RESEARCH ARTICLE

Drosophila Adaptation to Viral Infection through Defensive Symbiont Evolution

Vitor G. Faria¹‡, Nelson E. Martins¹‡, Sara Magalhães², Tânia F. Paulo¹, Viola Nolte³, Christian Schlötterer², Élio Sucena¹,4☯, Luis Teixeira¹*.*

¹ Instituto Gulbenkian de Ciência, Oeiras, Portugal, ² Centre for Ecology, Evolution and Environmental Changes (cE3c), Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal, ³ Institut für Populationsgenetik, Vetmeduni Vienna, Wien, Austria, 4 Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal

These authors contributed equally to this work.
‡ These authors share first authorship on this work.
* esucena@igc.gulbenkian.pt (ES); lteixeira@igc.gulbenkian.pt (LT)

Abstract

Microbial symbionts can modulate host interactions with biotic and abiotic factors. Such interactions may affect the evolutionary trajectories of both host and symbiont. Wolbachia protects Drosophila melanogaster against several viral infections and the strength of the protection varies between variants of this endosymbiont. Since Wolbachia is maternally transmitted, its fitness depends on the fitness of its host. Therefore, Wolbachia populations may be under selection when Drosophila is subjected to viral infection. Here we show that in D. melanogaster populations selected for increased survival upon infection with Drosophila C virus there is a strong selection coefficient for specific Wolbachia variants, leading to their fixation. Flies carrying these selected Wolbachia variants have higher survival and fertility upon viral infection when compared to flies with the other variants. These findings demonstrate how the interaction of a host with pathogens shapes the genetic composition of symbiont populations. Furthermore, host adaptation can result from the evolution of its symbionts, with host and symbiont functioning as a single evolutionary unit.

Author Summary

Animals live in close association with microbial partners that can shape many aspects of their lives. For instance, several insects carry bacteria that defend them against parasites and infectious diseases. The intracellular bacterium Wolbachia protects the fruit fly Drosophila melanogaster against viral infection. Natural populations of Drosophila carry different variants of Wolbachia, which differ from one another in the strength of this protection. Here we show that a population of Drosophila infected with viruses during several generations adapts to this challenge through turnover in Wolbachia composition. The Wolbachia variants that give higher protection to viruses, by increasing fly survival and fecundity upon infection, are strongly selected. This work demonstrates that the interaction of an animal with a pathogen can shape its associated microbial populations. We
show that adaptation to pathogens can be achieved not only through selection of resistance on the host proper but also through the evolutionary shaping of its microbial community.

Introduction

Animals and plants live in close association with numerous symbiotic bacteria that often cause strong phenotypic changes in their hosts [1]. For example, defensive symbionts can increase host resistance to pathogens and parasitoids [2–8]. In insects, several defensive symbionts are maternally transmitted [3–7], such that the fitness of the symbiotic bacteria is dependent on that of their female hosts. Therefore, one can expect that selection on host phenotypes, including resistance to (other) parasites, impacts the evolution of the bacterial symbiont population.

Host parasite burden can impact symbiont populations. For example, experimental evolution of the pea aphid Acyrthosiphon pisum or of Drosophila hydei in the presence of parasitoid wasps, caused an increase in the frequency of individuals carrying the protective symbionts Hamiltonella defensa and Spiroplasma, respectively [9,10]. Also, the recent spread of a Spiroplasma symbiont in natural populations of D. neotestacea in North America has been associated with the arrival of a parasitic nematode to this continent [7]. In agreement with this, the frequency of Spiroplasma in a D. neotestacea population increases in the presence of the parasitic nematode during experimental evolution [11]. These studies show changes in the prevalence of endosymbiont infection in host populations, but do not address selection at the level of the genetic diversity of the symbiont itself. However, some evidence suggests that this could be the case: 1) some defensive symbiont populations display genetic and phenotypic variability [12–18] and 2) variants or strains of endosymbionts change in frequency in natural populations or during experimental evolution [19–21]. Nonetheless, a clear link between the selective pressure exerted on hosts and the genetic changes observed in the symbionts has been missing. In this study, we establish a relation between host adaptation to parasites and changes in the genetic composition of endosymbiont populations.

Wolbachia is a maternally-transmitted bacterial endosymbiont widespread in arthropods [22]. In some natural hosts it induces strong protection against infection with several RNA viruses [3,4,23,24]. Importantly, genetic variation in the Wolbachia strain of Drosophila melanogaster (wMel), can be linked to the strength of antiviral protection [14,15]. Using experimental evolution, we have previously shown that D. melanogaster populations adapt to Drosophila C virus (DCV) challenge [25]. Resistance to this pathogen increases over twenty generations and we identified the genetic bases of this adaptation at the host level [25]. However, all individuals of the outbred founder population carried Wolbachia [26]. Therefore, we used this unique setup to ask if the genetic composition of the Wolbachia wMel populations also changed during host adaptation to DCV challenge and whether this change could impact on Drosophila fitness.

Results

We performed experimental evolution on four replicate populations of D. melanogaster under selection with systemic DCV infection (Virus-Selected) and four replicates with mock infection (Control) [26]. DCV infection was performed at every generation using the same virus strain, at the same dose. As previously described [25], we performed genome-wide sequencing of DNA from pools of each population (Pool-Seq) [27,28]. Using Pool-Seq on the Ancestral populations and on the Control and Virus-Selected populations after 20 generations [25], we determined the genetic diversity of Wolbachia in these populations. We found statistically
significant changes in the frequency of 125 single nucleotide polymorphisms (SNPs) between the Ancestral and the Virus-Selected populations (Fig 1A, S1 and S2 Figs, S1 Dataset). Of these, 111 were also significantly different between Control and Virus-Selected populations, but not between Control and Ancestral populations, showing that these changes in the genetic composition of the Wolbachia populations are mostly specific to the response to viral infection.

Phylogenetic analysis, based on whole genome sequencing of Wolbachia and mitochondria, indicate that in the recent past wMel has been strictly vertically transmitted [13,29,30]. Moreover, there is no evidence of fly lines simultaneously carrying wMel variants from distant haplotypes or recombination between these [29]. Therefore we inferred Wolbachia haplotypes in the Ancestral, Control and Virus-Selected populations from the Pool-Seq data (S1 Text). Overall, we identified diagnostic SNPs (i.e. SNPs present in all variants, and only in variants, of a specific clade) for three of the major clades of wMel (S1 Dataset) [13,14,21]. The Ancestral Wolbachia populations consisted of approximately 88% clade V variants and 12% of variants of clades I and III. In the Virus-Selected populations all these diagnostic SNPs became fixed with the nucleotide that matches clade V (S1 Dataset). In fact, in all the 123 SNPs that became fixed between Ancestral and Virus-Selected populations the fixed nucleotides match clade V. Moreover, between Ancestral and Control populations, the 8 SNPs that significantly changed have only been detected before in clades I and III variants, whereas Clade V specific SNPs did not significantly change in frequency between these populations (S1 Text and S1 Dataset). Therefore, we conclude that selection of D. melanogaster with a viral challenge changed the frequencies of wMel variants in the host populations and led to fixation of clade V wMel variants.

To confirm that the fixation of clade V variants was specific to the Virus-Selected populations, we analyzed individual flies from the Ancestral, Control and Virus-Selected populations as well as from a parallel selection regime in which Drosophila was challenged with systemic bacterial infection [26] (Fig 1B). We determined the wMel variant carried by 96 individual flies from each replicate population through restriction analysis of a PCR fragment containing a clade V diagnostic SNP. This analysis distinguishes flies carrying wMel variants of clades I/III or clade V. The frequencies of flies carrying clade V wMel variants in Ancestral, Control and Virus-Selected populations are in agreement with the Pool-Seq data (S1 Text) and clade V variants are only fixed in the Virus-Selected populations. We observed significant differences in frequencies between the Virus-Selected populations and the other tested populations but not between any other regimes (generalized linear mixed model (GLMM), Selection Regime effect, $\chi^2 = 31.648, p < 0.001$, Tukey HSD, $|z| > 3.437, p < 0.005$ for all comparisons with the Virus-Selected populations, $|z| < 2.067, p > 0.23$ for all other comparisons). These data argue against drift being responsible for the fixation of clade V variants since Bacteria-Selected populations had fewer surviving individuals for a larger number of generations than Virus-Selected populations (S1 Text and S2 Dataset) and wMel variants of clade V did not reach fixation in any of the Bacteria-Selected populations. Moreover, a time-course analysis of wMel clade V frequencies in the Virus-Selected regime, based on individual genotyping, also shows that changes in frequencies were parallel in all four replicates (Fig 1C). Finally, based on the frequencies of clade I/III and clade V variants in generations 0, 5, 10 and 20 we estimated a strong selection coefficient against clade I/III variants of 0.263 (0.177–0.349) (estimated log-linear slope using GLMM, Generation effect, $\chi^2 = 42.466, p < 0.001$) [31]. Therefore, fixation of clade V variants in all the Virus-Selected populations is unlikely due to drift, injury or a generic immune challenge, but the consequence of the specific adaptation to viral challenge.

To analyze the phenotype of clade V wMel variants against clade III variants we established eleven different isofemale lines carrying wMel from clade V and eleven different isofemale lines carrying wMel from clade III. These lines were established from the Control populations. To directly compare the differences between wMel from the different clades we set up
reciprocal crosses between eleven independent pairs of clade III and clade V isofemale lines. Since wMel is only maternally transmitted, the female progeny of each of these paired crosses differed in the wMel variant, but had the same host genotype. During the virus-selection protocol, reproduction of surviving adults took place five to seven days after DCV infection [25]. At five, six and seven days after DCV infection, flies carrying wMel clade III variants had lower survival than flies with clade V variants (Fig 2A, GLMM, wMel clade effect, $\chi^2_1 > 16.44$, $p < 0.001$ in all daily comparisons, see also analysis of S3A Fig, below). Analysis of the survival data until 20 days post-infection confirms an overall lower susceptibility upon viral infection of flies with wMel variants of clade V compared with flies carrying clade III variants (Fig 2B, mixed effect Cox model, wMel clade effect, $\chi^2_1 = 25.817$, $p < 0.001$, see also analysis of S3B Fig, below).
We also analyzed the reproductive output of flies with the different wMel variants (from the same reciprocal crosses), five to seven days post-infection with DCV (Fig 2C). Flies with clade III variants had fewer progeny than flies carrying clade V variants (linear mixed model (LMM), wMel clade effect, $\chi^2_1 = 39.217, p < 0.001$). This difference between variants is contingent on viral infection, since their reproductive output is not significantly different in the absence of infection (LMM, $\chi^2_1 = 2.321, p = 0.128$, S4 Fig). The differences between flies carrying wMel clade III variants and flies carrying clade V based on reproductive output and survival at five to seven days post-infection could explain the relative fitness of 0.723 (0.651–0.823) calculated from the above estimated selection coefficient ($w = 1 - s$).

Mitochondria are co-inherited with Wolbachia. Therefore, the phenotypic differences we observed between flies carrying different wMel variants could, hypothetically, be due to phenotypic differences of their associated mitochondria variants. If this were the case, selection could have acted on the mitochondria and indirectly affected frequencies of Wolbachia variants. To test for the contribution of mitochondria to the phenotypic differences observed, we repeated these assays with the same isofemale lines and matching isofemale lines from which wMel was removed by tetracycline treatment. We found a significant interaction between wMel/mitochondria clade (cytotype) and Wolbachia presence, both in survival 5, 6 or 7 days after infection and in overall survival (S3A and S3B Fig–clade by presence of Wolbachia, GLMM and Mixed effect Cox model, $p < 0.001$). Importantly, both models showed a significant difference in survival between flies carrying wMel clade V or wMel clade III, but not between flies of the same cytotypes without Wolbachia (pairwise comparisons between clades in Mixed effect Cox Model, $|z| = 5.739, p < 0.001$ and $|z| = 0.868, p = 0.385$, for flies with and without Wolbachia, respectively and in pairwise comparisons between clades using GLMM at 5, 6 or 7 days post-infection $|z| > 3.794, p < 0.001$ and $|z| < 1.678, p > 0.093$, for flies with and without Wolbachia, respectively). Analysis of differential reproductive output had similar results. There was a significant interaction between cytotype and Wolbachia presence (S3C Fig, LMM, clade by presence of Wolbachia, $\chi^2_1 = 4.2, p = 0.040$). Pairwise comparisons of reproductive output
between cytotypes with Wolbachia showed a significant difference \( t = 4.27, p < 0.001 \) but not between cytotypes in the absence of Wolbachia \( t = 1.2, p = 0.087 \). Overall, these data indicate that there is no significant difference in survival or reproductive output, upon viral infection, between flies only carrying different mitochondria. Therefore, the phenotypic differences we observe are due to differences between wMel variants and not between mitochondria variants.

Finally, we tested if lower fitness upon viral infection of flies carrying wMel clade III variants was associated with higher DCV load as different wMel variants have been shown to confer differential resistance to DCV infection [14] (S5A Fig). Flies carrying these variants had 5.4 fold higher levels of DCV compared with flies carrying clade V variants \( \chi^2 = 11.479, p < 0.001 \). The lower resistance to viruses of flies carrying Clade III variants, compared to Clade V, may explain their lower survival and fertility upon infection. Flies with Clade III variants also had lower Wolbachia levels when compared with flies carrying clade V variants (S5B Fig, LMM, \( \chi^2 = 16.292, p < 0.001 \)). This may explain lower antiviral resistance of these variants, in line with previous findings [14,15,32,33].

**Discussion**

Our data show that (a) the frequencies of Wolbachia variants specifically change when Drosophila populations evolve in the presence of viruses, (b) this exposure to DCV leads to fixation of clade V wMel variants, and (c) genetically identical individuals are more protected against DCV infection and display lower viral loads when they harbor these Clade V variants, relative to when they harbor other variants still present in the Control (and Ancestral) population. Moreover, the selection coefficient inferred from the evolutionary dynamics of clade V in DCV-exposed populations could be explained by the fitness advantage of clade V over clade III wMel variants in flies subjected to DCV infection. These results demonstrate that host infection by parasites can be a selective force leading to genetic changes in the endosymbiont population such that the most protective variants become fixed. In turn, this evolution can contribute to host adaptation to pathogens.

We have previously identified two regions in the D. melanogaster genome that mediate adaptation of this population to DCV infection [25]. Here we show that this adaptation also leads to change in wMel genetic diversity. There may be interactions between selection on the genomes of the symbiont and the host, which we did not test here. We have demonstrated before that the Virus-Selected population had a higher survival upon DCV infection than the Control populations even when Wolbachia was removed from these populations [25]. This indicates that, overall, the selected alleles confer an advantage in the presence of viruses independently of the presence of Wolbachia. However, it was recently shown that the strength of selection on host genetic variation is decreased in the presence of these protective symbionts [34]. Therefore, the presence or absence of Wolbachia interacts with selection at the host level. However, this does not address interactions between selection acting both at the level of the symbiont and the host. We show differences between the wMel variants using isofemale lines established from the Control populations, and therefore not evolved under Virus challenge. This indicates that the virus susceptibility phenotypes associated with the wMel variants are not dependent of selection at the level of the host genome. Moreover, we compared the phenotypes of the progeny of several independent reciprocal crosses between lines carrying different wMel variants. This setup controls for differences in host genetic background. It will be interesting in the future to investigate how genetic variation in the host impacts on the phenotypes of Wolbachia variants, and vice-versa.

Other wMel variants were shown to differ in survival upon viral infection [14]. wMel variants from clade VI confer more protection to viruses than variants from clade III or clade VIII.
Here clade V variants are more protective than clade III variants (and clade I variants are also counter-selected in the Virus-Selected populations). These results indicate that clade V and VI are more protective against viral infections and clade I, III and VIII, less protective. It will be important in the future to make a direct comparison of the antiviral protection conferred by these different variants and understand their dynamics in natural populations.

Previous work showed that variants that differ in protection to viruses also differed in the cost to the host in the absence of infection, indicating a trade-off between the two traits [14,15]. This led to the suggestion that the frequencies of different variants in natural populations might depend on the prevalence of viruses [14]. Here we demonstrate that an increase in viral burden does lead to changes in wMel variant frequencies. Moreover, the selection coefficient for specific wMel variants can be very high and promote their rapid fixation. wMel variants are strictly maternally transmitted and show no sign of recombination [13,29,30]. Therefore, as in these conditions specific haplotypes are fixed, the overall genetic diversity of wMel is strongly reduced (since mitochondria are co-inherited with Wolbachia this selection may also impact on their genetic diversity).

Viruses seem to impose strong natural selective pressure, as demonstrated by the fast evolutionary rates and signatures of positive selection in D. melanogaster genes involved in antiviral resistance [35]. Wolbachia can protect hosts against several positive sense single-stranded RNA viruses [3,4,23,24], including DCV, a natural pathogen of D. melanogaster [36–39]. However, approximately 25 different viruses have been found to infect natural populations of D. melanogaster [38,40,41]. Although most of them are positive sense single-stranded RNA viruses we do not know which represent the biggest burden to natural populations. Moreover, the effect of Wolbachia against most of these viruses is unknown, although it protects against the few that were tested (DCV, Cricket Paralysis virus, and Nora virus [3,4]). Different wMel variants also have different costs in the absence of infection and this is most probably an important factor in the dynamics of wMel in natural populations [14]. Our particular experimental evolution setup, with all the individuals being infected with DCV at every generation before reproduction, demonstrates that wMel selection upon viral infection is possible. In which conditions and to which degree this occurs in natural populations remains to be determined.

We can explain the strong selection coefficient for clade V over clade III wMel variants with the differences in the protection to viruses they confer to their hosts. Previous analyses of virus-infected hosts carrying different wMel variants or Wolbachia strains have shown differences in viral titers and survival [14,15,23,32,33,42]. Here we also show that flies carrying clade V variants have lower viral titers and higher survival when compared to flies carrying clade III variants. This higher survival most likely contributes to the selection of clade V variants. However, there is also a much higher fertility of flies carrying clade V wMel variants, upon viral infection, which likely also determines the strong selection coefficient. In fact, in natural populations this parameter might be more important for the protective effect of Wolbachia against viruses and the differential selection of wMel variants, than the effect on host survival.

Here, using experimental evolution, we provide direct proof that endosymbiont and host can form an evolutionary unit with adaptation relying on the evolution of both genomes. It is straightforward to extrapolate our results with maternally transmitted Wolbachia to interactions involving other defensive endosymbionts such as Spiroplasma, Regiella, and Hamiltonella [6,7,16]. The tight association between endosymbionts and their hosts make it probable that it is common for selection at the host phenotypic level to impact symbiont population genetics. It will be interesting in the future to assess to which degree this phenomenon occurs in interactions between hosts and microbes with different modes of transmission. One obvious example is the gut microbiota of mammals, which can protect the host against gut pathogens [8] and
show some degree of vertical transmission [43]. As research on microbiota-induced phenotypes and potential co-evolution with hosts increases, a central question arises on how selection on the microbiota-induced phenotypes impacts the population genetics of the microbes.

**Materials and Methods**

**Foundation, maintenance, and selection of populations**

We used an outbred population of *D. melanogaster* established in 2007 from 160 fertilized females, as described in [25,26]. The population was kept in laboratory conditions for more than 50 non-overlapping generations at high census. Before the initiation of experimental evolution, this population was serially expanded for two generations to allow the establishment of 36 new populations of which 12 were used in this work. All individual founders were naturally infected with *Wolbachia wMel* and the initial populations were 100% infected with *Wolbachia* (checked individually by PCR with *wsp* primers, as described in [44]).

Flies were kept in laboratory cages at constant temperature (25°C) and humidity (70%) in a light-darkness cycle (12h:12h). Flies were raised in standard cornmeal-agar medium. Each generation took three weeks and egg density per food cup was controlled.

**Virus-Selected populations**

Virus-Selected populations were infected every generation by pricking flies in the thorax with DCV (2 x 10^7 median tissue culture infective dose (TCID_{50}) per ml) [25]. DCV was grown and titrated as described in [3]. This dose caused in the initial population an average mortality of 66% 10 days after infection. Three hundred and ten males and 310 females were infected with DCV at every generation. Surviving individuals mated randomly in population cages and eggs were collected five to seven days post-infection. This selection protocol proceeded for 20 generations before Pool-Seq analysis.

Control populations were pricked at every generation with sterile solution. These populations were controlled to 600 adults at every generation.

**Bacteria-Selected populations**

Infection and selection protocol at every generation was the same as for the Virus-Selected populations. Flies were infected by pricking with *Pseudomonas entomophila* at a dose that causes an average mortality of 66% in the initial populations (OD600 = 0.01) [26].

**Whole-genome sequencing of populations (Pool-Seq)**

DNA extraction, library preparation and whole genome sequencing of pools of individuals was described in [25]. Briefly, 12 populations were sequenced (four per regime): Ancestral (generation 0), Control and Virus-Selected populations, the latter two at generation 20. Genomic DNA was extracted from a homogenate pool of 200 individuals of each population using a high-salt extraction protocol. Genomic DNA was sheared using a Covaris S2 device (Covaris, Inc.) and paired-end 100bp libraries were prepared using the TruSeq v2 DNA Sample Prep Kit (Illumina). Libraries were sequenced on a HiSeq 2000 (Illumina).

Raw reads were trimmed using Trimmomatic [45] (leading and trailing bases clipped if quality < 20, 3’ clipped if average quality of a window (4 bp) dropped below 20, minimum read length = 50) and then realigned to the reference *Wolbachia* genome (NC_002978.6 [46]) using bwa 0.6.2 [47], with the following parameters: maximum differences = 1%, maximum number of gaps = 2, maximum gap or deletion size = 12, seeding disabled. Alignments were converted to the sam/bam format using samtools [48] and sorted, filtered for quality, proper pairs and duplicate reads using bamtools [49]. Afterwards, SNPs were called simultaneously in all populations using freebayes (v 9.9.2) [50], in positions with a minimum count of the alternate allele of 2 and a minimum global alternate allele frequency of 2%. Only biallelic SNPs were considered.
Effects of the polymorphisms on putative coding sequences were predicted using SnpEff 4.11 [51], based on the ENSEMBL GCA_000008025.1.26 genome annotation.

**Determination of frequencies of clade V wMel variants**

We analyzed the frequency of clade V wMel variants by testing individual flies in Ancestral, Virus-Selected (at generations 5, 10, and 20), Control (at generation 20), and Bacteria-Selected (evolved against *Pseudomonas entomophila*, at generation 20) populations.

We extracted DNA from 96 individual female flies of each replicate population following the protocol in (http://www.drosdel.org.uk/molecular_methods.php#prep) [52]. Briefly, single flies were squashed in 100 mM Tris-EDTA-NaCl buffer (pH 7.7), 0.5% SDS and incubated at 65°C for 30 minutes. After protein and RNA precipitation with 6M LiCl / 5M KAc, DNA was precipitated using ice-cold isopropanol followed by ethanol cleaning. PCR amplification of the genomic region surrounding position 805,011 was performed using the primers 805011F (5’-AGTCGGGAGCATGAGGAAAGT-3’) and 805011R (5’-TTTCAGCATCAGTCGGCCTCGGC-3’). The polymorphism was detected by differential cleavage of amplified product with the enzyme BtsCI (NEB). Digestion was performed at 50°C for 60 minutes and the digestion product visualized in an agarose gel. The polymorphism at this position distinguishes wMel variants of clades I, II, III and IV from variants of clades V and VI. In our populations this SNP allows distinguishing clade V variants from clade I/III variants.

**Establishment of isofemale lines carrying wMel of clades III and V**

Ninety-six isofemale lines were founded from Control populations. The Pool-Seq data show that these populations only had wMel variants from clades III and V.

Each line was tested for three different wMel SNPs. Position 805,011 was tested as above. The SNPs at positions 655,839 and 1,027,577 distinguish clades I, II and III from clades IV, V, VI and VIII. PCR amplification of the genomic regions surrounding these positions were performed using the primers 655839F (5’-AGCACGTCTAGCAATCGCACAGCA-3’), 655839R (5’-GGCGTTTTAGGGGTGTTGGTGTGGTGTGGTGTTGGTGTTGGTGTTGGTGTTGGTGTTGGTG-3’), 1027577F (5’-TCCTGCATCAGTCCGCCACCACCA-3’), and 1027577R (5’-GGCAGCACTGATGGCGCAGACCA-3’). The PCR products were digested at 37°C for 60 minutes using the restriction enzymes MscI and HindIII (NEB) for positions 655,839 and 1,027,577, respectively. The results of the three enzymes were congruent allowing us to identify isofemale lines carrying clade V or clade III wMel variants.

We also tested for the insertion IS5-WD1310 by PCR, as described in [12]. This insertion is present in clade VI variants, absent in clade III and VIII variants, but unknown for variants of other clades, including clade V [12,14]. All flies were negative for this insertion.

After these analyses we selected eleven independent isofemale lines carrying clade V wMel variants and eleven independent isofemale lines carrying clade III wMel variants. Isofemale lines were kept in vials in similar conditions to the *D. melanogaster* populations.

**Generation of flies carrying different wMel variants for phenotypic characterization**

Eleven independent pairs of isofemale lines with wMel variants of clades III and V were crossed in a reciprocal scheme (female clade V x male clade III and female clade III x male clade V). The female progeny of these two crosses have an equivalent genetic background but different wMel variants (which is maternally transmitted). This female progeny was used for the phenotypic characterization and each reciprocal pair was considered a random effect in the statistical analysis ("cross genotype", see below).
Reproductive time-window and general husbandry conditions of these crosses were the same as for the experimental evolution protocol.

 Establishment of isofemale lines and generation of flies for the analysis of mitochondrial contribution to different phenotypes

To analyze the contribution of mitochondria associated with different wMel clades to the fitness-related phenotypes we established Wolbachia-free lines derived from the above selected isofemale lines carrying different wMel variants.

We treated ten clade III isofemale lines and ten clade V isofemale lines with tetracycline (as in [14]). Lines were raised in fly food with 0.05 mg/ml of tetracycline hydrochloride (Sigma) for two generations. After antibiotic treatment each treated line had their microbiota reconstituted with the microbiota associated with their original line. 150 μl of a bacterial inoculum of each of the original lines was added to each tetracycline-treated lines. Each inoculum was constituted of 5ml of sterile water mixed with 2 g of food from a 10 days old vial of the original stock, filtered to remove eggs and larvae.

All stocks were confirmed to be free of Wolbachia by PCR using primers specific for the Wolbachia gene wsp; wsp-81F (5’-TGGTCCAATAAGTGATGAAGAAAC-3’) and wsp-691R (5’-AAAAATTAAACGCTACTCCA-3’), as in [3]. Flies were raised without antibiotics for two generations before assays.

To compare the phenotype of different cytotypes in the presence or absence of Wolbachia we set up reciprocal crosses between lines carrying different wMel variants and reciprocal crosses between their matching isofemales lines after tetracycline treatment. Only ten reciprocal crosses of each kind were performed in this assay. The phenotypic assays were performed on the progeny of these crosses.

Fitness assays

For the survival assays, 100 females (3–6 days old) from each reciprocal cross, infected with DCV, were placed in vials (10 vials with 10 individuals each), at 25°C. The mortality was monitored daily for 20 days.

For the progeny assays, 20 couples (3–6 days old) from each reciprocal cross were infected with DCV and placed in vials 5 days after infection (1 couple per vial). Flies were allowed to lay eggs for two days and then removed (this protocol matches that of the experimental evolution). The progeny of each female corresponds to the number of pupae per vial. The same protocol was used for progeny quantification with females not exposed to DCV.

Wolbachia levels and viral titers

For the quantification of Wolbachia and viral titers in the progeny of the reciprocal crosses, we used three DCV-infected females of the progeny of each matched pair. Seven days post-infection total nucleic acid was extracted using MasterPure Complete DNA and RNA Purification Kit (Epicentre), according to manufacturers’ protocol, with some modifications. To purify DNA, 10 μl of each sample was treated with 1 μl of 10 mg/ml RNase A (Roche). To purify RNA, samples were treated with 1U DNase (Promega) per μg of total nucleic acid, in a total volume of 10 μl, at 37°C for 30 min; the reaction was stopped by adding 1 μl of RQ1 DNase stop solution, and incubated at 65°C to inactivate the DNase. RNA samples were then reverse transcribed to cDNA using M-MLV Reverse Transcriptase (Promega), according to manufacturers’ instructions. DNA and cDNA samples were used to quantify Wolbachia and DCV levels, respectively.
Quantification of Wolbachia levels and viral titers was performed by qPCR as described in [14]. For each reaction we used 6 μl of iQTM SYBR Green supermix (Bio-Rad), 0.5 μl of each primer solution at 3.6 μM and 5 μl of diluted DNA. Each plate contained three technical replicates of every sample for each set of primers. Relative amounts of wsp and DCV were calculated using the Pfaffl method [53] and Drosophila Rpl32 as a reference. Levels of wsp and DCV are relative to the wMel clade V samples.

Statistical analysis

Allele frequency comparisons. Allele frequencies were compared using a weighted binomial model. Let \( v_i \) be the frequency of the major allele in population \( i \) of a given selection regime:

\[
v_i = \log^{-1} (X_i^T \beta + e_i)
\]  

(1)

Where \( \beta \) is the vector of the Selection regime fixed effect and \( X_i \) is a row vector relating this fixed effect to population \( i \), weighted by the read depth or number of genotyped individuals. \( e_i \) is the residual error that captures overdispersion in the estimate of frequencies in each population.

In the pooled sequencing analysis, only positions in any of the populations with minor allele frequency > 2% were considered, and Benjamini & Hochberg adjusted p values (q-values) were considered significant if below a false discovery rate threshold of 0.1%.

In the comparisons of wMel variant frequencies between selection regimes, p values for multiple comparisons were adjusted using sequential Bonferroni correction.

In generation 20 all reads or all sampled individuals in the Virus-Selected populations were fixed to one of the alleles, leading to problems of convergence in the models. To correct for that, we assigned one read or one individual to the alternative allele.

Estimation of selection coefficient. Since Wolbachia is maternally transmitted, selection acts as in a haploid organism. The estimate for the fitness differential between the Wolbachia clades in the Virus-Selected populations was therefore calculated according to [31] (eqn 6.3):

\[
\log \left( \frac{p_t}{q_t} \right) = \log \left( \frac{p_0}{q_0} \right) + \log(w) \ast t
\]  

(2)

Where \( w \) is the relative fitness (1-s) of genotype \( p \) over genotype \( q \).

Assuming a small \( s (<0.5) \), \( (1+s)^t \sim e^s \). Therefore, we assessed statistical significance of the coefficient using mixed logistic regression. Let \( v_{i,t} \) be the frequency of a given Wolbachia genotype at generation \( t \) in population \( i \),

\[
v_{i,t} = \log^{-1}(s \ast t + v_{i,0} + e_{i,t})
\]  

(3)

The selection coefficient \( s \) is the slope of the regression coefficient given the initial frequencies \( (v_{i,0}) \), \( e_{i,t} \) is the residual error that captures overdispersion in the estimate of frequencies in the populations at each time point. Using the frequencies of wMel variants at generations 0, 5, 10 and 20 the selection coefficient against wMel I/III is 0.263 (0.177–0.349). This relatively high value is independent of the data at generation 20, when there is fixation of clade V, since the selection coefficient calculated with data from generations 0, 5, and 10 is 0.287 (0.183–0.391).

We tested for the presence of wMel in the progeny (96 individuals) of five females from different isofemale lines carrying clade III variants, and in the progeny (100 individuals) of five females from different isofemale lines carrying clade V variants. All individuals were positive.
for wMel showing that vertical transmission is virtually 100% and similar for variants of both clades. Therefore, we can compare these variants fitness using this multigenerational equation.

**Survival analysis.** To compare survival at days 5, 6 and 7 of flies with each Wolbachia variant after infection, we fitted a generalized linear mixed effects model. Let \( v_{ij} \) be the proportion of surviving flies in vial \( i \) of individuals of a given wMel variant, resulting from cross \( j \), at 5, 6 or 7 d.p.i.:

\[
v_{ij} = \logit^{-1}(X_i^\top \beta + c_j + \epsilon_{ij})
\]

Where \( \beta \) is the vector of fixed effects of wMel variant, \( X_i \) is the row vector relating the fixed effects of variant with vial, \( c_j \) is the random effect of fly cross genotype and \( \epsilon_{ij} \) is the residual error that captures overdispersion for each vial.

We also compared the full survival dynamics, until 20 days post-infection, using a mixed effects Cox model. This model accounted for both parental cross and between-vial variation in survival rates. The hazard of the \( i \)th individual of a given wMel strain, resulting from cross \( j \) in vial \( k \) was modeled as:

\[
H_{ijk}(t) = H_0(t)e^{X_i^\top \beta + c_j + \epsilon_{ijk}}
\]

Where \( H_0 \) is the baseline hazard at time \( t \), \( \beta \) is the vector of fixed effects of wMel variant, \( X_i \) is the row vector relating the fixed effects of variant with the individual fly, \( c_j \) is the random effect of cross genotype and \( \epsilon_{ijk} \) is the random effect of vial.

In both analyses, the effect of the wMel variant was compared using likelihood-ratio tests, with a model without the fixed effect term as the null model.

To analyze the effect of the mitochondria variants in the survival upon viral infection equivalent models were used taking into account the fixed effect of presence or absence of Wolbachia, and its interaction with the fixed effect variant.

**Reproduction tests.** To compare reproductive output of flies with different wMel variants after infection, we fitted a linear mixed model, where \( v_{ij} \) is the number of pupae after a 48h oviposition period by female \( i \) resulting from cross \( j \), with a particular wMel variant.

\[
v_{ij} = X_i^\top \beta + c_j + \epsilon_{ij}
\]

As above, \( \beta \) is the vector of fixed effects of wMel variant, \( X_i \) is the row vector relating the fixed effects of wMel variant with female \( i \), \( c_j \) is a random variable representing the deviation of the cross genotype (reciprocal cross pair) from the overall mean and \( \epsilon_{ij} \) is the random term that captures heterogeneity between different females of the same cross genotype.

The effect of the fixed factor was compared using likelihood-ratio tests.

To analyze the effect of the mitochondria variants in the reproductive output upon viral infection, a similar model was used taking into account the fixed effect of presence or absence of Wolbachia, and the interaction of this with the fixed effect variant.

In the second experiment (designed to test for the effect of mitochondria) there was a high number of females that did not reproduce. Therefore, we also analyzed these data using a hurdle model for count data in which two equations were used; one to compare the number of zero vs non-zero counts between the groups with a binomial model, and another to analyze the non-zero counts, assuming that these follow a zero-truncated negative binomial distribution.

This analysis gave a similar result to the linear mixed model used above. In the non-zero counts data there was an interaction between cytotype and Wolbachia presence (\( \chi^2_1 = 9.59, p = 0.002 \)). There was a significant difference in reproductive output between flies carrying wMel clade V or wMel clade III, but not between flies of the same cytotypes without Wolbachia (pairwise
comparisons between clades in generalized linear mixed model, \( t = 5.23, p < 0.001 \) and \( t = 1.71, p = 0.087 \), for flies with and without Wolbachia, respectively.

**Wolbachia and DCV titer quantification.** To compare Wolbachia or DCV titers after infection in flies with different wMel variants, we fitted a linear mixed model similar to the Eq (6), with \( v_{ij} \) being the \( \log(wsp) \) or \( \log(DCV) \) levels.

All statistical analyses were done in R version 3.1.2 [54]. Linear mixed models were fitted using the `lmer` function and generalized linear mixed models with the `glmer` function, both in the “lme4” package. Hurdle models were done with the `glmmADMB` function of the “glmmADMB” package. Multiple comparisons were done using the `lsmeans` function in the “lsmeans” package. Survival data were compared using the `coxme` function in “coxme” package.

**Accession numbers**
Trimmed fastq and assembled bam files are available via the European Nucleotide Archive (http://www.ebi.ac.uk/ena/about/search_and_browse), as project PRJEB8815, with reads accession numbers ERS684186-ERS684197 and ERS764859–ERS764870, respectively.

**Supporting Information**

S1 Text. Supplementary analysis of Pool-Seq data and frequencies of wMel variants. (DOCX)

S1 Fig. Frequencies of significantly differentiated SNPs in the Ancestral, Control and Virus-Selected populations. Frequencies of the major allele of Wolbachia single nucleotide polymorphisms (SNPs) in Ancestral (top), Control (middle) and Virus-Selected populations (bottom), determined by Pool-Seq. Shown are SNPs which significantly changed frequencies between Ancestral and Virus-Selected populations at generation 20. Panel columns 1 to 4 represent replicate populations. (TIF)

S2 Fig. Significantly different SNP frequencies between Ancestral, Control and Virus-Selected populations. (A) -log10 Benjamini & Hochberg adjusted \( p \) values for differences in frequencies of SNPs across the wMel genome, between the Ancestral and Control populations (top panel), Ancestral and Virus-Selected populations (mid panel) and Control and Virus-Selected populations (bottom panel). All the SNPs that were polymorphic in the Pool-Seq analysis are shown. (B) Venn diagram of the overlap of the significantly differentiated SNPs between Ancestral, Control and Virus-Selected populations. SNP frequencies were considered significantly different among treatments when Benjamini & Hochberg adjusted \( p \) values (q-values) were below a false discovery rate threshold of 0.1%. (TIF)

S3 Fig. Wolbachia variants, not mitochondrial variants, confer differential protection to viral infection. (A) Survival of flies of clade V (dark gray) and clade III (light gray) cytotypes five, six and seven days post infection with DCV (d.p.i.), in the presence or absence of wMel. (B) Cox hazard ratio between flies of clade V and clade III cytotypes, calculated from survival data until 20 d.p.i., in the presence (Wolb+) or absence of wMel (Wolb-). (C) Reproductive output of 5–7 d.p.i. flies of clade V and clade III cytotypes, in the presence (Wolb+) or absence of wMel (Wolb-). In all assays the female progeny of ten independent reciprocal crosses between isofemale flies of clade V (dark gray) and clade III (light gray) genotypes, were analyzed after systemic infection with DCV (2 x 10^7 TCID50/ml). *** \( p < 0.001 \). Means (± 95%
confidence intervals) are shown in all panels.

S4 Fig. *Drosophila* reproductive output is not influenced by the *Wolbachia* variant in the absence of DCV infection. Mean (±95% confidence interval) reproductive output of flies carrying Clade V and Clade III wMel variants in the absence of DCV infection. Reproductive output between flies carrying different wMel variants is not significantly different (GLMM, $\chi^2_1 = 2.322, p = 0.128$). The female progeny of eleven independent reciprocal crosses between isofemale flies, carrying Clade V and Clade III wMel variants, were assayed. Females 8–11 day-old laid eggs for 48h, matching the protocol of DCV infected flies.

S5 Fig. DCV and *Wolbachia* loads in flies carrying wMel variants from different clades. (A) Relative DCV levels at 7 d.p.i. and (B) relative *Wolbachia* titers 7 d.p.i. The female progeny of eleven reciprocal crosses between isofemale flies, carrying Clade V and Clade III wMel variants, were analyzed after systemic infection with DCV (2 x $10^7$ TCID$_{50}$/ml). $p < 0.001$ (***$p$*) in both comparisons.

S6 Fig. Frequencies of wMel Clade V diagnostic SNPs in the Ancestral, Control and Virus-Selected populations. Frequencies of wMel Clade V diagnostic SNPs in Ancestral, Control and Virus-Selected populations, determined by Pool-Seq. The data is discriminated for the four replicate populations of each condition.

S1 Dataset. Polymorphic positions in the wMel genome in Ancestral, Control and Virus-Selected populations. Frequencies of the 211 positions that were detected as being polymorphic (major allele frequency $\leq 0.98$) in any of the pooled samples. Shown are position in the reference wMel genome (AE017196) (POS), nucleotide in reference genome (REF), alternative nucleotide (ALT), major allele in Ancestral populations (MA_C0) and frequencies in the Ancestral (C0), Control (C20) or Virus-Selected (V20) columns (95% confidence intervals are shown in the corresponding _CI columns). The frequencies of these SNPs in each population are discriminated in the columns C0_1–4, C20_1–4, and V20_1–4. 133 of these polymorphisms significantly differentiated in at least one of the 3 possible comparisons between Ancestral, Control and Virus-Selected regimes (“Sign” in the C0_C20, C0_V20 and C20_V20 columns). Benjamini & Hochberg adjusted $p$ values (q-values) were considered significant if below a false discovery rate threshold of 0.1%. Columns Clades I-VIII show the allele(s) described for these clades in [13,14,21]. In all positions, the major allele in the Ancestral populations (MA_C0) matched the one described for clade V [21]. The column Annotation indicates in which predicted gene the SNP is located or if it is in an intergenic region. For each SNP within a gene the change in the codon (Codon) and if that leads to a synonymous or non-synonymous codon (Effect) is shown.

S2 Dataset. Mean survival across generations in the Virus and Bacteria Selection regimes. Estimates for the mortality after infection of individuals from Virus- and Bacteria-Selected populations, across 20 Generations of selection. Average of log-odds across all populations and both sexes (Mean), and separately for each of the four replicate populations (Pop_1–4) are shown. Female and male survival estimates are shown in the F_Pop_1–4 and M_Pop_1–4 columns, respectively. Number of total (N_Total), female (N_Females) and male (N_males) selected individuals, are shown. (NA) represent estimates of survival, which are not available.
(e.g. females at generations 0–3), despite having been selected. (NS) represent generations where selection was not done.

S3 Dataset. Frequencies of flies carrying wMel clade III or clade V in Ancestral populations and generation 20 of Control, Virus-Selected, and Bacteria-Selected populations (Data for Fig 1B).

S4 Dataset. Frequencies of flies carrying wMel clade III or clade V in Ancestral populations, and Virus-Selected populations at several generations (Data for Fig 1C).

S5 Dataset. Survival data for DCV-infected flies carrying different wMel clades (Data for Fig 2A and 2B).

S6 Dataset. Reproduction data for DCV-infected flies carrying different wMel clades (Data for Fig 2C).

S7 Dataset. Survival data for DCV-infected flies with different cytotypes, with and without wMel (Data for S3A and S3B Fig).

S8 Dataset. Reproduction data for DCV-infected flies with different cytotypes, with and without wMel (Data for S3C Fig).

S9 Dataset. Reproduction data for non-infected flies carrying different wMel clades (Data for S4 Fig).

S10 Dataset. Relative DCV titers in flies carrying different wMel clades (Data for S5A Fig).

S11 Dataset. Relative Wolbachia titers in flies carrying different wMel clades (Data for S5B Fig).

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Author Contributions

Conceptualization: SM ÉS LT.

Data curation: NEM.

Formal analysis: NEM.

Funding acquisition: CS ÉS LT.

Investigation: VGF NEM TFP VN.
Methodology: VGF NEM SM CS ÉS LT.

Project administration: ÉS LT.

Resources: CS ÉS LT.

Supervision: SM CS ÉS LT.

Visualization: VGF NEM LT.

Writing – original draft: VGF NEM ÉS LT.

Writing – review & editing: VGF NEM SM ÉS LT.

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