

RESEARCH ARTICLE

Diet-Induced Nutritional Stress and Pathogen Interference in *Wolbachia*-Infected *Aedes aegypti*

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Abstract

The pathogen interference phenotype greatly restricts infection with dengue virus (DENV) and other pathogens in *Wolbachia*-infected *Aedes aegypti*, and is a vital component of *Wolbachia*-based mosquito control. Critically, the phenotype's causal mechanism is complex and poorly understood, with recent evidence suggesting that the cause may be species specific. To better understand this important phenotype, we investigated the role of diet-induced nutritional stress on interference against DENV and the avian malarial parasite *Plasmodium gallinaceum* in *Wolbachia*-infected *Ae. aegypti*, and on physiological processes linked to the phenotype. *Wolbachia*-infected mosquitoes were fed one of four different concentrations of sucrose, and then challenged with either *P. gallinaceum* or DENV. Interference against *P. gallinaceum* was significantly weakened by the change in diet however there was no effect on DENV interference. Immune gene expression and H₂O₂ levels have previously been linked to pathogen interference. These traits were assayed for mosquitoes on each diet using RT-qPCR and the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit, and it was observed that the change in diet did not significantly affect immune expression, but low carbohydrate levels led to a loss of ROS induction in *Wolbachia*-infected mosquitoes. Our data suggest that host nutrition may not influence DENV interference for *Wolbachia*-infected mosquitoes, but *Plasmodium* interference may be linked to both nutrition and oxidative stress. This pathogen-specific response to nutritional change highlights the complex nature of interactions between *Wolbachia* and pathogens in mosquitoes.

Author Summary

Mosquito-transmitted disease severely impacts human health around the world. One novel form of control involves infecting medically important mosquito species with the naturally occurring bacterium *Wolbachia*, which restricts dengue and malaria transmission through a process called pathogen interference. The interference phenotype is still poorly understood, and potentially involves multiple physiological changes to the

mosquito. We examined the role of nutritional stress on pathogen interference in the dengue vector *Aedes aegypti*, in order to better understand factors that might lead to variable interference. We demonstrated that interference against malaria-causing *Plasmodium galinaceum* was dependent on mosquito nutritional status, however interference against dengue was not, implying that pathogen interference operates differently for different pathogens. We then examined mosquito immune processes that had been previously correlated with pathogen interference, and demonstrated that mosquito nutrition did not affect the expression of key mosquito immune genes, but did affect levels of reactive oxygen species. Our results highlight the complexity of the phenotype, and importantly suggest that adult nutrition may not be a key determinant of interference against DENV.

Introduction

Wolbachia pipiensis (Rickettsiaceae) is an obligate bacterial endosymbiont that shows great potential as a natural control agent for a range of clinically important mosquito-transmitted pathogens, including those responsible for diseases such as malaria and dengue in humans [1, 2]. *Wolbachia* naturally infect an estimated 40% of terrestrial insect species [3]. Infection often results in manipulation of host biology, with the nature and extent of these manipulations varying depending on the host and infecting *Wolbachia* strain [4, 5].

Wolbachia are maternally transmitted, and heavily infect host ovaries. The bacterium is often associated with extreme manipulation of the host reproductive process, furthering bacterial propagation at the expense of host fitness [6]. These manipulations allow the bacterium to naturally spread into uninfected insect populations, and to move across large distances [7]. The most common reproductive manipulation is cytoplasmic incompatibility (CI). CI-causing *Wolbachia* strains prevent or restrict viable egg production when uninfected females mate with *Wolbachia*-infected male insects, while *Wolbachia*-infected females can successfully breed with either infected or uninfected males. Infection can affect other host physiological processes including oogenesis [8], chemosensory perception [9] and parasitism [10]. Some strains form mutualistic relationships with their hosts, contributing resources [11], or enhancing fitness [12], while others are metabolically dependent on their hosts, and the resources they consume [13, 14].

Wolbachia naturally infect many mosquito species including *Aedes albopictus* and *Culex pipiens*, but not the primary dengue vector *Aedes aegypti* or most anopheline vectors of human malaria. Infections in *Ae. aegypti* have been generated in the laboratory via transinfection [15], through the injection of cytoplasm from the eggs of a *Wolbachia*-infected donor species into *Ae. aegypti* embryos [16–18]. These infections cause pathogen interference [19, 20], a *Wolbachia*-induced decrease in susceptibility to infection with pathogens including the dengue (DENV), chikungunya, yellow fever and West Nile viruses, filarial nematodes and some bacteria [17, 21–24]. Pathogen interference can result in decreased pathogen load, and largely prevent disseminated viral infection and salivary transmission [17, 25, 26]. Interference against DENV has been thoroughly studied in *Ae. aegypti* infected with the wMel and wMelPop *Wolbachia* strains, with the strength and prevalence of the interference phenotype dependent on the viral isolate and serotype [25].

Wolbachia can also interfere with *Plasmodium* infection in mosquitoes, however interaction between the bacterium and these parasites appears to be more variable. The only stable *Wolbachia* transinfection in an anopheline mosquito, wAlbB in *Anopheles stephensi*, reduced *Plasmodium falciparum* oocyst and sporozoite numbers [27]. Infection with wMelPop in *Ae. aegypti*

produced stronger interference against *P. gallinaceum* [23]. However, this effect may not be representative of how *Wolbachia* interacts with *Plasmodium* species that infect humans, given that the *Plasmodium* species that infect different animals are phylogenetically distinct [28], and that there are genetic, metabolic and immunological differences between anopheline and culicine mosquitoes [29, 30]. Prior to transinfection, *Wolbachia* infection in anophelines was studied using transient infection via somatic injection of *Wolbachia*. Some of these associations produced pathogen interference, however for *wAlbB* infections of *Anopheles gambiae*, *Plasmodium berghei* infection was enhanced [31]. This enhancement may be temperature dependent [32], and has also been observed for some native *Wolbachia* infections, including in *Culex pipiens* where *Wolbachia* protects the host against *Plasmodium*-induced mortality, but also increases susceptibility to infection [33–35]. Interestingly, such enhancement has never been observed for *Plasmodium* species that infect humans, or in a mosquito with a stable *Wolbachia* transinfection.

The process underlying pathogen interference remains poorly understood, while potential causes of enhancement are only hypothetical [31, 36]. Strong pathogen interference is typically associated with high *Wolbachia* density [17, 37]. The effect has been linked to activation of immune effector genes [22, 23, 38, 39], increased induction of reactive oxygen species (ROS) and related genes, which serve as part of the host defence against pathogens [38, 40], and competition for host cholesterol in *Drosophila melanogaster* [41]. Critically, none of these effects occur universally amongst the species and *Wolbachia* strains where pathogen interference has been observed, which suggests that the underlying mechanism may be complex, and that it could potentially be dependent on the length of the host-symbiont relationship [39, 42, 43].

Pathogen interference and CI serve as the basis for a form of *Wolbachia*-dependent mosquito control through mosquito population replacement [2, 44], which is currently being utilised for *Ae. aegypti* and dengue (www.eliminatedengue.com). This involves the release of *Wolbachia*-infected mosquitoes that mate with the wild population, where CI increases the *Wolbachia* infection frequency over successive generations [45]. High prevalence of pathogen interference in these mosquito populations would then potentially reduce disease transmission amongst humans [25]. Successful *Wolbachia* invasion is dependent on local environmental conditions, and a high proportion of infected individuals [44, 46]. Another critical factor is the competitiveness of released mosquitoes [47], with high fitness costs, as seen with the *wMelPop* strain [16, 48, 49], leading to rapid loss of infection in field populations [50]. In contrast, the *wMel* strain has minimal fitness costs [17], and a stably infected population has persisted for several years in the field [51], with no loss of pathogen interference observed since the initial release [52].

Nutritional status and diet are key factors in an insect's ability to resist infection with a pathogen [53–55]. Likewise, many pathogens are dependent on host nutritional resources, and can manipulate host metabolic process in order to facilitate infection [56–59]. Recent evidence has demonstrated that *Wolbachia* has a similar metabolic dependency [41, 60, 61], and this suggests that there is great potential for tripartite interactions between *Wolbachia*, pathogens, and host metabolism and nutrition to play a role in pathogen interference.

To that end, we have used dietary carbohydrate concentration as a platform to study the influence of host nutrition on the complexity and plasticity of pathogen interference and associated processes in female *wMel*-infected *Ae. aegypti*. We investigated the role of diet-induced nutritional stress on interference against DENV and *P. gallinaceum*, and levels of immune gene expression and H₂O₂, which have previously been linked to the phenotype. Through these experiments we sought to further understanding of how *Wolbachia* can influence pathogen infection.

Results

DENV infection

In all experiments described below, adult mosquitoes were fed one of four carbohydrate regimes (1%, 5%, 10% or 20% raw sugar solution). Two experimental infections with a recently circulating Brazilian DENV-3 isolate were performed to determine if altered carbohydrate diets affected pathogen interference against DENV. Mosquitoes were fed on the carbohydrate regimes for 7 days post-eclosion, and then orally challenged with DENV. In both replicates, no DENV RNA was amplified from any *wMel* sample at either 7 (Fig 1A & 1C) or 14 days post-infection (Fig 1B & 1D), for any diet. In contrast, the Tet infection rate varied between 35% and 82%, depending on diet, and the duration of infection. Prevalence (proportion infected with DENV-3) was consequently significantly higher for Tet mosquitoes than for *wMel*, for each diet (Fisher's exact test; $P = 0.0033 - <0.0001$). As no *wMel* mosquitoes became infected, only the viral load data for Tet mosquitoes were compared statistically. There was a significant difference in viral load due to host nutrition at 7 dpi for both replicates, characterized by higher DENV levels on the 1% diet (Kruskal Wallis; $R_1 - P = 0.0489$; $R_2 - P = 0.0084$). At 14 dpi there were higher DENV levels on the 1% diet in the first replicate (Kruskal Wallis; $P = 0.0015$), but no effect in the second replicate.

Plasmodium gallinaceum infection

Three replicate *P. gallinaceum* infection experiments were performed to assess the impact of dietary carbohydrate levels on the ability of the *wMel* *Wolbachia* strain to interfere with *Plasmodium* infection (Fig 2). Prevalence (proportion of mosquitoes infected) and intensity (number of oocysts in infected mosquito midguts) of *Plasmodium* infection were measured at 7–8 days post-infection, and data were compared independently for each experiment using binomial regression to determine the effects of *Wolbachia* infection and diet (S1 Table). In both experiment 1 (Fig 2A) and 2 (Fig 2B), *Wolbachia* was a significant factor affecting prevalence (Binomial models; $E_1 - P = 0.0014$; $E_2 - P < 0.0001$). There was a strong inhibitory effect of *wMel* on the 10% diet in both experiments, however this attenuated as dietary carbohydrate levels changed, as evidenced by a significant effect of diet (Binomial models comparing each diet against the 10% diet; $E_1 - 1\%$ diet and 20% diet— $P < 0.0001$; $E_2 - 1\%$, 5% and 20% diets— $P < 0.0001$). Interestingly, pairwise comparisons of prevalence for *wMel* and Tet mosquitoes on each diet revealed that some level of interference was maintained for all diets except the 1% (Fisher's exact test; $E_1 - P < 0.05$, $E_2 - P < 0.001$).

In experiment 3 (Fig 2C), the prevalence of *Plasmodium* infection observed for the 10% diet was greater than in the other two experiments, although the parasitemia level was lower. In this experiment there was no overall effect of *Wolbachia* on prevalence (Binomial regression; $P = 0.646$), however there was still a significant effect of diet (Binomial models; 1% diet— $P < 0.05$; 5% and 20% diets— $P < 0.001$).

Plasmodium intensity data were compared independently for each experiment using binomial negative regression, and we observed a significant effect of *Wolbachia* infection only in experiment 2 (Binomial negative regression against 10% diet; $P < 0.0001$). As for prevalence, a change in diet led to increased intensity of infection for all three experiments when compared to the 10% diet (Binomial negative regression: $E_1, E_2 -$ All diets: $P < 0.0001$, $E_3 - 1\%$ and 5%: $P < 0.0001$, 20%: $P > 0.05$, all comparisons in reference to the 10% diet). However, pairwise comparisons for each diet indicated that a significant interference effect due to *Wolbachia* was induced only for low carbohydrate diets, as *wMel* infection reduced the intensity of infection on the 1% diet in all 3 experiments (Mann Whitney U test; $E_1 - P = 0.0151$; $E_2 - P < 0.0001$; $E_3 - P = 0.0231$), and for the 5% diet in experiments 2 and 3 (Mann Whitney U test; $E_2 - P < 0.0001$; $E_3 - P = 0.0376$).

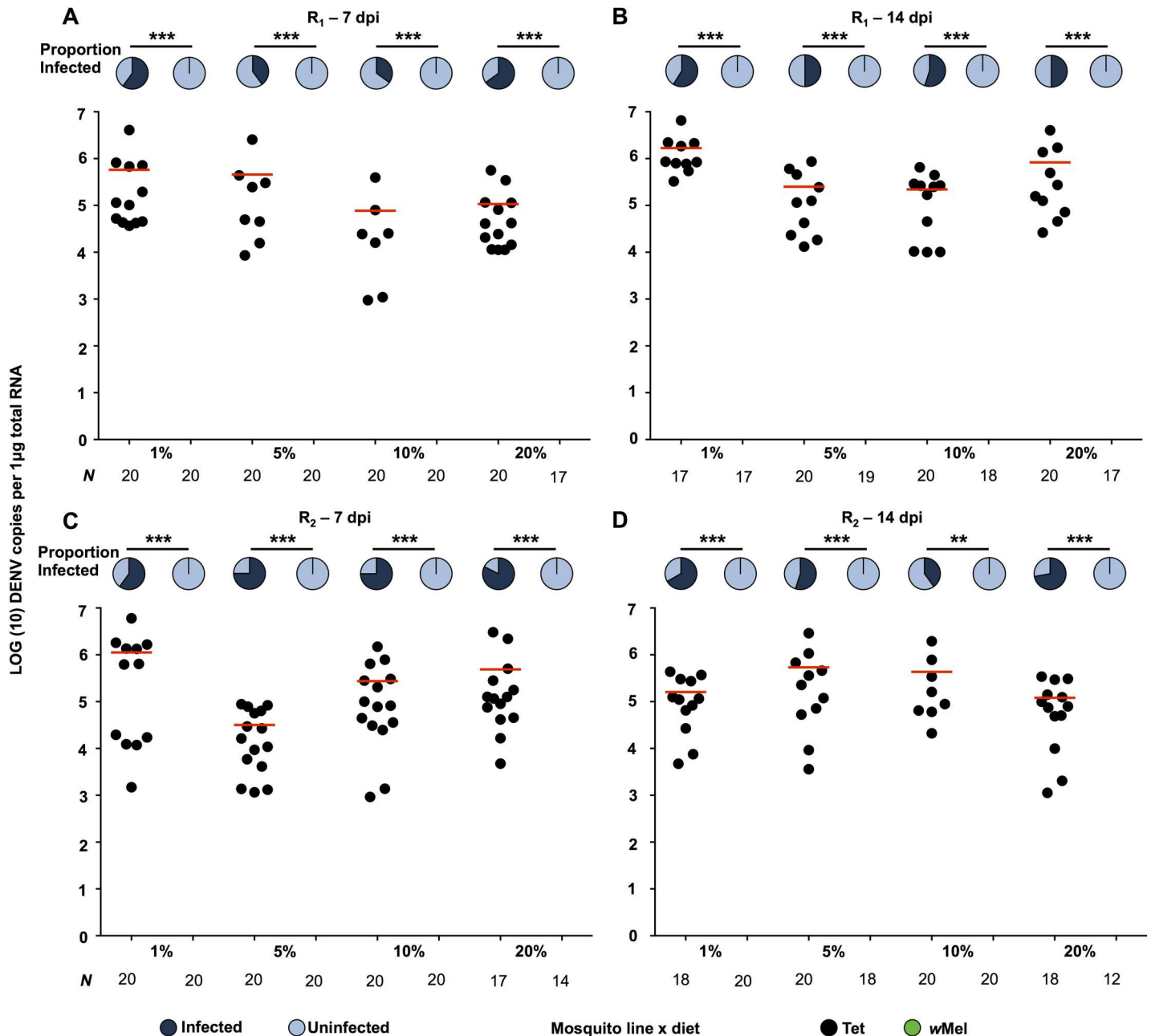


Fig 1. Interference against DENV-3 in wMel-infected *Ae. aegypti* fed on different carbohydrate regimes. DENV-3 prevalence and intensity data for wMel (+*Wolb*) and Tet (-*Wolb*) mosquitoes fed on one of four carbohydrate diets after experimental oral infection (R₁—A - 7dpi, B - 14dpi; R₂—C - 7dpi, D - 14dpi), as determined by RT-qPCR quantification using a DENV-specific TaqMan probe. Pie charts represent prevalence of infection (dark blue—proportion infected, light blue—proportion uninfected), and dot plots represent viral load in infected mosquitoes. Horizontal lines in each treatment represent mean viral load. *P* values: ** < 0.01, *** < 0.001, Prevalence—Fisher’s exact test.

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Longevity assay

To obtain a broad indicator of fitness changes due to host nutrition, we compared the effects of altered dietary carbohydrates on the longevity of *Wolbachia*-infected wMel mosquitoes using Cox Regression. Average mosquito survival was greater with higher dietary carbohydrate levels (S1 Fig). Average (\pm s.e.m.) survival time on the control diet was 29.48 ± 1.15 days,

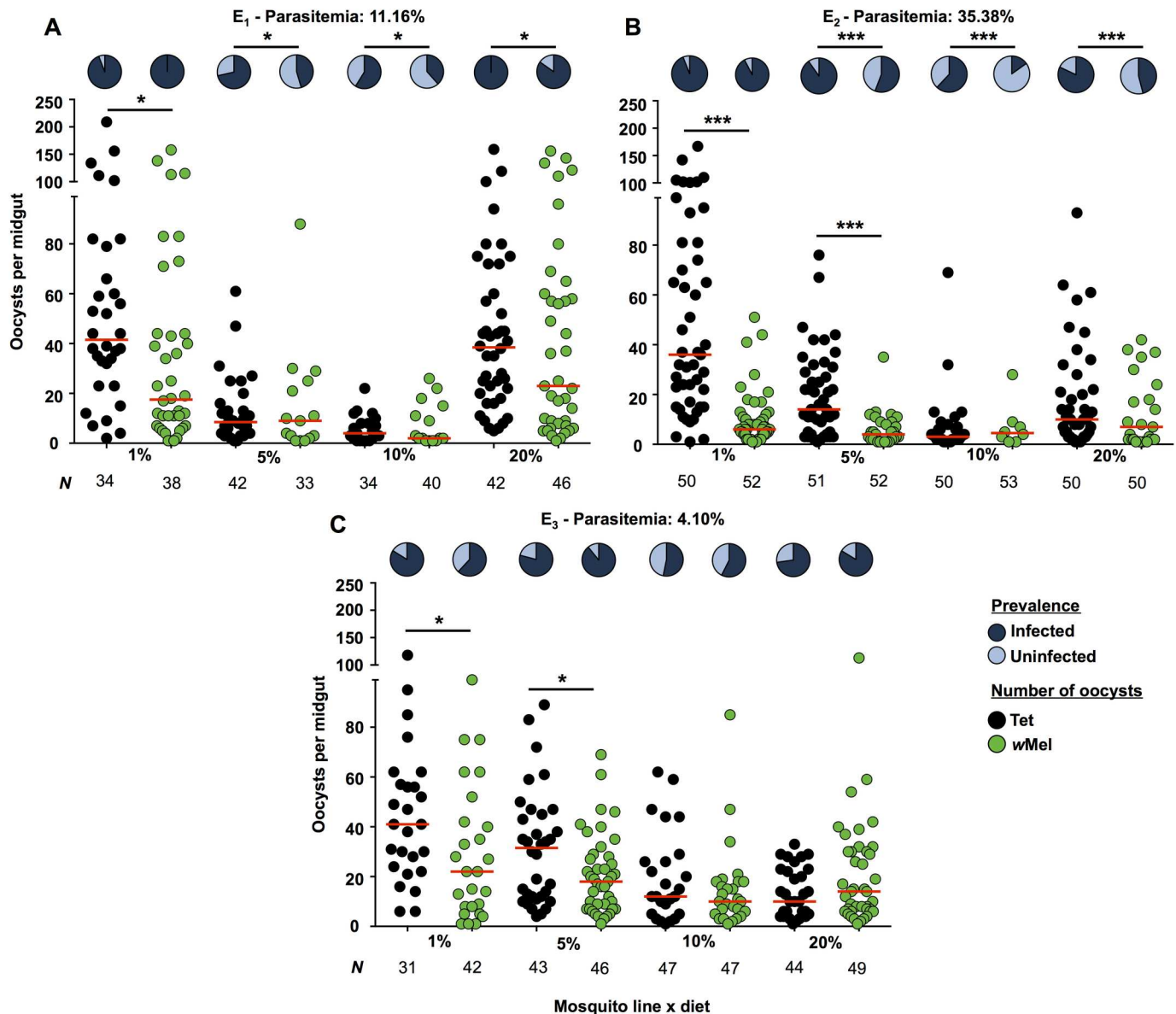


Fig 2. Interference against *P. gallinaceum* in wMel-infected *Ae. aegypti* fed on different carbohydrate regimes. In three experimental replicates (E_1 —A, E_2 —B, E_3 —C), wMel (+*Wolb*) and Tet (-*Wolb*) mosquitoes were fed one of four different carbohydrate regimes and then fed on a single *Plasmodium gallinaceum*-infected chicken. Prevalence of infection was determined by counting the proportion of mosquito's that had oocysts in their midguts at 7–8 days post-infection (pie charts: proportion infected—dark blue, proportion uninfected—light blue). Intensity of infection was calculated as the number of oocysts per midgut for Tet (black circles), and wMel mosquitoes (green circles). Red bars represent median oocysts for *P. gallinaceum*-infected mosquitoes. *P* values: * < 0.05, *** < 0.001, Prevalence—Fisher's exact test, Intensity—Mann Whitney *U* test.

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which was 12.28 days and 4.26 days longer than the average survival times for mosquitoes reared on the 1% and 5% diets, but 5.57 days shorter than the average for the 20% diet. Diet was a significant factor affecting mosquito longevity (Cox Regression; $P < 0.0001$). The 1% ($B = 4.41$, 95% CI = 3.32–5.88, $P < 0.0001$) and 5% ($B = 1.38$, 95% CI = 1.06–1.79, $P = 0.016$) regimes were associated with significantly higher hazard ratios than the control diet (10%), however the 20% diet led to a lower hazard ratio than the control ($B = 0.60$, 95% CI = 0.46–0.78, $P < 0.0001$).

Wolbachia levels

Expression levels of the *Wolbachia* gene *wsp* were quantified relative to the host *rps17* in mosquitoes after 7 days of feeding on the different dietary regimes, in order to determine if the different regimes altered *Wolbachia* levels in *wMel* mosquitoes (Fig 3). These data were compared statistically using univariate general linear models, which indicated that there was no statistically significant effect of diet (GLM; $P = 0.468$).

Immune gene activation

We looked at whether changing dietary carbohydrate levels affected the expression of four genes associated with immune activation by *Wolbachia*.

These genes, Cecropin E (*cece*) and Defensin C (*defc*), both antimicrobial peptides stimulated by the Toll and IMD immune pathways, C-type lectin galactose binding 5 (*ctlga5*), a carbohydrate-binding protein involved in bacterial recognition, and Transferrin (*tsf*), an iron transport protein, were all strongly upregulated by *wMel* infection in Australian mosquitoes [39]. Expression data for each gene were analysed independently using general linear models

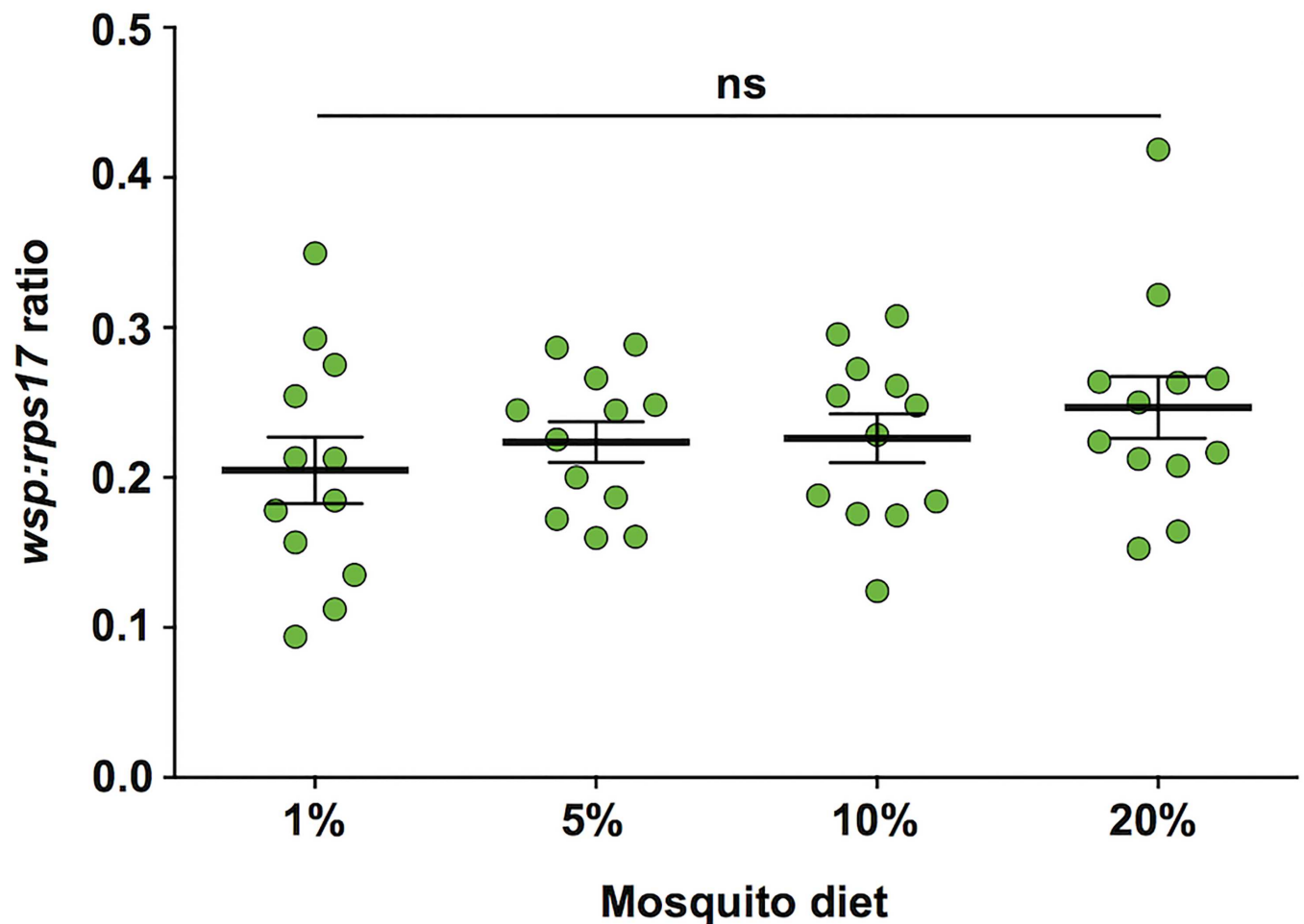


Fig 3. *Wolbachia* levels in *wMel*-infected *Ae. aegypti* fed on different carbohydrate regimes. Expression levels of *Wolbachia* surface protein (*wsp*) were quantified relative to the mosquito *rps17* gene for 12 pairs of female mosquitoes from each diet using RT-qPCR. Each circle represents one pair of mosquitoes. Solid black lines represent mean expression (\pm s.e.m.). P values: ns > 0.05

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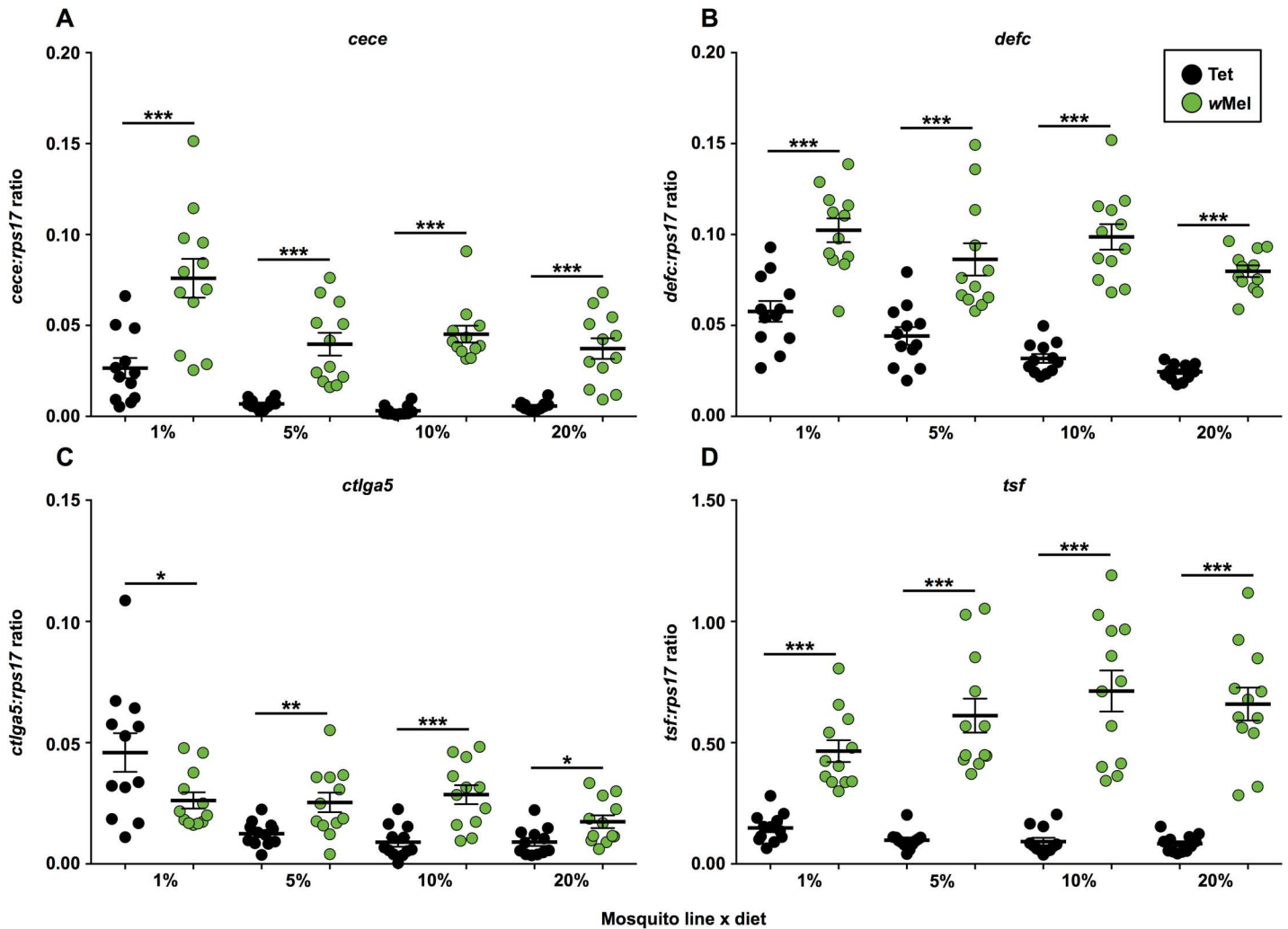


Fig 4. Immune activation in wMel-infected *Ae. aegypti* fed on different carbohydrate regimes. Levels of 4 key immune genes, Cecropin E (*cece*) (A), Defensin C (*defc*) (B), C-type lectin, galactose binding 5 (*ctlga5*) (C), and Transferrin (*tsf*) (D), were quantified through RT-qPCR for wMel (+*Wolb*) and Tet (-*Wolb*) mosquitoes, after 7 days feeding on their respective carbohydrate diets. Expression levels were normalized against host *rps17* expression levels. Each circle represents one pair of either Tet (black circles) or wMel (green circles) mosquitoes, with 12 samples examined for each treatment. Solid black lines represent mean expression (\pm s.e.m.). *P* values: Student's *t* tests, * < 0.05 , ** < 0.01 *** < 0.001 .

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to determine if *Wolbachia* infection status or nutrition had a major effect (Fig 4). Data for all immune assays were obtained from mosquitoes fed on carbohydrates for 7 days. These mosquitoes were not blood fed or infected with a pathogen.

Levels of *cece* (Fig 4A) and *defc* (Fig 4B) were significantly affected by both *Wolbachia* infection and diet (GLM; $P < 0.0001$), however there was no effect of interaction between diet and *Wolbachia* infection. Analysis of individual treatments revealed that *cece* and *defc* levels were higher in wMel mosquitoes than in Tet for all diets (Student's *t* tests; $P < 0.001$). Levels of *cece* were increased on the 1% diet for both wMel and Tet mosquitoes, while *defc* expression in Tet mosquitoes was increased on the 1% and 5% diets, although average expression levels were still lower than for wMel.

Wolbachia did not have a significant effect on *ctlga5* expression in the overall GLM model, however both diet and the *Wolbachia* x diet interaction (GLM; $P < 0.0001$) were significant factors. Expression levels of *ctlga5* were higher in wMel mosquitoes than in Tet for all diets

except the 1% (Fig 4C; student's *t* tests—5% & 10%; $P < 0.01$, 20%; $P < 0.05$), where levels in *wMel* mosquitoes remained high, but Tet levels were slightly higher (Student's *t* test; $P = 0.0324$). There was a decrease in expression for *wMel* mosquitoes on the 20% diet, where levels were on average 39.06% lower than for the 10% diet.

Expression levels of *tsf* (Fig 4D) were not significantly affected by diet in the overall model, but were affected by both *Wolbachia* (GLM; $P < 0.0001$) and *Wolbachia* x diet (GLM; $P = 0.015$). *tsf* levels were significantly higher for *wMel* than Tet for all 4 diets (Student's *t* tests; $P < 0.0001$). However, *tsf* expression in *wMel* mosquitoes on the 1% diet was significantly lower than for the 10% and 20% diets (Student's *t* test; $P < 0.05$).

Immune pathway regulation

The expression of 8 genes with putative regulatory roles in the mosquito IMD (*caspar* and *rel2*), JAK-STAT (*domeless* and *pias*), JNK (*ap-1* and *jnk*) and Toll (*cactus* and *rel 1A*) immune pathways was examined in order to determine whether diet x *Wolbachia* interactions had a broader effect on host immunity (S2 Fig). We observed no effect of *Wolbachia*, diet, or *Wolbachia* x diet interaction in the expression of these genes. The one exception to this was for *pias*, a putative negative regulator of the JAK-STAT immune pathway, where *Wolbachia* but not diet or the *Wolbachia* x diet interaction was a significant predictor in the overall model (GLM; $P = 0.044$). In biological terms, this translated to higher *pias* expression in *wMel* mosquitoes compared to Tet, but only for the 10% diet.

Stress response & ROS induction

Expression levels of *duox-2* (Fig 5A), an important gene in mosquito reactive oxygen species production, were unaffected by *wMel* infection, diet, or diet x *Wolbachia* interaction (GLM; $P < 0.05$). Likewise levels of *nos* (Fig 5B), which is involved in nitric oxide production, were unaffected by the presence of *Wolbachia* (GLM; $P < 0.05$). However, we observed a significant increase in *nos* expression associated with lower carbohydrate diets in the overall model (GLM; $P < 0.0001$), and independently for both Tet (GLM; $P < 0.0001$) and *wMel* mosquitoes

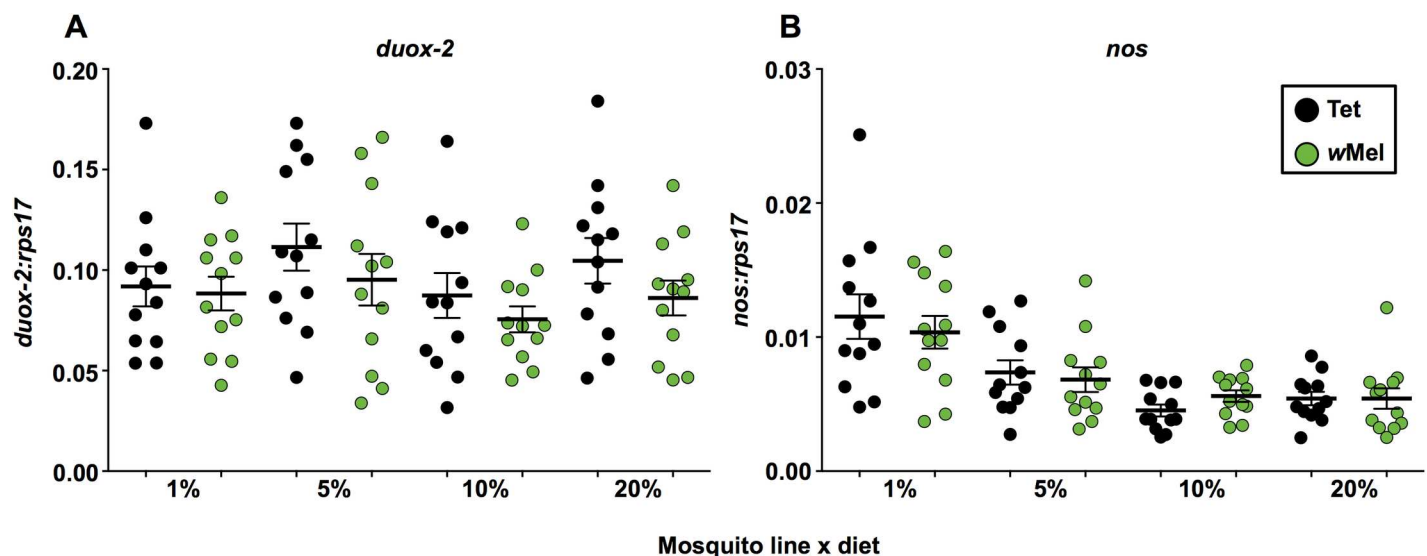


Fig 5. Levels of key oxidative stress response genes in *wMel*-infected *Ae. aegypti* fed on different carbohydrate regimes. Levels of Dual Oxidase 2 (*duox-2*) (A) and Nitric Oxide Synthase (*nos*) (B) were quantified for Tet (-*Wolb*) and *wMel* (+*Wolb*) mosquitoes using RT-qPCR. Gene expression values were normalized against host *rps17* expression. Each circle represents one pair of either Tet (black circles) or *wMel* (green circles) mosquitoes, with 12 samples examined per treatment. Solid black lines represent mean expression (\pm s.e.m.). *P* values: General linear models, ** < 0.01 , *** < 0.001 .

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(GLM; $P = 0.001$). Average *nos* levels were 45.92% higher for *wMel* mosquitoes on the 1% diet than those on the 10% diet (student's *t* test; $P = 0.0013$). For Tet mosquitoes, the 1% diet had on average 60.74% higher *nos* levels than the 10% diet (student's *t* test; $P = 0.0005$), and those from the 5% diet had on average 38.56% higher *nos* levels (student's *t* test; $P = 0.0099$).

H_2O_2 levels were quantified in pairs of female mosquito after spending 7 days feeding on the different carbohydrate diets (Fig 6). *Wolbachia* infection (GLM; $P < 0.0001$), diet (GLM; $P < 0.0001$) and *Wolbachia* x diet (GLM; $P = 0.0006$) were all significant factors affecting H_2O_2 levels in mosquitoes. H_2O_2 levels in Tet mosquitoes did not change due to diet however mean H_2O_2 levels in *wMel* mosquitoes were positively correlated with dietary carbohydrate concentration. ROS induction due to *Wolbachia* infection was observed for each of the three highest concentration diets, where significantly higher levels were observed in *wMel* mosquitoes (Student's *t* tests; 5% diet— $P = 0.0046$; 10% diet— $P = 0.0040$; 20% diet— $P = 0.0082$), however on the 1% diet there was no effect of *Wolbachia* (Student's *t* test; $P = 0.2034$).

Discussion

A contrasting effect of host nutrition on DENV & *Plasmodium* interference

Pathogen interference in *Wolbachia*-infected *Ae. aegypti* restricts or prevents infection and transmission of DENV and other pathogens [22, 23, 25]. Interference is fundamental to

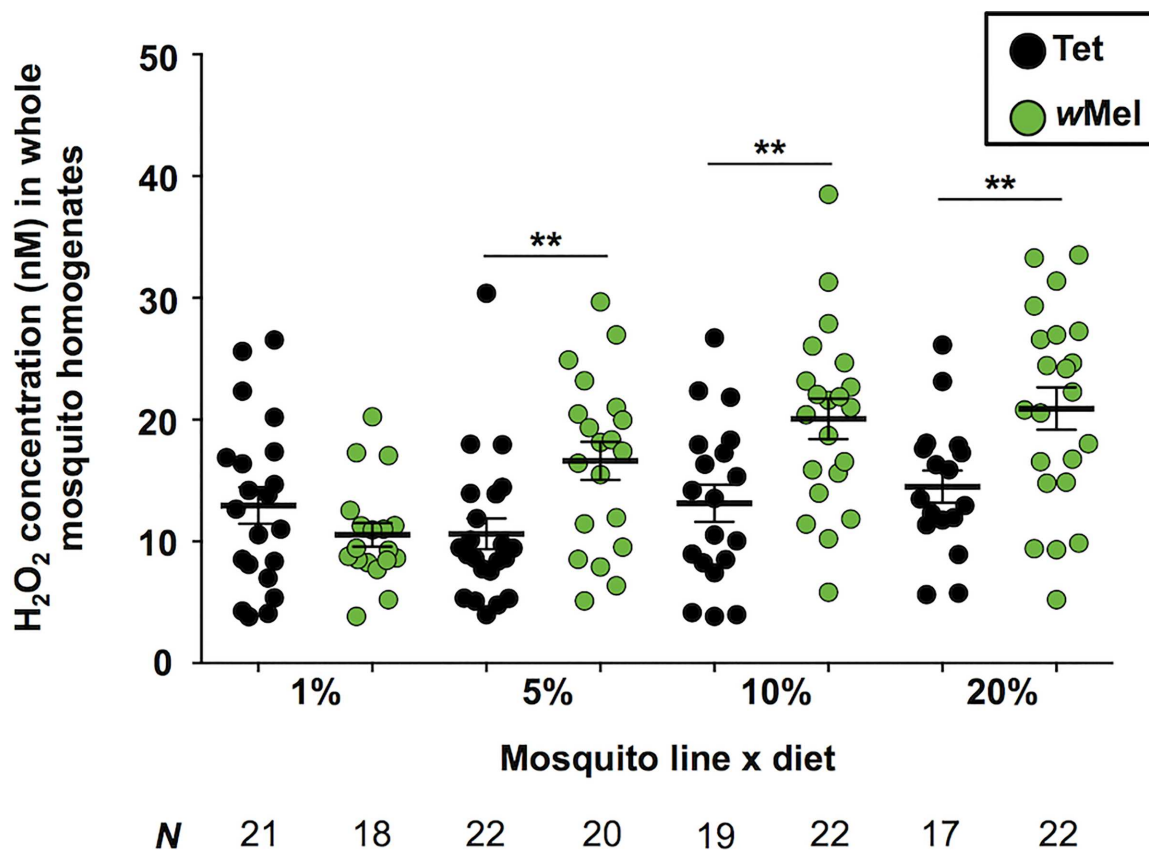


Fig 6. ROS induction in *wMel*-infected *Ae. aegypti* fed on different carbohydrate regimes. Levels of reactive oxygen species (H_2O_2) were quantified for whole *wMel* (+*Wolb*) and Tet (-*Wolb*) mosquitoes from all 4 diets using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. Each circle represents one pair of either Tet (circles) or *wMel* (green circles) mosquitoes, with 17–22 samples examined for each treatment. Solid black lines represent mean expression (\pm s.e.m.). *P* values: Student's *t* tests, ** < 0.01 .

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transmission-blocking strategies that use *Wolbachia* to combat mosquito-transmitted disease [44], yet the underlying biological processes remain poorly understood. Competition for nutrients is important to interference in *Drosophila* [41], but no link with host nutrition had previously been made in *Ae. aegypti*. To that end, we fed *wMel* (+*Wolb*) and Tet (-*Wolb*) *Ae. aegypti* mosquitoes with 1 of 4 carbohydrate diets (1%, 5%, 10% or 20% sucrose solution), and challenged them with either DENV-3 or *P. gallinaceum*.

We observed strong interference to both pathogens on the 10% (control) diet. For *P. gallinaceum*, *wMel* infection reduced the prevalence of infection but did not affect intensity, while no *wMel* mosquitoes became infected with DENV. Pathogen interference against *P. gallinaceum* had not previously been described for *wMel*-infected *Ae. aegypti*. This effect was not as strong as for *wMelPop*-infected *Ae. aegypti* where there was greatly reduced prevalence and intensity of infection [23], although that strain has a higher bacterial density, which likely promotes stronger pathogen interference [17, 25]. Our DENV interference results were similar to results from other DENV isolates, where mosquitoes were reared on 10% sucrose [17, 25].

Altering host nutritional status by feeding 1%, 5% or 20% sucrose led to increased prevalence of *P. gallinaceum* infection in *wMel* mosquitoes, which could be interpreted as less effective pathogen interference. The effect was most striking on the 1% diet, where *P. gallinaceum* prevalence for both Tet and *wMel* mosquitoes was near 100%. This increased prevalence could have been driven by starvation, similar to what is seen with *Plasmodium* infection in mosquitoes that experience larval nutritional stress [55, 62]. These data suggest that there are certain nutritional states or biological conditions that favour *Plasmodium* infection to the point where an inhibitory effect by *Wolbachia* is not possible. The fact that we also observed less effective interference on the 20% diet indicated that our results could not be solely explained by a starvation effect, and could have been due to a broader modulatory effect of host nutrition. Changing nutritional status also increased the median oocyst count for both Tet and *wMel* mosquitoes, particularly on the 1% and 20% diets, however there was still a statistically significant effect of *wMel* infection for the latter. Interestingly, *wMel* limited the increase in the intensity of infection on the 1% and 5% diets, suggesting that the interference effects of *wMel* at the intensity level occurred with the change in host nutritional status.

We observed greater overall *P. gallinaceum* intensity, and a different effect of *Wolbachia* on *P. gallinaceum* prevalence in one experiment. Infection with *P. gallinaceum* is typically subject to high variability, with great differences in prevalence and pathogen levels resulting from mosquito, parasite and avian genetic factors, and environmental factors [33, 63, 64]. Each experiment involved different chickens, with different genetic and immune responses that could have influenced the course of infection [65]. Across the three experiments, a stronger pathogen interference effect was associated with higher parasitemia, with no effect of *Wolbachia* observed in the experiment with the lowest parasitemia. While we did observe some variation between experiments, our results did suggest that host nutritional status can alter the response of *wMel* to *P. gallinaceum* under some conditions, but also that this interference does not occur under all experimental conditions, and may only be induced during more severe infection.

In contrast, we saw no effect of host nutrition on DENV interference as no *wMel* mosquitoes became infected on any diet across two experiments. This indicated that DENV interference is not affected by the change in host nutritional status, starvation or dietary excess. Furthermore, the different response to host nutritional status between the two pathogens suggests that there are potentially host biological factors that differentially affect interference against *P. gallinaceum* and DENV.

Effect on processes associated with pathogen interference

We sought to determine if nutritional stress affected *Wolbachia* density, the expression of key immune genes and ROS levels, all of which have previously been linked to pathogen interference in either mosquitoes or *Drosophila*. These processes were characterized after mosquitoes fed on the different carbohydrate regimes for 7 days, the same time at which mosquitoes were infected with a pathogen in our experimental infection assays. These mosquitoes were not blood fed or infected with a pathogen in order to characterize basal changes due to diet and *Wolbachia*, and to avoid metabolic and transcriptional changes induced by blood feeding [56, 66].

High *Wolbachia* levels appear to be a key driver of pathogen interference [17, 67], and reduction of bacterial density can lead to weaker interference [68]. Critically, we saw no effect of diet on *Wolbachia* expression. This could potential indicate that the loss of interference against *P. gallinaceum* was not associated with a change in *Wolbachia* density. Although it is possible that such a change could occur in response to feeding on *Plasmodium*-infected blood, or that changes in *Wolbachia* levels at the tissue level led to a loss of bacterial density. An alternative explanation is that there was amelioration of another biological process linked to the phenotype.

Pathogen interference in mosquitoes has been strongly associated with the increased expression of key immune effector genes [22, 23, 38, 39]. We observed that high expression levels of four of these genes, *cece*, *defc*, *ctlga5* and *tsf* were consistently associated with *Wolbachia* infection for all diets. This could imply that a loss of immune gene activation did not underlie the less effective interference for *P. gallinaceum* that we observed on some diets, however it should be noted that we only measured basal immune gene levels, not in the context of *Plasmodium* infection, and this could potentially have led to different results. We did observe slight decreases in the expression of *tsf* on the 1% diet, and *defc* and *ctlga5* on the 20% diet in *wMel* mosquitoes, and it is possible that our results could be explained by a similar effect across a large number of immune genes.

Similarly, we saw no effect of diet on the expression of regulatory genes in the IMD, JAK-STAT, JNK, and Toll mosquito immune pathways that might explain our results. Given that *Plasmodium* and DENV infections stimulate different immune pathways [69–73], it was possible that a diet-induced change in regulatory gene expression could stimulate higher infection levels. However, we only saw an effect of *Wolbachia* on the expression of *pias*, a negative regulator of the JAK-STAT pathway, and this change—higher expression in *wMel* mosquitoes than Tet only for the 10% diet—did not adequately explain our results, as *wMel* mosquitoes had similar *pias* levels across all diets. These results do not preclude an immune basis for the *Plasmodium*-specific response if it were to occur through genes or pathways other than those we measured.

Diet can influence levels of ROS and oxidative stress in insects [74, 75], and we observed a clear effect of mosquito diet on ROS induction, with equivalent H₂O₂ levels in *wMel* and Tet mosquitoes from the 1% diet, and higher dietary carbohydrate levels associated with higher mean H₂O₂ levels in *wMel* mosquitoes. In contrast, H₂O₂ levels in Tet mosquitoes were unaffected by diet, suggesting that there was a *Wolbachia*-specific interaction between nutritional and oxidative stress. The ROS induction phenotype is strongly correlated with pathogen interference in both mosquitoes and *Drosophila* [38, 40]. However, it is not universal amongst all host-strain associations where pathogen interference occurs, as is the case for *wMel*-infected *Ae. albopictus*, where there is interference against DENV and Chikungunya virus infection [43, 76, 77]. The fact that ROS induction occurs for *wMel*-infected *Ae. aegypti* suggests that its absence in *Ae. albopictus* is more likely due to the host mosquito than the *wMel* strain,

potentially because of the residual effects of co-adaptation with its native *Wolbachia* strains *wAlbA* and *wAlbB*.

The fact that loss of ROS induction occurred for the 1% diet, where *wMel* and Tet mosquitoes has a similar susceptibility to *P. gallinaceum* infection is particularly interesting. ROS induction is part of the natural response to *Plasmodium* infection, with higher oxidative stress levels promoting parasite melanisation [78, 79]. Interestingly, levels of *tsf* in *wMel* mosquitoes were also decreased for that diet. This gene is involved in iron transport and changes in its expression could have contributed to decreased ROS production and may be indicative of broader alterations to host oxidative stress response under conditions of starvation in *Wolbachia*-infected mosquitoes. Critically, as less effective *Plasmodium* interference, and high H₂O₂ levels were observed for the 20% diet, changes to ROS induction are unlikely to be the sole factor causing the differential effect of host nutrition that we observed on *Plasmodium* and DENV infection.

The stimulation of mitochondrial and oxidative stress gene expression by *Wolbachia* has been implicated in ROS induction, activation of the Toll immune pathway, and pathogen interference [22, 38, 39]. In *wAlbB*-infected *Ae. aegypti*, this effect was linked to a 23-fold increase in the expression of *duox-2*, which is thought to be an important enzyme for ROS production [38]. However in our experiments, and in *wMel*-infected *Ae. albopictus*, *Wolbachia* did not affect *duox-2* levels, potentially because the gene lacks peroxidase activity, and therefore cannot directly stimulate ROS [43]. As we observed ROS induction without an effect of *Wolbachia* on *duox-2*, this implies that ROS induction occurs via a different process, potentially via the *duox-1* gene. Likewise, *duox-2* expression could not explain our ROS induction results, given the lack of an effect of host nutrition.

The enzyme *nos* is involved in the production of nitric oxide and reactive nitrogen species, and high *nos* levels have been linked to the inhibition of both *Plasmodium* and DENV in mosquitoes [80–82]. We observed no change in *nos* expression due to *Wolbachia*, indicating that this gene was unlikely to contribute to pathogen interference. Interestingly, we observed higher *nos* levels on the 1% and 5% diets for both *wMel* and Tet mosquitoes, where the prevalence of *P. gallinaceum* infection was greater. This suggests that there is a link between nutritional stress and *nos* expression, and that *nos* levels can be induced under conditions of starvation without a strong effect on *P. gallinaceum* infection. It is possible that levels of H₂O₂, *nos* or the immune genes that we examined could have been changed in response to blood feeding or severity of *Plasmodium* infection, as both factors are linked to oxidative stress response [56, 66, 83, 84]. Additionally, there could have been systemic change in the mosquito oxidative stress and immune responses as a result of these processes, and this may have contributed to the response of *Wolbachia* to pathogens, even under conditions of starvation.

Potential causes of diet-induced changes

We observed that changing host nutrition affected response to *Plasmodium* interference, ROS induction and *nos* expression. Furthermore there was differential fitness due to diet in the form of a longevity cost for low carbohydrate diets, which is not unexpected as dietary composition and insulin signalling affect lifespan in *Wolbachia*-infected insects [13, 85], and because *Wolbachia* increases the rate of resource depletion during starvation in larvae [86]. Starvation can stimulate immune response [53, 87, 88], as we observed with immune gene and *nos* expression on the 1% diet. It also reduces the availability of arginine and therefore affects levels of nitric oxide, and consequently affects the prevalence and intensity of *Plasmodium* infection [55, 89]. As such, it is possible that starvation-induced perturbations of the oxidative stress or nitric oxide response were the primary determining factor explaining our *Plasmodium* results

from the 1% diet. Dietary excess is another form of nutritional stress, and in insects it causes obesity, alters the metabolism and biosynthesis of fats and carbohydrates, and alters oxidative stress response [90–93], which could explain some of the results for the 20% diet.

Metabolic interaction and competition for resources between the host and *Wolbachia* affects host gene expression, metabolic homeostasis, and physiological processes linked to metabolism [22, 39, 49, 60, 85, 94]. Resource competition leads to less effective pathogen interference in *D. melanogaster* [41, 60], and could underlie diet-based differences in *Plasmodium* interference, particularly on the 20% diet. Both *Plasmodium* and DENV exploit host carbohydrate metabolism [54, 95–97], and infection alters host carbohydrate homeostasis [57, 59, 98]. However, *Plasmodium* are probably more heavily reliant on host sugars, which they use for glycolysis, carbohydrate metabolism, and fatty acid II synthesis [99], and for development [95], and thus could be more highly affected by competition with *Wolbachia*. As the type of carbohydrate intake can influence susceptibility to *Plasmodium* infection in mosquitoes, there could be similar effects on the ability of *Wolbachia* to interfere with infection [95].

The composition of the host microbial community can be affected by host diet [100, 101], can alter host metabolic profile [102–104], and can affect response to pathogen infection [105–108]. Interestingly, the microbiota induce production of ROS, which can influence susceptibility to *Plasmodium* infection, and offers a potential explanation for the diet-induced changes we observed in oxidative stress response [109]. There is evidence of interaction between *Wolbachia* and the microbiota, in the form of mutual exclusion between *Wolbachia* and *Asaia* in anophelines [110], and a microbial influence on the vertical transmission of *Wolbachia* in transiently infected *An. stephensi* [111]. But the full extent of the interactions between *Wolbachia* and host microbiota are unclear, and there is certainly scope for a nutrition-driven interaction, that could affect a range of physiological processes including pathogen interference.

Implications for pathogen interference

wMel-infected mosquitoes have been present in the field for several years, where they maintain high levels of interference against different DENV isolates [52]. The issue of nutritional stress and pathogen interference is particularly important in the field where mosquitoes are released in locations with complex environmental and nutritional factors, and high levels of endemic dengue transmission [50, 112]. Adult *Ae. aegypti* nutritional needs are fulfilled by blood feeding when human hosts are available [113], and plant sugars when they are not. Recent work suggests that repeated blood feeding does not affect interference against DENV in *Ae. aegypti* [114]. While a sucrose-based diet is unlikely to be perfectly reflective of mosquito carbohydrate intake in the field, our diets did induce varying levels of nutritional stress, which could be similar to what mosquitoes in a heterogeneous environment might experience. What our results suggest is that DENV interference appears to be quite robust in the face of variable host nutritional status, and such an effect would be greatly beneficial if it were to occur in the field *Wolbachia*-infected mosquitoes. These data should be further clarified using different DENV serotypes, genetic isolates, and viral titres, as well as for other types of host diet, as these factors can all influence pathogen interference [25].

Our results did show that changes in host diet led to significantly weaker pathogen interference against *P. gallinaceum* under some host nutritional conditions, and that this may correspond to altered oxidative stress response. Yet because *Wolbachia*-infected *Ae. aegypti* are unlikely to become infected with *Plasmodium* in the field this does not represent a large issue for current control efforts. Potential problems could arise if a similar nutrition-based interaction were to occur in *Wolbachia*-infected anophelines. Critically, *P. gallinaceum* does not infect humans, and the effect we observed here may not occur for the mosquitoes and parasites

responsible for human malaria, given their different immune and metabolic interactions [29, 30]. Pathogen interference has been observed against *P. falciparum* in *wAlbB*-infected *An. stephensi* [27], and future studies should determine the extent to which this phenotype is subject to environmental factors including nutrition, as this will have implications for future malaria control programs involving *Wolbachia*.

Perhaps the most interesting idea resulting from our data is the reinforcement and extension of the hypothesis of a complicated pathogen interference phenotype. Previous data indicates that the associated processes are not universal, with ROS induction being strain specific, and immune activation apparently specific to mosquitoes [37, 39, 43]. We have demonstrated that interference can also be pathogen specific, with diet-induced nutritional stress, and potentially starvation, affecting interference against *P. gallinaceum* but not DENV. It is also clear that host nutritional status can affect the ROS induction effect that has been linked to interference, and this should be further examined in the context of blood feeding, and experimental *Plasmodium* and DENV infection in order to characterize the effects of *Wolbachia* in a more natural nutritional state. These findings highlight the complicated nature of the phenotype, with the implication being that there is unlikely to be a ‘magic bullet’ explaining all occurrences of the phenotype. Rather, pathogen interference may arise through combinations of contributory factors with additive effects, and different pathways to interference occurring for different host-strain-pathogen combinations. The identity of these factors is currently unclear. However, given the breadth of *Wolbachia*’s effects on mosquito molecular biology, there are many potential candidates that have not yet been studied in great detail.

Materials and Methods

Mosquitoes and dietary manipulation

Two *Ae. aegypti* lines were used in these experiments. The first was infected with the *Wolbachia* strain *wMel* (*wMel*). This line was derived from the *wMel*-transinfected line, originally generated in *Ae. aegypti* with an Australian genetic background [17]. The *wMel* infection was introgressed into a Brazilian genetic background by breeding infected females with uninfected, field-collected males over nine generations, as previously described [112]. A subset of these mosquitoes were treated with tetracycline to clear the *Wolbachia* infection and then had their gut microbiota recolonised by introducing larval water from untreated mosquitoes into rearing trays, as previously described, with this line serving as a *Wolbachia*-uninfected control line (Tet) [112]. 50 wildtype, *Wolbachia*-uninfected F₁-F₂ males were introduced into colony cages for both *wMel* and Tet lines each generation, in order to limit the occurrence of inbreeding and genetic divergence between the lines. These mosquitoes were collected near Rio de Janeiro, Brazil, and reared under laboratory conditions until eclosion, as described below. No wildtype males were introduced into experimental cages. *wMel* mosquitoes used in these experiments were from G₁₄–G₂₉ post introgression into the Brazilian genetic background. Tet mosquitoes were from G₁₀–G₂₅ post microbial recolonization.

All mosquitoes in these experiments were reared under standard laboratory conditions in a climate-controlled insectary (temperature—27 ± 1 °C, RH -70 ± 10%, photoperiod—12 hours light: dark). Mosquito larvae were hatched in 3L RO water containing ½ of a tetramin tropical tablet (Tetramin) ground into powder. Larval density was reduced to 50 per litre 24 hours after hatching. Larvae were then fed ½ a tetramin tropical tablet as required, with food levels equating to 1mg of food per larva per day. Pupae were sexed, collected and moved to small cylindrical cages (diameter— 16cm, height— 18cm) for experiments, with a maximum adult density of 150 per cage.

Adult mosquitoes were maintained on one of four different carbohydrate diets for the entirety of each experiment. The control diet was 10% sucrose, which was the same

concentration provided to colony mosquitoes. The other three diets consisted of 1%, 5% and 20% sugar solution, with each inducing dietary stress either through starvation or excess. All diets were prepared by dissolving raw, granular cane sugar into RO water. Sucrose cups in experimental cages were changed every two days to prevent microbial contamination, with the solutions prepared fresh each time.

DENV culture, infections and quantification

The virus used in these assays, DENV-3 MG20 (375) was originally isolated from infected patient blood in Brazil in 2012. The virus was cultured in C6-36 cells, titred using both the TCID-50 and plaque forming assay methods according to previously described methods [23]. Titre estimates were 10^{10} – 10^{13} infectious units/mL and 1.9×10^6 infectious units/mL, respectively. Viral aliquots were stored at -80°C until the day of feeding. Cages of approximately 60 female mosquitoes were reared on carbohydrate diets as described above, and were starved overnight prior to feeding. Virus was mixed with freshly drawn blood from a willing volunteer at a 1:1 ratio. Blood used for feeding was screened for dengue virus using the Dengue NS1 Ag Strip Test (BioRad Laboratories). Mosquitoes were fed through glass feeders with pig intestine, using a heated waterbath system at a temperature of 37°C for 1 hour. Afterwards, non-blood fed, and semi-fed mosquitoes were removed and carbohydrate diets were re-introduced to cages. Half of the cage was collected at 7 days post-infection, and the other half collected at 14 days post-infection. Two independent feeding experiments were performed, using different aliquots from the same batch of virus.

Whole mosquito samples were stored at -80°C , and total RNA was extracted using the TRIzol protocol (ThermoFisher Scientific cat 15596–026) according to manufacturer's instructions. Mosquitoes were homogenized in 200 μL TRIzol using a mini beadbeater (BioSpec products). Samples were quantified using a NanoDrop 2000 UV-Vis spectrophotometer (ThermoFisher Scientific), and 1 μg of total RNA was used for first strand cDNA synthesis using the M-MLV reverse transcriptase assay according to manufacturer's instructions (Promega cat: C118A). cDNAs were then diluted 1:10 in nuclease-free water and stored at -30°C . Absolute DENV levels were quantified in duplicate for each cDNA, using a TaqMan-based assay with primers and a probe generalized to all four DENV serotypes (S2 Table). Each reaction contained the following: 2.5 μL of cDNA, 2.50 μL of TaqMan Universal Master Mix (ThermoFisher Scientific cat: 4304437), 0.50 μL each of forward and reverse primers (10 μM), 0.25 μL of DENV probe (10 μM), and 3.75 μL of nuclease-free water. For a standard curve, we utilised a cloned DENV fragment, as previously described [23]. Serial dilutions of this fragment were run in triplicate between the concentrations of 10^7 and 10^3 copies for each plate. The run profile was 10 mins to denature at 95°C , followed by 40 amplification/cycles of 15 sec at 95°C followed by 1 min at 60°C using a Viiia 7 Real-Time PCR System (ThermoFisher Scientific). DENV copies per sample were normalised per 1 μg of total RNA. 12–20 samples were quantified per treatment.

Plasmodium gallinaceum stock and infections

The *Plasmodium gallinaceum* stock used in these experiments was a long-term laboratory line (Brumpt, 1937, strain 8A). Cultures were maintained in the laboratory stored in chicken blood at -80°C , and through regular passage in 1–2 week old *Gallus gallus* chicks. Chicks were obtained at 1–2 day olds from Rivelli Poultry Farms, Mateus Leme, MG, Brazil, and were maintained in the FIOCRUZ Animal Facility during the course of experiments. Chicks were infected with *P. gallinaceum* infected blood drawn from previously infected chickens by trained personnel. Blood parasitemia levels were monitored during the course of infection by

counting infected cells in a Giemsa-stained blood smear, with the blood obtained through toenail clipping. In each experiment, approximately 70 female mosquitoes from each of the 8 treatments (4 diets x 2 *Wolbachia* infection statuses) were fed on the different carbohydrate diets for 7 days. Mosquitoes were starved overnight and then allowed to feed on the same chick for 15 minutes per cage, with cages fed in random order. Blood parasitemia levels in the chicks on the day of feeding varied between experiments (E_1 : 11.16%, E_2 : 35.38%, E_3 : 4.10%). *Plasmodium* stocks used in these experiments had been passaged a maximum of three times. Post-feeding, the appropriate diets were re-introduced to cages, and non-blood fed, and semi-fed mosquitoes were removed. There were no noticeable effects of *Plasmodium* feeding on mosquito survival. At 7–8 days post-blood feeding, midguts were dissected in sterile 1x PBS before staining in 2% mercurochrome for 10 mins. Oocysts were visualised and counted via light microscopy. Mosquito numbers ranged between 33–53 per treatment across the three experiments.

Wolbachia and immune gene transcription assays

12 pairs of 7–8 day-old, female, *wMel* and Tet mosquitoes were collected after 7 days on their respective diets. Paired samples were used to reduce within treatment variation. This corresponded to the time when the mosquitoes in the pathogen infection assays were infected with either *P. gallinaceum* or DENV, however samples in these experiments were not infected with a pathogen. RNA extractions and first strand cDNA synthesis were performed as described above. The levels of 14 immune-related genes were quantified for all samples, while *Wolbachia* expression levels were quantified for only the *wMel* samples using the *wsp* gene (S3 Table). Primer sequences used in these assays were either designed using Primer 3 V0.4.0 (<http://bioinfo.ut.ee/primer3-0.4.0/>), or as previously described [22, 23, 38], (S2 Table). Prior to use in experiments, each primer pair was assayed for specificity by melt curve analysis, with all pairs displaying only one peak. Additionally, we assayed primer efficiency by examining amplification performance with dilutions of cDNA samples. All primer pairs had an efficiency of between 90–100% at the dilution used in the experiments described below.

The immune transcription assays comprised of three parts. The first was an examination of genes previously shown to be highly upregulated by *wMel* infection in *Ae. aegypti* with an Australian genetic background [39]. Four genes were examined: cecropin e (*cece*), defensin c (*defc*), transferrin (*tsf*) and c-type lectin galactose binding 5 (*ctlga5*). The second looked at regulatory genes in 4 different mosquito immune pathways. Eight genes were examined: *rel2* and *caspar* from the IMD pathway, *domeless* and *pias* from the JAK/STAT pathway, *ap-1* and *jnk* from the JNK pathway, and *rel1a* and *cactus* from the Toll pathway. The third part looked at two genes linked to stress response in mosquitoes. These were *duox-2*, which is linked to oxidative stress, and nitric oxide synthase (*nos*), which is linked to stress and *Plasmodium* infection. All genes were quantified in duplicate relative to the host ribosomal protein S17 (*rps17*). Each reaction contained the following: 2.50μL of cDNA, 7.50μL of SYBR Green PCR Master Mix (ThermoFisher Scientific cat 4309155), 0.75μL each of forward and reverse primers (10μM), and 4.50μL of nuclease-free water. The run profile was the same as described above. Mean normalised expression values were calculated for each gene using Q-Gene [115].

ROS quantification assays

17–22 pairs of 7–8 day-old, female, *wMel* and Tet mosquitoes were collected after 7 days on their respective diets. H_2O_2 levels in these samples were quantified using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (ThermoFisher Scientific cat A22188). Samples were collected on ice and then immediately homogenized in 200μL of 1x reaction buffer, using a

mini beadbeater (BioSpec products), and then centrifuged for 2 mins at 14,200 x g, at 4°C. 50µL the supernatant was used to run the H₂O₂ assay, according to manufacturers instructions. Assays were run in black Nunc MicroWell 96-well Optical Bottom plates (ThermoFisher Scientific), and quantified using a Synergy 2 Multi-Mode Reader (BioTek), with an excitation wavelength of 545nm and an emission wavelength of 590nm.

Longevity assay

A longevity assay was conducted to provide a basic measurement of the effects of host nutritional status on the fitness of *wMel*-infected *Ae. aegypti* mosquitoes. *wMel* larvae were reared as described above, and then female pupae were sexed and transferred to experimental cages, separated by carbohydrate diet. Pupal cups were removed from cages after 48 hours, so that all mosquitoes shared a similar age and development time. There were 3 replicate cages per diet, each containing approximately 45 females. Survival was monitored daily for the duration of the experiment, with dead mosquitoes removed from cages. Cage positions were rotated daily in order to normalize environmental variance.

Ethics Statement

Maintenance of chickens, infections with *P. gallinaceum* and feeding of mosquitoes were conducted according to protocols that were reviewed and approved by The Commission of Ethical Animal Use (CEUA)/ FIOCRUZ (License—LW 38/12). This complied with Brazilian law 11794/08 which governs the use of animals for scientific purposes and principles as dictated by the Brazilian Society of Science on Laboratory Animals (SBCAL), and The National Council of Animal Experimentation Control (CONCEA).

The human blood used in these experiments was drawn from one willing, adult volunteer by trained medical personnel, after obtaining written consent. This process was conducted according to established guidelines, and approved by The Committee for Ethics in Research (CEP)/ FIOCRUZ (License—CEP 732.621). Our use of human blood was in accordance with Brazilian laws 196/1996 and 01/1988, which govern human ethics issues in scientific research in compliance with the National Council of Ethics in Research (CONEP).

Statistical Analysis

P. gallinaceum infection data were analysed in two components; prevalence and intensity of infection. Uninfected mosquitoes were not considered in intensity analyses. Prevalence data were compared using binomial regression, and oocyst data by binomial negative regression, as there was overdispersion within the data set [116, 117]. Due to the fact that different *P. gallinaceum*-infected chickens were used in each experimental infection, the three experiments were analysed independently.

The test variable in these analyses was either infection status or oocyst number, while explanatory variables in the models were *Wolbachia* infection status, and dummy variables considering the effect of each diet, compared to the control 10% diet. A general effect of diet was not considered in the model, as we believed that the effect would differ between diets. *Wolbachia* x diet interaction terms were included, however these were generally not significant, and the models fit the data better after they were excluded (S1 Table).

Pairwise comparisons of differences in prevalence of *Plasmodium* infection due to *Wolbachia* for individual diets were calculated using Fisher's exact test. Pairwise comparisons of oocyst levels for each diet were made using Mann-Whitney U tests. DENV prevalence data were compared by treatment using Fisher's exact test. Viral intensity data for Tet mosquitoes were compared using one-way ANOVA.

Longevity data were compared statistically using Cox Regression. Expression data for immune activation genes, immune pathway regulators, stress response genes, *wsp* levels, and H₂O₂ levels were compared independently using univariate general linear models. When significant effects were observed, interactions between treatments were compared post-hoc using student's *t*-tests and then using a false discovery rate of 0.05 as a multiple test correction. Statistical tests were applied only if the data fit the underlying assumptions. Statistical analyses were performed using R, SPSS V17 (IBM) and Prism 6.0g (Graphpad). Figures were prepared using Prism V 6.0g, Microsoft PowerPoint for Mac 2011, and GIMP v 2.8.14.

Gene Accession Numbers

From VectorBase (<https://www.vectorbase.org>) unless noted.

ap-1 (AAEL011650-RA), *c*-type lectin galactose binding 5 (AAEL005641-RA), *cactus* (AAEL000709-RB), *caspar* (AAEL003579), *cecropin e* (AAEL000611-RA), *defensin c* (AAEL003832-RA), *domeless* (AAEL012471-RA), *duox-2* (AAEL007562-RA), *jnk* (AAEL008634-RA), *nitric oxide synthase* (AAEL009745-RA), *pias* (AAEL015099-RA), *rel1a* (AAEL007696-RA), *rel2* (AAEL007624-RA), *ribosomal protein S17* (AAEL004157), *transferrin* (AAEL015458-RA), *wolbachia surface protein* (GenBank accession: EU395833.1).

Supporting Information

S1 Fig. Longevity of *w*Mel-infected *Ae. aegypti* fed on different carbohydrate regimes. The average survival time (\pm s.e.m.) of *w*Mel-infected *Ae. aegypti* was monitored daily across three cages per diet, with each containing 32–46 female mosquitoes. Mosquitoes were maintained on either 1%, 5%, 10% or 20% sucrose diets throughout the experiment. Data were compared by Cox Regression.

(TIF)

S2 Fig. The expression of genes regulating the IMD, JAK-STAT, JNK and Toll immune pathways in *w*Mel-infected *Ae. aegypti* fed on different carbohydrate regimes. Expression levels of the IMD pathway regulatory genes *rel 2* (A) and *caspar* (B), the JAK-STAT pathway regulatory genes *domeless* (C) and *pias* (D), the JNK pathway regulatory genes *ap-1* (E) and *jnk* (F), and the Toll pathway regulatory genes *rel 1A* (G) and *cactus* (H) were quantified for Tet (black circles) or *w*Mel (green circles) mosquitoes fed one of four carbohydrate diets. Gene expression values were normalized against host *rps17* expression. Each circle represents one pair of mosquitoes, with 12 samples examined for each treatment. Solid black lines represent mean expression (\pm s.e.m.). *P* value: Student's *t* test, * < 0.05.

(TIF)

S1 Table. Statistical Output from *Plasmodium gallinaceum* infection experiments.

(DOCX)

S2 Table. List of Primers and Probes.

(DOCX)

S3 Table. Mean normalised expression data from RT-qPCR experiments.

(XLSX)

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