelPop was most probably isolated in the laboratory because husbandry conditions buffered the cost to flies of pathogenic bacteria and because low host population numbers increased drift. Our results demonstrate that a single mutation (a duplication) can profoundly alter endosymbiont replication. This conversion of a mutualist into a pathogen by a single genomic event suggests that virulent mutations in microbial symbionts may be frequent and constantly counter-selected. Therefore, symbiont titers may be at a labile equilibrium achieved in the course of co-evolution and to a large extent selected at the level of the symbiont.

Materials and Methods

Fly Strains

D. melanogaster w^{1118} stock with *Wolbachia w*MelPop was kindly provided by Markus Riegler and Scott O'Neill. *w*MelPop OPL stock was kindly provided by William Sullivan and Laura Serbus. Both *w*MelPop stocks are derived from Min and Benzer original stock [19]. DrosDel isogenic background (*iso*) flies with no *Wolbachia* and with *w*MelCS_b or *w*MelPop were described before [8,24,99]. The *w*MelPop and mitochondria of this DrosDel isogenic background line derive from the w^{1118} stock [8].

DNA Extractions

DNA was extracted from individual flies (*w*MelPop) or pools of ten flies (*w*MelCS_b controls in the selection experiments). Each fly or pool of flies was squashed in 250 μl of 0.1 M Tris HCl, 0.1 M EDTA, and 1% SDS (pH 9.0) and incubated 30 min at 70°C. Next, 35 μl of 8 M CH_3CO_2K was added, and samples were mixed by shaking and incubated for 30 min on ice. Samples were then centrifuged for 15 min at 13,000 rpm at 4°C, and the supernatant was diluted 100× for qPCR.

RNA Extractions and cDNA Synthesis

For each sample, ten 3- to 6-d-old flies were pooled and homogenized with a plastic pestle in 1 ml of Trizol Reagent (Invitrogen). RNA was extracted according to manufacturer's protocol and resuspended in 50 μl of DEPC-treated water (Ambion). RNA concentrations were determined using a NanoDrop ND-1000 Spectrophotometer. cDNA was prepared from 1 μg of total DNAse-treated RNA using Random Primers and M-MLV Reverse Transcriptase (all Promega). Primers were pre-incubated with template RNA for 5 min at 70°C. Next, the enzyme was added, and reactions were placed at 25°C for 10 min, 37°C for 60 min, and 80°C for 10 min. cDNA was diluted 100× for qPCR.

Real-Time Quantitative PCR

The real-time qPCR reactions were carried out in the CFX384 Real-Time PCR Detection System (Bio-Rad) as described before [8]. Briefly, each of the reactions was performed with 6 μl of iQ SYBR Green Supermix (Bio-Rad), 0.5 μl of each primer (3.6 mM), and 5 μl of diluted DNA.

We performed at least two technical replicates per biological sample for each set of primers. Primer sequences were described before [8]. The following thermal cycling protocol was applied: initial 2 min at 50°C, denaturation for 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at 59°C, and 30 s at 72°C. Melting curves were examined to confirm the specificity of amplified products. Ct values were obtained using Bio-Rad CFX Manager software with default threshold settings. Ct values were subjected to a quality check—samples with standard deviation between technical replicates exceeding one were discarded. Relative amounts of transcripts and genes were calculated by the Pfaffl method [100]. To apply the method, the efficiency of each of the primer pairs was predetermined in a separate experiment. For the Octomom expression data, values were normalized to *gmk* expression. For the determination of the number of genomic Octomom copies, values were normalized to the single-copy *wsp* gene. For *Wolbachia* quantification, *wsp* levels were normalized to *Drosophila Rpl32*.

Sequencing of the *WD0514–WD0507* Junction

The *WD0514–WD0507* junction was amplified using specific primers (Link_seq_1 and Link_seq_2), and Sanger sequencing was performed with these primers and the primers annealing inside the junction (Link_seq_3–7) by Source Bioscience. Primer sequences are listed in S3 Table.

Selection Experiments

Selection for high- and low-copy Octomom *w*MelPop lines in w^{1118} and *iso* backgrounds was initiated with females from a single vial of each background. For each background, ten single females were separated into individual vials and allowed to lay eggs for 5 d before being sacrificed for determination of *Wolbachia WD0513* copy number. The offspring of the female with the highest and the lowest Octomom copy number was used to start the next generation. This general procedure was repeated at every generation of selection. Three replicates of high- and low-copy Octomom selection lines for each background were established at generation two. From that point on, we selected one female/line/generation with the desired Octomom copy number (based on real-time qPCR). Female age for egg laying (0–2 d) and qPCR (5–7 d) was controlled from generation four and two for the *iso* and w^{1118} lines, respectively. At generation seven of the w^{1118} lines, we started to also select for one-copy Octomom *w*MelPop. At this point we selected the female with *Wolbachia* with *WD0513* copy number closest to one for this selection regime, and the female with *Wolbachia* with *WD0513* copy number closest to two for the low-copy Octomom lines.

From generation two to generation 13 of the w^{1118} selection lines and from generation two to generation 22 of the *iso* selection lines, we were selecting from among six to ten females. From these generations on, we selected from three females per line.

At generation 14 of the w^{1118} lines and generation 18 of the *iso* lines, the selection was not performed.

Preparation of Flies for Phenotypic Analyses

For phenotypic analyses of flies carrying *w*MelPop with different Octomom copy numbers, single females were placed in vials, allowed to lay eggs for 5 d, and sacrificed to determine *WD0513* copy number. The progeny of females carrying *Wolbachia* with the specified Octomom copy numbers were selected for the phenotypic analyses. All lifespan assays were performed at 25°C and 29°C, the temperature regimes applied in the first report on *w*MelPop phenotypes [19].

In order to directly compare flies with *w*MelPop with the full range of Octomom copy numbers, flies with *w*MelCS_b, and flies without *Wolbachia*, we used hybrids between w^{1118} and *iso* genetic backgrounds (S1 and S2 Tables). Females with the desired *Wolbachia* status, which is transmitted to the next generation, were crossed with males from the other genetic background. Since females were used in the phenotypic analyses, their genetic backgrounds were all equal and heterozygous between w^{1118} and *iso*, irrespective of the direction of the crosses. The mitochondria from these two lines should be identical since they share a very recent common ancestor [8]. We used females with high Octomom copy number from both genetic backgrounds to control for the possible influence of the direction of the cross and maternal effects potentially associated with different backgrounds.

Lifespan and *Wolbachia* Levels Experiments

Females whose mothers' Octomom copy number was assessed by qPCR were collected at eclosion (ten per tube), allowed to mate for 24 h (five males per tube), separated from males, and either checked for survival at 25°C or 29°C every day or kept at 25°C and sacrificed at the indicated time points for *Wolbachia* density quantification. Females were maintained on a standard cornmeal diet without live yeast and were passed to fresh vials every 3 d. The mothers of females used for phenotypic analyses were derived from selection lines at the generations indicated in S2 Table.

Virus Production and Infection

DCV was produced and titrated as described before [8,24]. Infections were performed by pricking 1- to 2-d-old female flies with virus at 10^9 $TCID_{50}$ (median tissue culture infectious dose)/ml. After infection, flies were kept in vials without live yeast, ten flies per vial, at 18°C. It has been shown previously that *w*MelPop is not pathogenic to the flies at this temperature [41]. Flies were checked for survival daily and passed to fresh vials every 5 d.

Statistical Analysis

Survival data were analyzed by Cox proportional hazard mixed effects models. Octomom copy number was considered a fixed effect, and replicate tube (containing ten flies) within the same experiment was considered random. Model fitting was done using the coxme package in R [101]. Tukey´s test was applied for pairwise comparisons of Cox hazard ratios between flies with all *w*MelPop lines, flies with *w*MelCS_b, and flies without *Wolbachia*.

Analysis of growth curves of *w*MelPop lines with different Octomom copy number was performed with log-linear model fits (lm in R). The slopes of different fitted regression lines were compared and corrected for multiple comparisons (Bonferroni correction).

Spearman correlation between Octomom copy number and median time to death was performed in R (cor.test).

Comparison of the expression of several *Wolbachia* genes between *w*MelCS_b and *w*MelPop (S10 Fig.) was done with the *t*-test in R (t.test) and was corrected for multiple comparisons with the Bonferroni correction.

Comparison of *wsp* and *WD0511* gene expression between fly lines carrying different *Wolbachia* (Fig. 4) was performed with a log-linear model fit (lm in R), and the different lines were compared pairwise with a Tukey's test.

Western Blot

Ten mated females from high- and low-copy *iso* selection lines, whose mothers were individually tested for Octomom copy number, were aged for 10 d before protein extraction. Flies without *Wolbachia* were used as a negative control. Anti-WSP rabbit polyclonal antibody was kindly provided by Kostas Bourtzis [102,103] and pre-absorbed in fixed *Wolbachia*-free *D. melanogaster* embryos. Anti-beta-tubulin mouse monoclonal E7 antibody was acquired from the Developmental Studies Hybridoma Bank [104].

Supporting Information

S1 Data. Relative *WD0513* copy number in single females carrying *w*MelPop from different stocks (data for Fig. 1A).
(XLSX)

S2 Data. Relative *WD0507* and *WD0513* copy number from individual flies carrying *w*MelPop (data for Fig. 1B).
(XLS)

S3 Data. Relative *WD0513* copy number in *w*MelPop in w^{1118} flies throughout selection (data for Fig. 2).
(XLSX)

S4 Data. Lifespan data for flies carrying *w*MelPop with different Octomom copy numbers (data for Fig. 3A).
(XLS)

S5 Data. Lifespan data for flies carrying *w*MelPop from forward and reverse selection (data for Fig. 3B).
(XLS)

S6 Data. Time-course analysis of relative levels of *Wolbachia w*MelPop with different Octomom copy numbers (data for Fig. 3C).
(XLS)

S7 Data. Survival data for DCV-infected flies carrying *w*MelPop with different Octomom copy numbers, *w*MelCS_b, or no *Wolbachia* (data for Fig. 3E).
(XLS)

S8 Data. Relative expression of *wsp* and *WD0511* in flies carrying *w*MelPop with different Octomom copy numbers or *w*MelCS_b (data for Fig. 4).
(CSV)

S9 Data. Relative *WD0507*, *WD0510*, *WD0513*, *rpoD*, and *gmk* copy numbers from individual flies carrying *w*MelPop (data for S1 Fig.).
(XLS)

S10 Data. Relative *WD0513* copy number in *w*MelPop in *iso* flies throughout selection (data for S2 Fig.).
(XLSX)

S11 Data. Lifespan data for *iso* flies carrying *w*MelPop with different Octomom copy numbers (data for S5A Fig.).
(XLS)

S12 Data. Lifespan data for *iso* flies carrying *w*MelPop with different Octomom copy numbers (data for S5B Fig.).
(XLS)

S13 Data. Lifespan data for w^{1118} flies carrying *w*MelPop with different Octomom copy numbers (data for S5C Fig.).
(XLS)

S14 Data. Lifespan data for w^{1118} flies carrying *w*MelPop with different Octomom copy numbers (data for S5D Fig.).
(XLS)

S15 Data. Lifespan data for flies carrying *w*MelPop with different Octomom copy numbers, *w*MelCS_b, or no *Wolbachia* (data for S5E Fig.).
(XLS)

S16 Data. Lifespan data for flies carrying *w*MelPop with different Octomom copy numbers, *w*MelCS_b, or no *Wolbachia* (data for S5F Fig.).
(XLS)

S17 Data. Lifespan data for flies carrying *w*MelPop with different Octomom copy numbers, *w*MelCS_b, or no *Wolbachia* (data for S5G Fig.).
(XLS)

S18 Data. Survival data of DCV-infected flies carrying *w*MelPop with different Octomom copy numbers, *w*MelCS_b, or no *Wolbachia* (data for S5H Fig.).
(XLS)

S19 Data. Relative *WD0513* copy number in *iso* flies carrying *w*MelPop throughout reverse selection (data for S6A Fig.).
(XLSX)

S20 Data. Lifespan data for flies carrying *w*MelPop from forward and reverse selection (data for S6B Fig.).
(XLS)

S21 Data. Lifespan data for flies carrying *w*MelPop from forward and reverse selection (data for S6C Fig.).
(XLS)

S22 Data. Lifespan data for flies carrying *w*MelPop from forward and reverse selection (data for S6D Fig.).
(XLS)

S23 Data. Median time to death and Octomom copy number in experiments shown in Figs. 3A and S5A–G (data for S7 Fig.).
(XLS)

S24 Data. Relative *WD0513* copy number in flies carrying *w*MelPop in the absence of selection (data for S8 Fig.).
(XLSX)

S25 Data. Relative *WD0513* copy number in single females carrying *w*MelPop from different stocks (data for S9A Fig.).
(XLSX)

S26 Data. Lifespan data for flies carrying *w*MelPop OPL, *w*MelCS_b, or no *Wolbachia* (data for S9B Fig.).
(XLS)

S27 Data. Relative expression of Octomom genes and other *Wolbachia* genes in flies carrying *w*MelCS_b or *w*MelPop (data for S10 Fig.).
(XLS)

S1 Fig. Different Octomom genes are amplified to the same extent in individual *w*MelPop flies. Octomom gene copy number variability relative to *wsp* between *w*MelPop *iso* flies. qPCR was performed on DNA from single females from *iso* line three (Fig. 1A) for *WD0507*, *WD0510*, and *WD0513* (A) and *rpoD* and *gmk* (B). *w*MelCS_b flies were used for copy number normalization. Supporting data can be found in S9 Data.
(TIF)

S2 Fig. Selection for *w*MelPop with high and low Octomom copy number in *iso* flies. The bars for generation zero correspond to the data for *iso* line three from Fig. 1A. The female with the highest or lowest *WD0513* copy number was always the founder of the next generation. After the first generation, three females with high and low copy number gave rise to three replicate lines that were maintained separately for the subsequent generations. The boxes extend from the 25th to 75th percentile, and the whiskers include all values. Dashed lines separate the generations. Gen, generation; Rep, replicate. Supporting data can be found in S10 Data.
(TIF)

S3 Fig. PCR of the predicted *WD0514–WD0507* junction in flies harboring *w*MelPop with a single Octomom copy. *w*MelCS_b was used as a negative control, and *w*MelPop with two and ten Octomom copies were used as positive controls for the *WD0514–WD0507* junction. Flies without *Wolbachia* (*iso*) were used as a negative control for *wsp*. Two samples of each *Wolbachia* variant were used.
(TIF)

S4 Fig. Alignment of the sequences containing the *w*MelPop unique SNP site from *w*MelCS_b and *w*MelPop selection lines with one, two and a high number of Octomom copies. CLUSTAL O (1.2.1) multiple sequence alignment [105–107] was used to align the sequences surrounding the *w*MelPop unique SNP at position 943,443 in the w^{1118} selection lines. Position 943,443 is highlighted in yellow.
(TIF)

S5 Fig. Octomom copy number determines *w*MelPop phenotypes. (A and B) One hundred *iso* females from high- and low-copy selection regimes were checked for survival at 25°C every day. Mixed effects Cox model fit, high versus low copy number for both replicates, $p < 0.001$. Supporting data can be found in S11 and S12 Data. (C and D) One hundred w^{1118} females from high- and low-copy selection regimes were checked for survival at 25°C (C) or 29°C (D) every day. Mixed effects Cox model fit, high versus low copy number at both temperatures, $p < 0.001$. Supporting data can be found in S13 and S14 Data. (E–G) Sixty–seventy females carrying *w*MelPop with different Octomom copy numbers were monitored daily for survival at 29°C (E) or at 25°C (F and G). Females are the progeny from crosses between *iso* and w^{1118} lines. Letters refer to groups with significantly different survival curves according to Tukey's test of all pairwise comparisons of Cox hazard ratios. The experiment at 29°C is a replicate of the one presented in Fig. 3A. Supporting data can be found in S15–S17 Data. (H) One hundred females with different *w*MelPop Octomom copy numbers were pricked with DCV (10^9 $TCID_{50}$/ml), and survival was followed daily. Females are the progeny from crosses between *iso*

and w^{1118} lines. Letters refer to groups with significantly different survival curves according to Tukey's test of all pairwise comparisons of Cox hazard ratios. This experiment is a replicate of the one shown in Fig. 3E. Supporting data can be found in S18 Data.
(TIF)

S6 Fig. Phenotypic responses to reverse selection. (A) At generation 17 of the selection for *w*MelPop *iso* lines with high and low *WD0513* copy number (S2 Fig.), the selection was reversed. This reverse selection was performed in all three replicate lines from the high- and low-copy selection regimes by selecting the female with the highest *WD0513* abundance from each low-copy line and the female with the lowest *WD0513* abundance from each high-copy line (forward selection also continued, as shown in S2 Fig.). The boxes extend from the 25th to 75th percentile, and the whiskers include all values. Dashed lines separate the generations. Gen, generation; Rep, replicate. Supporting data can be found in S19 Data. (B and C) Lifespan of females of reversely selected high-copy lines was compared with that of high-copy females under forward selection at generation 22. Fifty females per line were used. (B) High-copy line one (nine Octomom copies) versus reverse high-copy line one (five copies) (C) High-copy line three (ten copies) versus reverse high-copy line three (six copies). Tukey's test on the mixed effects Cox model fit, high versus low copy number, $p < 0.001$ and $p = 0.0321$ for lines one and three, respectively. Supporting data can be found in S20 and S21 Data. (D) Lifespan of females from forward selection low-copy line three (3.5 Octomom copies) and the corresponding reverse selection line (eight copies) at generation 22. Fifty females per line were used. Tukey's test on the mixed effects Cox model fit, high versus low copy number, $p < 0.001$. Supporting data can be found in S22 Data.
(TIF)

S7 Fig. Negative correlation between Octomom copy number and host longevity. Median time to death (days) for lifespan experiments performed (Figs. 3A and S5A–G) is plotted as a function of Octomom copy number (relative *WD0513* copy number). These data refer to flies with two different genetic backgrounds and experiments performed at two different temperatures. The two variables are negatively correlated (Spearman correlation $rho = -0.701$, $p < 0.001$). Supporting data can be found in S23 Data.
(TIF)

S8 Fig. Release of selection pressure leads to a change in Octomom copy number. Selection was released in *w*MelPop *iso* flies at generation 26. The progeny of single females from generation 26 were kept without selection for Octomom copy number for five generations by passing all the flies to a new tube every 20 d. After these five generations, ten females per line were scored for *WD0513* copy number in their *Wolbachia* bacteria. Plotted are the original selection lines at generation 26, the same selected lines at generation 31 (the high-copy-number line was selected for ten Octomom copies from generation 29 onwards), and released selection lines at generation 31. The mothers of selected lines are represented by triangular data points, the mothers of the released selection lines are represented by blue circular data points. Lines are medians of the points at each generation/treatment. Octomom copy number decreased in three out of four lines released from selection. The only line that did not show a decrease started with two copies of Octomom. Supporting data can be found in S24 Data.
(TIF)

S9 Fig. Lack of Octomom amplification and virulent phenotype in a different *w*MelPop stock. (A) Comparison of *WD0513* copy number within different *w*MelPop *iso* and w^{1118} stocks kept in the Teixeira lab (Fig. 1A) with *w*MelPop stock obtained from another lab (*w*MelPop OPL [original Popcorn line]). DNA from single females was extracted for qPCR.

*w*MelCS_b *iso* flies were used for copy number normalization, and *wsp* was used as a reference gene. Lines are medians of the replicates. Supporting data can be found in S25 Data. (B) Life-span of females without *Wolbachia*, with *w*MelCS_b, and with *w*MelPop OPL. Females are the progeny from crosses between flies of the *iso* and the *w*MelPop OPL genetic backgrounds. One hundred females were collected at eclosion, allowed to mate for 24 h, separated from males, and scored daily for survival at 29°C. Letters refer to groups with significantly different survival curves according to Tukey's test of all pairwise comparisons of Cox hazard ratios. Supporting data can be found in S26 Data.
(TIF)

S10 Fig. Octomom amplification leads to higher expression of Octomom genes. Expression of genes in the Octomom region (*WD0507–WD0514*), in the flanking repeated region (*WD0506/WD0515*), in the immediately adjacent region (*WD0505* and *WD0519*), and in other locations of the chromosome (*wsp* and *rpoD*) in *w*MelCS_b (A) and *w*MelPop (B) (both in DrosDel isogenic background). The expression levels of *WD0506–WD0513* are higher in *w*MelPop than in *w*MelCS_b (*t*-test, $p < 0.001$ for all). The expression levels of Octomom gene *WD0514* and genes outside Octomom (*wsp*, *rpoD*, *WD0505*, and *WD0519*) are not significantly different between the two *Wolbachia* variants. Relative expression for each gene is calculated using *gmk* as a reference gene and is relative to that of *w*MelCS_b samples. RNA was extracted from eight samples of ten 3- to 6-d-old *iso* males, and real-time qPCR was performed on cDNA with specific primers. Lines are medians of the replicates. Cycle threshold values for the genes *WD0507*, *WD0513*, and *WD0514* are high, indicating low gene expression levels for these genes. These cycle threshold values fall in a nonlinear section of the standard curve, making the quantification inaccurate. Moreover, cycle threshold values for some reactions were below the detection limit. Supporting data can be found in S27 Data.
(TIF)

S1 Table. Genetic background of females used in reciprocal crosses to generate w^{1118} × *iso* hybrids (Figs. 3A, 3C, 3E, 4, and S5E–H).
(DOCX)

S2 Table. Selection generation number origin of mothers of the flies used for phenotypic analyses.
(DOCX)

S3 Table. Oligonucleotide primers used for amplification and sequencing of the *WD0514–WD0507* junction.
(DOCX)

S1 Text. Sequence of the new *WD0514–WD0507* junction. The sequencing of the PCR band (Fig. 1C) was performed with primers Link_seq_1–7 (S3 Table).
(TXT)

Acknowledgments

We are thankful to Casey Bergman for discussions and comments on the manuscript. We also thank Isabel Gordo, Miguel Soares, and Jonathan Howard for comments on the manuscript. We are grateful to Nelson Martins and Krzysztof Kus for help in the statistical analysis. We are also grateful to all the colleagues that made *D. melanogaster* lines and reagents available.

Author Contributions

Conceived and designed the experiments: EC LT. Performed the experiments: EC LT. Analyzed the data: EC LT. Wrote the paper: EC LT.

References

1. Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42: 165–190. doi: 10.1146/annurev.genet.41.110306.130119 PMID: 18983256
2. Jaenike J (2012) Population genetics of beneficial heritable symbionts. Trends Ecol Evol 27: 226–232. doi: 10.1016/j.tree.2011.10.005 PMID: 22104387
3. Engelstädter J, Hurst GDD (2009) The ecology and evolution of microbes that manipulate host reproduction. Annu Rev Ecol Evol Syst 40: 127–149.
4. Jaenike J (2009) Coupled population dynamics of endosymbionts within and between hosts. Oikos 118: 353–362.
5. Unckless RL, Boelio LM, Herren JK, Jaenike J (2009) Wolbachia as populations within individual insects: causes and consequences of density variation in natural populations. Proc Biol Sci 276: 2805–2811. doi: 10.1098/rspb.2009.0287 PMID: 19419989
6. Mouton L, Henri H, Charif D, Boulétreau M, Vavre F (2007) Interaction between host genotype and environmental conditions affects bacterial density in Wolbachia symbiosis. Biol Lett 3: 210–213. PMID: 17251124
7. Kondo N, Shimada M, Fukatsu T (2005) Infection density of Wolbachia endosymbiont affected by co-infection and host genotype. Biol Lett 1: 488–491. PMID: 17148240
8. Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, et al. (2013) Wolbachia variants induce differential protection to viruses in Drosophila melanogaster: a phenotypic and phylogenomic analysis. PLoS Genet 9: e1003896. doi: 10.1371/journal.pgen.1003896 PMID: 24348259
9. Weldon SR, Strand MR, Oliver KM (2013) Phage loss and the breakdown of a defensive symbiosis in aphids. Proc Natl Acad Sci U S A 280: 201221003.
10. Hurst GDD, Johnson AP, Schulenburg JHGVD, Fuyama Y (2000) Male-killing Wolbachia in Drosophila: a temperature-sensitive trait with a threshold bacterial density. Genetics 156: 699–709. PMID: 11014817
11. Sinkins SP, Braig HR, O'Neill SL (1995). Wolbachia pipientis: bacterial density and unidirectional cytoplasmic incompatibility between infected populations of Aedes albopictus. Exp Parasitol 81: 284–291. PMID: 7498425
12. Bourtzis K, Nirgianaki A, Markakis G, Savakis C (1996) Wolbachia infection and cytoplasmic incompatibility in Drosophila species. Genetics 144: 1063–1073. PMID: 8913750
13. Breeuwer JAJ, Werren JH (1993) Cytoplasmic incompatibility and bacterial density in Nasonia vitripennis. Genetics 135: 565–574. PMID: 8244014
14. Bressac C, Rousset F (1993) The reproductive incompatibility system in Drosophila simulans: DAPI-staining analysis of the Wolbachia symbionts in sperm cysts. J Invertebr Pathol 61: 226–230. PMID: 7689622
15. Bordenstein SR, Marshall ML, Fry AJ, Kim U, Wernegreen JJ (2006) The tripartite associations between bacteriophage, Wolbachia, and arthropods. PLoS Pathog 2: e43. PMID: 16710453
16. Boyle L, O'Neill SL, Robertson HM, Karr TL (1993) Interspecific and intraspecific horizontal transfer of Wolbachia in Drosophila. Science 260: 1796–1799. PMID: 8511587
17. Martinez J, Longdon B, Bauer S, Chan Y-S, Miller WJ, et al. (2014) Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of Wolbachia strains. PLoS Pathog 10: e1004369. doi: 10.1371/journal.ppat.1004369 PMID: 25233341
18. Chrostek E, Marialva MSP, Yamada R, O'Neill SL, Teixeira L (2014) High anti-viral protection without immune upregulation after interspecies Wolbachia transfer. PLoS ONE 9: e99025. doi: 10.1371/journal.pone.0099025 PMID: 24911519
19. Min KT, Benzer S (1997) Wolbachia, normally a symbiont of Drosophila, can be virulent, causing degeneration and early death. Proc Natl Acad Sci U S A 94: 10792–10796. PMID: 9380712
20. Zug R, Hammerstein P (2012) Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS ONE 7: e38544. doi: 10.1371/journal.pone.0038544 PMID: 22685581

21. Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008) How many species are infected with Wolbachia?—a statistical analysis of current data. FEMS Microbiol Lett 281: 215–220. doi: 10.1111/j.1574-6968.2008.01110.x PMID: 18312577
22. Werren JH, Baldo L, Clark ME (2008) Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741–751. doi: 10.1038/nrmicro1969 PMID: 18794912
23. Hosokawa T, Koga R, Kikuchi Y, Meng X-Y, Fukatsu T (2010) Wolbachia as a bacteriocyte-associated nutritional mutualist. Proc Natl Acad Sci U S A 107: 769–774. doi: 10.1073/pnas.0911476107 PMID: 20080750
24. Teixeira L, Ferreira A, Ashburner M (2008) The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol 6: e2. doi: 10.1371/journal.pbio.1000002 PMID: 19222304
25. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) Wolbachia and virus protection in insects. Science 322: 702. doi: 10.1126/science.1162418 PMID: 18974344
26. Osborne SE, Leong YS, O'Neill SL, Johnson KN (2009) Variation in antiviral protection mediated by different Wolbachia strains in Drosophila simulans. PLoS Pathog 5: e1000656. doi: 10.1371/journal.ppat.1000656 PMID: 19911047
27. Glaser RL, Meola MA (2010) The native Wolbachia endosymbionts of Drosophila melanogaster and Culex quinquefasciatus increase host resistance to West Nile virus infection. PLoS ONE 5: e11977. doi: 10.1371/journal.pone.0011977 PMID: 20700535
28. Hoffmann AA, Clancy DJ, Merton E (1994) Cytoplasmic incompatibility in Australian populations of Drosophila melanogaster. Genetics 136: 993–999. PMID: 8005448
29. Hoffmann A, Hercus M, Dagher H (1998) Population dynamics of the Wolbachia infection causing cytoplasmic incompatibility in Drosophila melanogaster. Genetics 148: 221–231. PMID: 9475734
30. Solignac M, Vautrin D, Rousset F, Solignac M, Vautrin DRF (1994) Widespread occurence of the proteobacteria Wolbachia and partial incompatibility in Drosophila melanogaster. C R Acad Sci III 317: 461–470.
31. Ferreira ÁG, Naylor H, Esteves SS, Pais IS, Martins NE, et al. (2014) The Toll-Dorsal pathway is required for resistance to viral oral infection in Drosophila. PLoS Pathog 10: e1004507. doi: 10.1371/journal.ppat.1004507 PMID: 25473839
32. Rancès E, Johnson TK, Popovici J, Iturbe-Ormaetxe I, Zakir T, et al. (2013) The toll and Imd pathways are not required for wolbachia-mediated dengue virus interference. J Virol 87: 11945–11949. doi: 10.1128/JVI.01522-13 PMID: 23986574
33. Obbard DJ, Jiggins FM, Halligan DL, Little TJ (2006) Natural selection drives extremely rapid evolution in antiviral RNAi genes. Curr Biol 16: 580–585. PMID: 16546082
34. Johnson KN, Christian PD (1999) Molecular characterization of Drosophila C virus isolates. J Invertebr Pathol 73: 248–254. PMID: 10222177
35. Kapun M, Nolte V, Flatt T, Schlötterer C (2010) Host range and specificity of the Drosophila C virus. PLoS ONE 5: e12421. doi: 10.1371/journal.pone.0012421 PMID: 20865043
36. Brun G, Plus N (1978) The viruses of Drosophila. In: Ashburner M, Wright TRF, editors. The genetics and biology of Drosophila. New York: Academic Press. pp. 625–702.
37. Zug R, Hammerstein P (2014) Bad guys turned nice? A critical assessment of Wolbachia mutualisms in arthropod hosts. Biol Rev Camb Philos Soc. E-pub ahead of print.
38. Osborne SE, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL, Johnson KN (2012) Antiviral protection and the importance of Wolbachia density and tissue tropism in Drosophila. Appl Environ Microbiol 78: 6922–6929. doi: 10.1128/AEM.01727-12 PMID: 22843518
39. Frentiu FD, Robinson J, Young PR, McGraw EA, O'Neill SL (2010) Wolbachia-mediated resistance to dengue virus infection and death at the cellular level. PLoS ONE 5: e13398. doi: 10.1371/journal.pone.0013398 PMID: 20976219
40. Lu P, Bian G, Pan X, Xi Z (2012) Wolbachia induces density-dependent inhibition to dengue virus in mosquito cells. PLoS Negl Trop Dis 6: e1754. doi: 10.1371/journal.pntd.0001754 PMID: 22848774
41. Reynolds KT, Thomson LJ, Hoffmann AA (2003) The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent Wolbachia strain popcorn in Drosophila melanogaster. Genetics 164: 1027–1034. PMID: 12871912
42. McGraw EA, Merritt DJ, Droller JN, O'Neill SL (2002) Wolbachia density and virulence attenuation after transfer into a novel host. Proc Natl Acad Sci U S A 99: 2918–2923. PMID: 11880639
43. Werren JH (1997) Wolbachia run amok. Proc Natl Acad Sci U S A 94: 11154–11155. PMID: 9326576

44. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, et al. (2009) A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium. Cell 139: 1268–1278. doi: 10.1016/j.cell.2009.11.042 PMID: 20064373

45. Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. Science 326: 134–136. doi: 10.1126/science.1177531 PMID: 19797660

46. Bian G, Xu Y, Lu P, Xie Y, Xi Z (2010) The endosymbiotic bacterium Wolbachia induces resistance to dengue virus in Aedes aegypti. PLoS Pathog 6: e1000833. doi: 10.1371/journal.ppat.1000833 PMID: 20368968

47. Kambris Z, Blagborough AM, Pinto SB, Blagrove MSC, Godfray HCJ, et al. (2010) Wolbachia stimulates immune gene expression and inhibits plasmodium development in Anopheles gambiae. PLoS Pathog 6: e1001143. doi: 10.1371/journal.ppat.1001143 PMID: 20949079

48. Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL (2011) Wolbachia infections are virulent and inhibit the human malaria parasite Plasmodium falciparum in Anopheles gambiae. PLoS Pathog 7: e1002043. doi: 10.1371/journal.ppat.1002043 PMID: 21625582

49. Bian G, Joshi D, Dong Y, Lu P, Zhou G, et al. (2013) Wolbachia invades Anopheles stephensi populations and induces refractoriness to Plasmodium infection. Science 340: 748–751. doi: 10.1126/science.1236192 PMID: 23661760

50. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, et al. (2011) The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. Nature 476: 450–453. doi: 10.1038/nature10355 PMID: 21866159

51. Van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, et al. (2012) Impact of Wolbachia on infection with chikungunya and yellow fever viruses in the mosquito vector Aedes aegypti. PLoS Negl Trop Dis 6: e1892. doi: 10.1371/journal.pntd.0001892 PMID: 23133693

52. Blagrove MSC, Arias-Goeta C, Failloux A-B, Sinkins SP (2012) Wolbachia strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in Aedes albopictus. Proc Natl Acad Sci U S A 109: 255–260. doi: 10.1073/pnas.1112021108 PMID: 22123944

53. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. Nature 476: 454–457. doi: 10.1038/nature10356 PMID: 21866160

54. Frentiu FD, Zakir T, Walker T, Popovici J, Pyke AT, et al. (2014) Limited dengue virus replication in field-collected Aedes aegypti mosquitoes infected with Wolbachia. PLoS Negl Trop Dis 8: e2688. doi: 10.1371/journal.pntd.0002688 PMID: 24587459

55. Hoffmann AA, Iturbe-Ormaetxe I, Callahan AG, Phillips BL, Billington K, et al. (2014) Stability of the wMel Wolbachia infection following invasion into Aedes aegypti populations. PLoS Negl Trop Dis 8: e3115. doi: 10.1371/journal.pntd.0003115 PMID: 25211492

56. McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, et al. (2009) Stable introduction of a life-shortening Wolbachia infection into the mosquito Aedes aegypti. Science 323: 141–144. doi: 10.1126/science.1165326 PMID: 19119237

57. McMeniman CJ, O'Neill SL (2010) A virulent Wolbachia infection decreases the viability of the dengue vector Aedes aegypti during periods of embryonic quiescence. PLoS Negl Trop Dis 4: e748. doi: 10.1371/journal.pntd.0000748 PMID: 20644622

58. Sun LV, Riegler M, Neill SLO (2003) Development of a physical and genetic map of the virulent Wolbachia strain wMelPop. J Bacteriol 185: 7077–7084. PMID: 14645266

59. Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, et al. (2004) Phylogenomics of the reproductive parasite Wolbachia pipientis wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biol 2: e69. PMID: 15024419

60. Riegler M, Sidhu M, Miller WJ, O'Neill SL (2005) Evidence for a global Wolbachia replacement in Drosophila melanogaster. Curr Biol 15: 1428–1433. PMID: 16085497

61. Riegler M, Iturbe-Ormaetxe I, Woolfit M, Miller WJ, O'Neill SL (2012) Tandem repeat markers as novel diagnostic tools for high resolution fingerprinting of Wolbachia. BMC Microbiol 12 (Suppl 1): S12. doi: 10.1186/1471-2180-12-S1-S12 PMID: 22375862

62. Woolfit M, Iturbe-Ormaetxe I, Brownlie JC, Walker T, Riegler M, et al. (2013) Genomic evolution of the pathogenic Wolbachia strain, wMelPop. Genome Biol Evol 5: 2189–2204. doi: 10.1093/gbe/evt169 PMID: 24190075

63. Elde NC, Child SJ, Eickbush MT, Kitzman JO, Rogers KS, et al. (2012) Poxviruses deploy genomic accordions to adapt rapidly against host antiviral defenses. Cell 150: 831–841. doi: 10.1016/j.cell.2012.05.049 PMID: 22901812

64. Andersson DI, Hughes D (2009) Gene amplification and adaptive evolution in bacteria. Annu Rev Genet 43: 167–195. doi: 10.1146/annurev-genet-102108-134805 PMID: 19686082

65. Kroll JS, Loynds BM, Moxon ER (1991) The Haemophilus influenzae capsulation gene cluster: a compound transposon. Mol Microbiol 5: 1549–1560. PMID: 1664907

66. Mekalanos JJ (1983) Duplication and amplification of toxin genes in Vibrio cholerae. Cell 35: 253–263. PMID: 6627396

67. Mavingui P, Laeremans T, Flores M, Romero D, Martínez-Romero E, et al. (1998) Genes essential for nod factor production and nodulation are located on a symbiotic amplicon (AMPRtrCFN299pc60) in Rhizobium tropici. J Bacteriol 180: 2866–2874. PMID: 9603874

68. Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, et al. (2012) Population genomics of the Wolbachia endosymbiont in Drosophila melanogaster. PLoS Genet 8: e1003129. doi: 10.1371/journal.pgen.1003129 PMID: 23284297

69. Early AM, Clark AG (2013) Monophyly of Wolbachia pipientis genomes within Drosophila melanogaster: geographic structuring, titre variation and host effects across five populations. Mol Ecol 22: 5765–5778. doi: 10.1111/mec.12530 PMID: 24118111

70. Ilinsky Y (2013) Coevolution of Drosophila melanogaster mtDNA and Wolbachia genotypes. PLoS ONE 8: e54373. doi: 10.1371/journal.pone.0054373 PMID: 23349865

71. Bouchon D, Rigaud T, Juchault P (1998) Evidence for widespread Wolbachia infection in isopod crustaceans: molecular identification and host feminization. Proc Biol Sci 265: 1081–1090. PMID: 9684374

72. Le Clec'h W, Raimond M, Guillot S, Bouchon D, Sicard M (2013) Horizontal transfers of feminizing versus non-feminizing Wolbachia strains: from harmless passengers to pathogens. Environ Microbiol 15: 2922–2936.

73. Sasaki T, Kubo T, Ishikawa H (2002) Interspecific transfer of Wolbachia between two lepidopteran insects expressing cytoplasmic incompatibility: a Wolbachia variant naturally infecting Cadra cautella causes male killing in Ephestia kuehniella. Genetics 162: 1313–1319. PMID: 12454075

74. Toft C, Andersson SGE (2010) Evolutionary microbial genomics: insights into bacterial host adaptation. Nat Rev Genet 11: 465–475. doi: 10.1038/nrg2798 PMID: 20517341

75. Degnan PH, Yu Y, Sisneros N, Wing RA, Moran NA (2009) Hamiltonella defensa, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. Proc Natl Acad Sci U S A 106: 9063–9068. doi: 10.1073/pnas.0900194106 PMID: 19451630

76. Burke GR, Moran NA (2011) Massive genomic decay in Serratia symbiotica, a recently evolved symbiont of aphids. Genome Biol Evol 3: 195–208. doi: 10.1093/gbe/evr002 PMID: 21266540

77. Gil R, Belda E, Gosalbes MJ, Delaye L, Vallier A, et al. (2008) Massive presence of insertion sequences in the genome of SOPE, the primary endosymbiont of the rice weevil Sitophilus oryzae. Int Microbiol 11: 41–48. PMID: 18683631

78. Sloan DB, Moran NA (2013) The evolution of genomic instability in the obligate endosymbionts of whiteflies. Genome Biol Evol 5: 783–793. doi: 10.1093/gbe/evt044 PMID: 23542079

79. Plague GR, Dunbar HE, Tran PL, Moran NA (2008) Extensive proliferation of transposable elements in heritable bacterial symbionts. J Bacteriol 190: 777–779. PMID: 17981967

80. Klasson L, Walker T, Sebaihia M, Sanders MJ, Quail MA, et al. (2008) Genome evolution of Wolbachia strain wPip from the Culex pipiens group. Mol Biol Evol 25: 1877–1887. doi: 10.1093/molbev/msn133 PMID: 18550617

81. Klasson L, Westberg J, Sapountzis P, Näslund K, Lutnaes Y, et al. (2009) The mosaic genome structure of the Wolbachia wRi strain infecting Drosophila simulans. Proc Natl Acad Sci U S A 106: 5725–5730. doi: 10.1073/pnas.0810753106 PMID: 19307581

82. Ellegaard KM, Klasson L, Näslund K, Bourtzis K, Andersson SGE (2013) Comparative genomics of Wolbachia and the bacterial species concept. PLoS Genet 9: e1003381. doi: 10.1371/journal.pgen.1003381 PMID: 23593012

83. Foster J, Ganatra M, Kamal I, Ware J, Makarova K, et al. (2005) The Wolbachia genome of Brugia malayi: endosymbiont evolution within a human pathogenic nematode. PLoS Biol 3: e121. PMID: 15780005

84. Oakeson KF, Gil R, Clayton AL, Dunn DM, von Niederhausern AC, et al. (2014) Genome degeneration and adaptation in a nascent stage of symbiosis. Genome Biol Evol 6: 76–93. doi: 10.1093/gbe/evt210 PMID: 24407854

85. Baldo L, Bordenstein S, Wernegreen JJ, Werren JH (2006) Widespread recombination throughout Wolbachia genomes. Mol Biol Evol 23: 437–449. PMID: 16267140

86. Dunbar HE, Wilson ACC, Ferguson NR, Moran NA (2007) Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. PLoS Biol 5: e96. PMID: 17425405

87. Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. Science 325: 992–994. doi: 10.1126/science.1174463 PMID: 19696350

88. Klasson L, Kambris Z, Cook PE, Walker T, Sinkins SP (2009) Horizontal gene transfer between Wolbachia and the mosquito Aedes aegypti. BMC Genomics 10: 33. doi: 10.1186/1471-2164-10-33 PMID: 19154594

89. Woolfit M, Iturbe-Ormaetxe I, McGraw EA, O'Neill SL (2009) An ancient horizontal gene transfer between mosquito and the endosymbiotic bacterium Wolbachia pipientis. Mol Biol Evol 26: 367–374. doi: 10.1093/molbev/msn253 PMID: 18988686

90. Korochkina S, Barreau C, Pradel G, Jeffery E, Li J, et al. (2006) A mosquito-specific protein family includes candidate receptors for malaria sporozoite invasion of salivary glands. Cell Microbiol 8: 163–175. PMID: 16367875

91. Iturbe-Ormaetxe I, Burke GR, Riegler M, Neill SLO (2005) Distribution, expression, and motif variability of ankyrin domain genes in Wolbachia pipientis. J Bacteriol 187: 5136–5145. PMID: 16030207

92. Hacker J, Blum-Oehler G, Mühldorfer I, Tschäpe H (1997) Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol Microbiol 23: 1089–1097. PMID: 9106201

93. Schmidt H, Hensel M (2004) Pathogenicity islands in bacterial pathogenesis. Clin Microbiol Rev 17: 14–56. PMID: 14726454

94. Carrington LB, Leslie J, Weeks AR, Hoffmann AA (2009) The popcorn Wolbachia infection of Drosophila melanogaster: can selection alter Wolbachia longevity effects? Evolution 63: 2648–2657. doi: 10.1111/j.1558-5646.2009.00745.x PMID: 19500146

95. McGraw EA, O'Neill SL (2013) Beyond insecticides: new thinking on an ancient problem. Nat Rev Microbiol 11: 181–193. doi: 10.1038/nrmicro2968 PMID: 23411863

96. Jin C, Ren X, Rasgon JL (2009) The virulent Wolbachia strain wMelPop efficiently establishes somatic infections in the malaria vector Anopheles gambiae. Appl Environ Microbiol 75: 3373–3376. doi: 10.1128/AEM.00207-09 PMID: 19329661

97. Suh E, Mercer DR, Fu Y, Dobson SL (2009) Pathogenicity of life-shortening Wolbachia in Aedes albopictus after transfer from Drosophila melanogaster. Appl Environ Microbiol 75: 7783–7788. doi: 10.1128/AEM.01331-09 PMID: 19820149

98. Schneider DI, Riegler M, Arthofer W, Merçot H, Stauffer C, et al. (2013) Uncovering Wolbachia diversity upon artificial host transfer. PLoS ONE 8: e82402. doi: 10.1371/journal.pone.0082402 PMID: 24376534

99. Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, et al. (2004) The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. Genetics 167: 797–813. PMID: 15238529

100. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45. PMID: 11328886

101. R Project for Statistical Computing (2012) R [computer program]. Available: http://www.R-project.org/. Accessed 9 January 2015.

102. Zabalou S, Charlat S, Nirgianaki A, Lachaise D, Merçot H, et al. (2004) Natural Wolbachia infections in the Drosophila yakuba species complex do not induce cytoplasmic incompatibility but fully rescue the wRi modification. Genetics 167: 827–834. PMID: 15238531

103. Veneti Z, Clark ME, Zabalou S, Karr TL, Savakis C, et al. (2003) Cytoplasmic incompatibility and sperm cyst infection in different Drosophila-Wolbachia associations. Genetics 164: 545–552. PMID: 12807775

104. Chu DTW, Klymkowsky MW (1989) The appearance of acetylated α-tubulin during early development and cellular differentiation in Xenopus. Dev Biol 136: 104–117. PMID: 2680681

105. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7: 539. doi: 10.1038/msb.2011.75 PMID: 21988835

106. McWilliam H, Li W, Uludag M, Squizzato S, Park YM, et al. (2013) Analysis tool web services from the EMBL-EBI. Nucleic Acids Res 41: W597–W600. doi: 10.1093/nar/gkt376 PMID: 23671338

107. Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, et al. (2010) A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res 38: W695–W699. doi: 10.1093/nar/gkq313 PMID: 20439314