




RESEARCH ARTICLE

REVISED Pluripotency of *Wolbachia* against Arboviruses: the case of yellow fever [version 2; peer review: 2 approved]

Previously titled: Pluripotency of *Wolbachia* against Arbovirus: the case of yellow fever

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Abstract

Background: Yellow fever outbreaks have re-emerged in Brazil during 2016-18, with mortality rates up to 30%. Although urban transmission has not been reported since 1942, the risk of re-urbanization of yellow fever is significant, as *Aedes aegypti* is present in most tropical and sub-tropical cities in the World and still remains the main vector of urban YFV. Although the YFV vaccine is safe and effective, it does not always reach populations at greatest risk of infection and there is an acknowledged global shortage of vaccine supply. The introgression of *Wolbachia* bacteria into *Ae. aegypti* mosquito populations is being trialed in several countries (www.worldmosquito.org) as a biocontrol method against dengue, Zika and chikungunya. Here, we studied the ability of *Wolbachia* to reduce the transmission potential of *Ae. aegypti* mosquitoes for *Yellow fever virus* (YFV).

Methods: Two recently isolated YFV (primate and human) were used to challenge field-derived wild-type and *Wolbachia*-infected (wMel +) *Ae. aegypti* mosquitoes. The YFV infection status was followed for 7, 14 and 21 days post-oral feeding (dpf). The YFV transmission potential of mosquitoes was evaluated via nano-injection of saliva into uninfected mosquitoes or by inoculation in mice.

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1. Andrew van den Hurk , University of Queensland, St. Lucia, Australia 2. Jayme A. Souza-Neto  , São Paulo State University (UNESP), Botucatu, Brazil Bianca C. Carlos , São Paulo State University		

Results: We found that *Wolbachia* was able to significantly reduce the prevalence of mosquitoes with YFV infected heads and thoraces for both viral isolates. Furthermore, analyses of mosquito saliva, through indirect injection into naïve mosquitoes or via interferon-deficient mouse model, indicated *Wolbachia* was associated with profound reduction in the YFV transmission potential of mosquitoes (14dpf).

Conclusions: Our results suggest that *Wolbachia* introgression could be used as a complementary strategy for prevention of urban yellow fever transmission, along with the human vaccination program.

Keywords

Wolbachia, Aedes aegypti, Yellow fever virus, vector competence

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Any reports and responses or comments on the article can be found at the end of the article.



This article is included in the [World Mosquito Program](#) gateway.

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Author roles: **Rocha MN:** Conceptualization, Formal Analysis, Methodology, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Duarte MM:** Methodology, Resources, Visualization; **Mansur SB:** Methodology; **Silva BDMe:** Methodology; **Pereira TN:** Methodology; **Adelino TÉR:** Methodology; **Giovanetti M:** Methodology; **Alcantara LCJ:** Methodology; **Santos FM:** Methodology; **Costa VRdM:** Methodology; **Teixeira MM:** Conceptualization, Formal Analysis, Supervision, Writing – Review & Editing; **Iani FCdM:** Methodology, Writing – Original Draft Preparation; **Costa VV:** Formal Analysis, Methodology, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Moreira LA:** Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED Amendments from Version 1**Background:**

- We added the text: "Although the YFV vaccine is safe and effective, it does not always reach populations at greatest risk of infection and there is an acknowledged global shortage of vaccine supply".

Introduction:

- More details on *Wolbachia* were added.

Methods

- Sample collection – We added Adungo *et al.*, 2016 and cited Domingo *et al.*, 2012.
- Mosquitoes and infections – We added the number of mosquitoes analyzed per group and the text: "*Wolbachia* density was analyzed in the three time-points, being 40 mosquitoes at 7dpf, 39 at 14dpf and 38 mosquitoes after 21dpf."
- Mosquito saliva transmission assay – The word "Individual" was added in the sentence "Individual saliva..." and the following text "Usually, with one saliva sample it is possible to inject 15 mosquitoes, but due mortality, 8 mosquitoes were analyzed from each nano-injected saliva sample".
- Viral detection in mosquitoes and mice – We added the following text "Head and thorax samples from YFV-challenged mosquitoes were analyzed in duplicate through RT-qPCR and viral and *Wolbachia* quantification were performed in comparison with serial dilution of a standard curve of the respective genes cloned into the pGEMT plasmid (Promega). Therefore, it was possible to calculate the number of copies per tissue. As a mosquito control gene, we used the RPS 17S sequence of *Ae. aegypti* (Moreira, 2009)."

Results:

- Figure 3 legend – added *p* values.
- Figure 4 legend was corrected.

Discussion

- We added extra text to include the rationale behind giving a second blood meal to infected mosquitoes.
- In the end we added text regarding possible evolution on the system, as requested by the reviewer.

See referee reports**Introduction**

Arboviruses impose a substantial disease burden on the human population^{1,2}. Most recently, the *Zika virus* re-emerged in 2014, and unexpectedly caused serious congenital infections in pregnant women and Zika fetal syndrome in affected newborns in several American countries in 2016 and 2017³. *Chikungunya virus* caused massive epidemics in the Americas in 2014 and still circulates in several countries⁴.

The *Yellow fever virus* (YFV) is a member of the *Flaviviridae* family and transmitted by sylvan mosquitoes of the genus *Haemagogus* and *Sabethes*, in South America and *Aedes aegypti* in urban settings^{5–8}. Monkeys are important reservoirs of YFV in sylvan environments. Encroachment by humans into environments where competent mosquito vectors and infected monkeys co-exist is the commonest reason for spillover of YFV transmission to human populations. Although the last reported cases of urban transmission in Brazil occurred in 1942, in

2016–2017, the country faced major outbreaks of the disease mainly in the states of Minas Gerais, Espírito Santo and Rio de Janeiro. In 2018, the epidemic also extended to São Paulo State⁹. According to the Brazilian Ministry of Health, from July 2017 to April 2018, there were 1,127 YFV cases with 328 deaths, with no evidence of urban transmission. Although the YFV vaccine is safe and effective, it does not always reach populations at greatest risk of infection and there is an acknowledged global shortage of vaccine supply¹⁰.

Recent studies have shown that anthropophilic mosquitoes, such as *Aedes aegypti* and *Aedes albopictus*, as well as Brazilian enzootic mosquitoes, such as *Haemagogus leucocelaenus* and *Sabethes albiprivus*, were highly susceptible to American and African YFV strains^{11–13}. Therefore, the possible resurgence of urban epidemics of YFV in South America has to be constantly monitored by public health authorities¹³. Population control of *Ae. aegypti* mosquitoes using insecticides has been a mainstay of vector-borne disease control methods for decades but is undermined by widespread insecticide resistance. A promising innovative strategy, based on a bacterium called *Wolbachia pipiens*, has been trialed in many countries. *Wolbachia* is a maternally transmitted bacterial endosymbiont and is naturally present in at least 40% of all insect species¹⁴. The World Mosquito Program is deploying *Wolbachia* as a self-sustaining disease control agent on the basis that *Wolbachia* reduces the transmission potential of *Ae. aegypti* mosquitoes for dengue¹⁵, Zika¹⁶ and chikungunya viruses¹⁷.

Here, we studied the ability of *Wolbachia* to suppress YFV infectivity in *Ae. aegypti* mosquitoes. Two virus isolates were used: one from a human clinical sample and another one of primate origin. We found that *Wolbachia* had a major impact on virus replication in mosquitoes, as well as reduced the potential of YFV transmission via saliva, as indirectly determined via mosquitoes or a mouse model.

Methods**Sample collection**

The first sample named M377_IV[Human|MinasGerais_PadreParaíso|2017-02-04 (YFV377H) was isolated from human serum, positive for YFV by RT-qPCR (CT = 28.95) in February, 2017 from Padre Paraíso city (northeast of Minas Gerais state). The other sample named M127_IV[Primate|MinasGerais_NovaLima|2018-01-15 (YFV127P) was isolated from the liver of a non-human primate found dead in January 2018, in Nova Lima city, in the center-south of Minas Gerais state, positive for YFV via RT-qPCR (CT = 17.19). Sequencing of both isolates was performed and is described below. Viral isolation was confirmed by two methodologies: indirect immunofluorescence (IFA) and RT-qPCR. IFA was performed with a monoclonal YFV antibody donated by Evandro Chagas Institute (Arbovirology and Hemorrhagic Fevers Section) and conjugated goat anti-mouse IgG labeled with fluorescein FITC (MP Biomedicals) according to Adungo *et al.* 2016¹⁸ with modifications. Images were obtained using an Olympus microscope model BX51 with DP72 camera and DP-2BSW software. Viral molecular confirmation was performed using RNA extracted from the culture supernatant of each isolate, followed by amplification of the genetic material

as described below in the viral detection section according to Domingo *et al.* 2012¹⁹. For mosquito infections, the YFV isolates were replicated in C636 cells (*Ae. albopictus*) cultured in Leibovitz 15 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) for 5 days at 28°C. Viral load was confirmed by RT-qPCR and later through plaque assays (PFU) in VERO cells (CCL81) grown in DMEM medium (Gibco) and 3% Carboxymethylcellulose (Sigma) supplemented with 2% FBS (Gibco) at 37°C and 5% CO₂²⁰.

Nucleic acid isolation and virus genome sequencing

Viral RNA was isolated from 200 µL of each sample using MagNA Pure 96 (Roche) following manufacturer's recommendations. To confirm the viral presence in isolates, RT-qPCR was performed, according to Domingo *et al.* 2012¹⁹.

A real-time nanopore sequencing strategy with previously developed primers²¹, was applied to both RT-qPCR-positive samples. For these samples, extracted RNA was converted to cDNA using GoScript™ Reverse Transcriptase (Promega) and random hexamer priming. Whole-genome amplification by multiplex PCR was attempted using GoTaq® qPCR Master Mix (Promega), the 500bp sequencing primer scheme and 35 cycles using the adapted protocol²¹. Electrophoresis (2% agarose gel) was used to confirm the expected bands and to purify the specific amplicons using Invitrogen™ E-Gel™ SizeSelect, followed by quantification using fluorimetry with the Qubit dsDNA High Sensitivity assay on the Qubit 3.0 instrument (Life Technologies).

Template was amplified with end point PCR to increase template concentration following the Ion Plus Fragment Library Kit recommendation and PCR products were cleaned-up using AmpureXP purification beads (Beckman Coulter). Emulsion PCR was performed to amplify the library using Ion PGM™ Hi-Q™ View OT2 Kit (Thermo Fisher Scientific) and the Ion OneTouch 2 system (Thermo Fisher Scientific). Ion Sphere particles (ISPs) were enriched using the Ion OneTouch ES (Thermo Fisher Scientific). Enriched ISPs were sequenced using the Ion Torrent Personal Genome Machine (PGM) and the Ion PGM Hi-Q Sequencing kit (Thermo Fisher Scientific), with the Ion 314 chip. All procedures above followed manufacturer's instructions.

Consensus genome sequences from fastq file were produced by alignment of two-direction reads by using a reference YFV genome. Quality control on raw sequence data have been performed using FastQC²². Bowtie 2 was used for mapping reads to a reference using Galaxy²³. Only positions with $\geq 20\times$ genome coverage were used to produce consensus sequences. Regions with lower coverage and those in primer-binding regions were masked with N characters.

In order to identify the origin of the YFV genome from the samples, we performed a maximum likelihood (ML) phylogenetic analysis using the newly two nucleotide sequences recovered in this study plus 125 reference YFV complete genome sequences from each different genotype (South American I n=84; South American II n=2; West African n=23; East African n=16) already

published in peer-reviewed journals, for which sampling year and geographic location is available. Full details of the reference sequences used are provided in Extended data: [Table S1](#).

Consensus sequences were aligned using MAFFT v.7²⁴. Maximum likelihood phylogenetic trees were estimated using IqTree²⁵ under a GTR + Γ_4 nucleotide substitution model. Statistical support for phylogenetic nodes was estimated using a bootstrap approach (100 replicates).

The phylogenetic signal has been investigated with the likelihood mapping method by analyzing groups of four sequences, randomly chosen, called quartets. Likelihood mapping analyses was performed with the program TREE-PUZZLE by analyzing 10,000 random quartets²⁶.

Mosquitoes and infections

Wild type *Aedes aegypti* mosquitoes collected in the neighborhood of Urca, Rio de Janeiro-RJ, Brazil in 2018 were reared in the laboratory for five generations and confirmed for the absence of *Wolbachia* (WT). *Wolbachia* wMel strain-containing mosquitoes (wMel +) were obtained from the colony maintained by the World Mosquito Program (WMP) Brazil laboratories in Belo Horizonte, which is backcrossed every five generations with Urca male mosquitoes. They were reared in a controlled environment at $27 \pm 2^\circ\text{C}$ and $60 \pm 10\%$ relative humidity. Four to six days-old female mosquitoes were starved for 20 to 24 hours and subsequently offered YFV virus culture supernatant mixed with washed human red blood cells (RBCs) (2:1 ratio). The viral titer offered to mosquitoes was 4×10^5 PFU/mL for YFV377H and 1.4×10^6 PFU/mL for YFV127P. RBCs were washed three times for removal of potential YFV vaccine antibodies. Mosquitoes were allowed to feed for one hour and then, engorged females were selected and maintained in triple containment, under BSL-2 conditions. Sucrose solution (10%) was offered *ad libitum* during the extrinsic incubation period. Viral load was analyzed at 7, 14 and 21 days post feeding (dpf), via RT-qPCR and number of mosquitoes analyzed per group were presented in [Figures 3B, C and D](#) and ranged from 17 to 20. Additionally, a subset of mosquitoes (at 7dpf) received an extra blood meal and were collected at 14dpf, when *Wolbachia* density and viral load was determined. *Wolbachia* density was analyzed in the three time-points, being 40 mosquitoes at 7dpf, 39 at 14dpf and 38 mosquitoes after 21dpf. The blood used in the infective feedings was obtained from a blood bank (Hemominas) through an agreement signed between both institutions (OF.GPO/CCO-Nr224/16). As a laboratory routine each blood bag is previously tested for dengue, Zika, chikungunya, mayaro and yellow fever, through RT-qPCR to rule out any cross-infection that could interfere with the results.

Mosquito saliva transmission assays

In order to check the ability of mosquitoes to transmit the virus, saliva samples from infected mosquitoes were individually collected at 14 dpf. After removal of legs and wings, mosquitoes had their proboscis introduced into 10 µL tips, containing 50% Fetal Bovine Serum (FBS) (Gibco) and 30% sugar solution and allowed to salivate for 30 minutes. Mosquitoes and solution containing

in comparison with serial dilution of a standard curve of the respective genes cloned into the pGEMT plasmid (Promega)^{16,27}. Therefore, it was possible to calculate the number of copies per tissue. As a mosquito control gene, we used the RPS 17S sequence of *Ae. aegypti* (Moreira 2009)¹⁵. Viable viral loads were quantified by titration assay in permissive Vero cells as described in Costa *et al.*, 2012²⁹.

Statistical analysis

All statistical analyses were performed on Prism (Graphpad Version 7.04). Initially the D'Agostino and Person normality test was performed. *Wolbachia* density data as well as viral load were compared using the non-parametric Mann-Whitney test. Statistical analyzes for the mouse data were performed with ANOVA one-way test. The significance level was set for *p* values less than 0.05.

Results

Viral isolation and sequencing

Two plasma samples (one human and one from a non-human primate) were isolated from the diagnostic service of Fundação Ezequiel Dias, the State Reference Laboratory of Minas Gerais, Brazil. Viral isolation was confirmed by indirect immunofluorescence (IFA), showing the typical signal of fluorescence for both isolates (Figure 1B and C). Both samples were successfully sequenced with PGM (Personal Genome Machine) technology with adapted overlapping multiplex PCR protocol, as shown in Table 2. The phylogenetic analysis showed that the isolates obtained from the two samples (M377_IV and M127_IV) belonged to the South American genotype I and clustered closely with strong bootstrap support (>90%) with the recent sequences, isolated in Minas Gerais, from the current outbreak (Figure 2)³⁰.

Wolbachia density

Absolute quantification of *Wolbachia* in mosquitoes were analyzed in the heads and thoraces of *Wolbachia*-positive mosquitoes (wMel +) after challenge with YFV. There was no difference in *Wolbachia* density among heads and thoraces, collected at 7 or 14 days post feeding (dpf), as shown in Figure 3A. However, *Wolbachia* density presented a slight reduction at 21dpf, which was statistically significant in relation to 14dpf (*p* = 0.0062, Mann Whitney). The median at 14dpf was 2.04×10^6 copies per head/thorax whereas at 21dpf, it decreased to 1.37×10^6 .

Wolbachia reduces susceptibility of *Ae. aegypti* to YFV infection

In mosquitoes without *Wolbachia* (WT) the prevalence of YFV infection of heads and thoraces was 30–45% at 7dpf, and 80–89% at 14dpf. For those mosquitoes that received a 2nd blood meal, the prevalence was 89 to 94% at 14dpf and 85 to 100% at 21dpf. There was no significant difference between infection rates resulting from the human or primate virus isolates (Figure 3). In heads and thoraces of *Wolbachia*-positive mosquitoes (wMel +) the infection rate ranged from 0 to 15% at 7dpf, 11 to 16% at 14dpf, 20 to 32% at 14dpf when mosquitoes received a second blood meal, and 20 to 25% at 21dpf (Figure 3). Again, there was no major difference between viral isolates.

The infection rate observed at 7dpf was low for both viral isolates (Figure 3B). At day 7, the presence of *Wolbachia* was already associated with a marked decrease in viral titers in mosquitoes (Figure 3B). At 14dpf, there was a significant increase in the number of viral copies in WT mosquitoes (Figure 3C). Further increase on viral load was observed when mosquitoes received a second blood meal 7 days after the infective meal and were analyzed at 14 dpf. This increase was statistically significant for both isolates (*p* < 0.01, Mann Whitney). This may have been due to the fact that the second blood supplied extra important nutrients for viral replication. At 21dpf, the infection reached 100% for the human isolate with a median of 3.15×10^7 viral copies. For the primate isolate, although the infection rate was lower (85%), the viral load was higher with a median of 5.61×10^7 viral copies per head/thoraces. Regardless of the strain of virus used, viral loads were remarkable lower in presence of *Wolbachia* at all time points (Figure 3B–D). In addition, there was no increase in viral load in wMel + mosquitoes after supplying a second blood meal (Figure 3C).

Virus transmission through saliva

Next, we evaluated the ability of orally infected mosquitoes to transmit the virus. We first collected saliva from infected mosquitoes at 14 dpf, from both groups of mosquitoes and virus isolates. We then injected a number of saliva samples into eight naïve (WT) mosquitoes and, after five days, we checked whether those mosquitoes became infected through RT-qPCR, demonstrating that a particular saliva was infectious. As shown in Figure 4, when saliva samples originated from wMel + mosquitoes, no mosquitoes became infected. This assay shows, indirectly, the potential of *Wolbachia* to completely abrogate YFV transmission potential of *Ae. aegypti* mosquitoes. Nevertheless, saliva originating from WT mosquitoes was able to infect 20% of the naïve-injected mosquitoes.

Similar experiments were performed by injecting saliva samples from either the WT or wMel + groups into 4-week-old A129^{-/-} mice, which are susceptible to arboviral infections^{31,32}. Results showed that there was no major impact on clinical and laboratory parameters, which is consistent with the relatively low number of viable virus injected (Figure 5A–D). However, there were viable viruses, as assessed by plaque assay, recovered from the paw of mice inoculated with saliva from WT mosquitoes. Indeed, there was culturable virus when both P (primate) and H (human) strains were used. In contrast, none of the samples from the wMel + groups were positive on the plaque assay (Figure 5E–H). Consistently with the mosquito saliva findings above, there were higher number of viral RNA copies in draining lymphnode and liver from mice injected with WT saliva than mice inoculated with wMel + saliva (Figure 5 I–K). Virus isolated from the primate (YFV127P) showed greater presence in liver while the human strain (YFV377H) was more localized at the lymphoid tissue (Figure 5).

Collectively these results suggest that *Wolbachia*-positive mosquitoes can efficiently suppress YFV replication and reduce virus transmission through saliva.

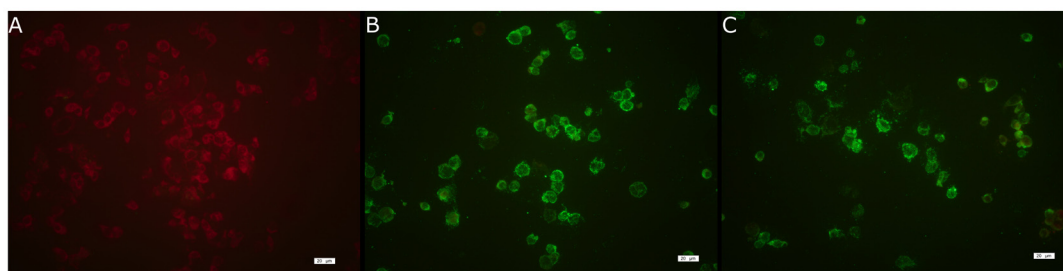


Figure 1. Yellow fever virus (YFV) immunofluorescence in C636 cells. (A) Control cells without virus, (B) cells infected with YFV 377 H and (C) cells with YFV127 P. Green fluorescence depicts YFV in cells marked with a monoclonal YFV antibody conjugated goat anti-mouse IgG labeled with fluorescein FITC.

Table 2. Main results obtained by sequencing.

Sample ID	Acession number (GenBank)	CT value	Coverage	Mean depth	N° of reads	Mapped reads	Mean mapping quality
M377_IV	MK249065	13.82	92.5%	4,004 X	218,811	216.613 (99%)	37
M127_IV	MK249066	16.68	93%	6,640 X	361,806	358.522 (99%)	37.02

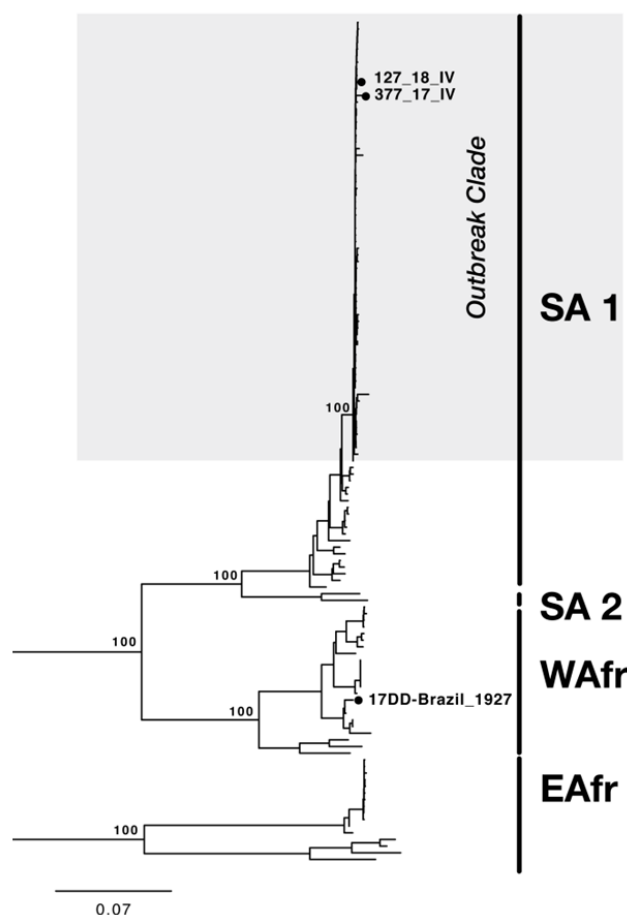


Figure 2. Maximum likelihood phylogeny obtained using two novel complete Yellow fever virus sequences plus 126 YFV reference sequences from each different genotype (South American I; South American II; West African; East African). ML showing the two newly genomes belongs to South American I (SA1) genotype. SA2, WAfr, and EAfr indicate the South America II, West Africa, and East Africa genotypes, respectively. The scale bar is in units of substitutions per site (s/s). Node labels indicate bootstrap support values. 17DD, the vaccine strain used in Brazil.

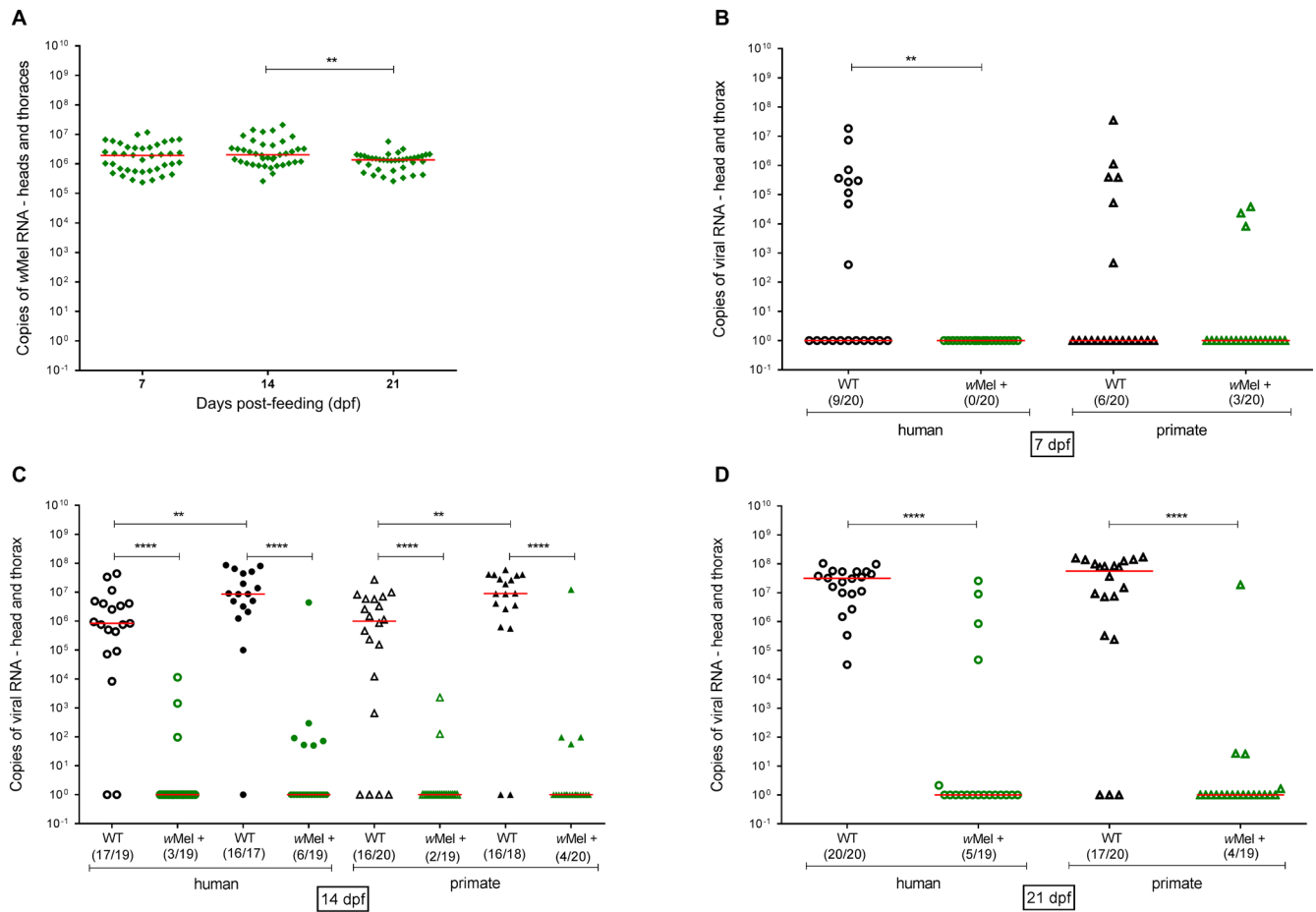


Figure 3. Interference of *Wolbachia* towards *Yellow fever virus* and *Wolbachia* absolute quantification. Wild type (WT) or positive (wMel +) were orally infected with two YFV isolates and virus dissemination in mosquitoes was analyzed at different times post infection. (A) YFV infected mosquitoes' heads and thoraxes were analyzed for *Wolbachia* density at different times post-infection through real time RT-qPCR, based on a *Wolbachia* standard curve. Red lines indicate the median wMel copies (Mann-Whitney U test, ** $p=0.0062$). (B) Analysis of copies of viral RNA on 7dpf -WT x wMel+ (** $p=0.0028$) and YFV Human x Primate WT ($p=0.43$), (C) 14dpf YFV Human and Primate WT x wMel+ (**** $p<0.0001$), YFV Human x Primate WT ($p=0.75$), YFV Human x Primate extra blood meal WT ($p=0.78$), YFV Human WT x WT extra blood meal (** $p=0.0061$) and YFV Primate WT x WT extra blood meal (** $p=0.0056$) and (D) 21 dpf -WT x wMel+ (**** $p=0.0001$) and YFV Human x Primate ($p=0.51$). Empty black circles and triangles are WT mosquitoes, whereas empty green circles and triangles depict mosquitoes with wMel +. Black filled circles and triangles are mosquitoes that received a second blood meal. The red line indicates the median YFV copies.

Discussion

The ability of *Wolbachia* to reduce the susceptibility of *Ae. aegypti* to disseminated arbovirus infection has been repeatedly demonstrated for dengue¹⁵, Zika¹⁶, chikungunya¹⁷, West Nile³³ and mayaro viruses²⁷. We have shown that wMel was able to significantly reduce the infectivity of YFV to mosquitoes, independently of the source of the virus (both human and primate). Previously, it has been shown that two strains of *Wolbachia* (wMelPop and wMel) were able to significantly reduce YFV mosquito infection, although with virus isolated from human cases from Nigeria and Bolivia, in 1987 and 1999, respectively³⁴. Here we evaluated the effect of *Wolbachia* (wMel strain) towards two recently isolated *Yellow fever virus*, originating from the 2017–2018 outbreaks in Brazil. The *Yellow fever virus* isolates used here have different origins, one originating from a non-human primate found in the city of

Nova Lima and another originated from a human case in the city of Padre Paraíso, both in the state of Minas Gerais. Although these cities are located more than 500 km apart, they belong to the same genotype. Besides working with recently isolated virus from human and primate sources, the difference in the present study refers to the way in which this population of mosquitoes have been infected. Furthermore, this study was performed with orally infected mosquitoes, which is closer to natural conditions, in comparison to the previous study which infected mosquitoes through thorax injection, in order to improve mosquito infection³⁵.

The use of *Wolbachia* as an arbovirus control strategy has been developed by the not-for-profit initiative, the World Mosquito Program. The approach offers the prospect of a natural and sustainable method for arbovirus control^{35–38}. The impact

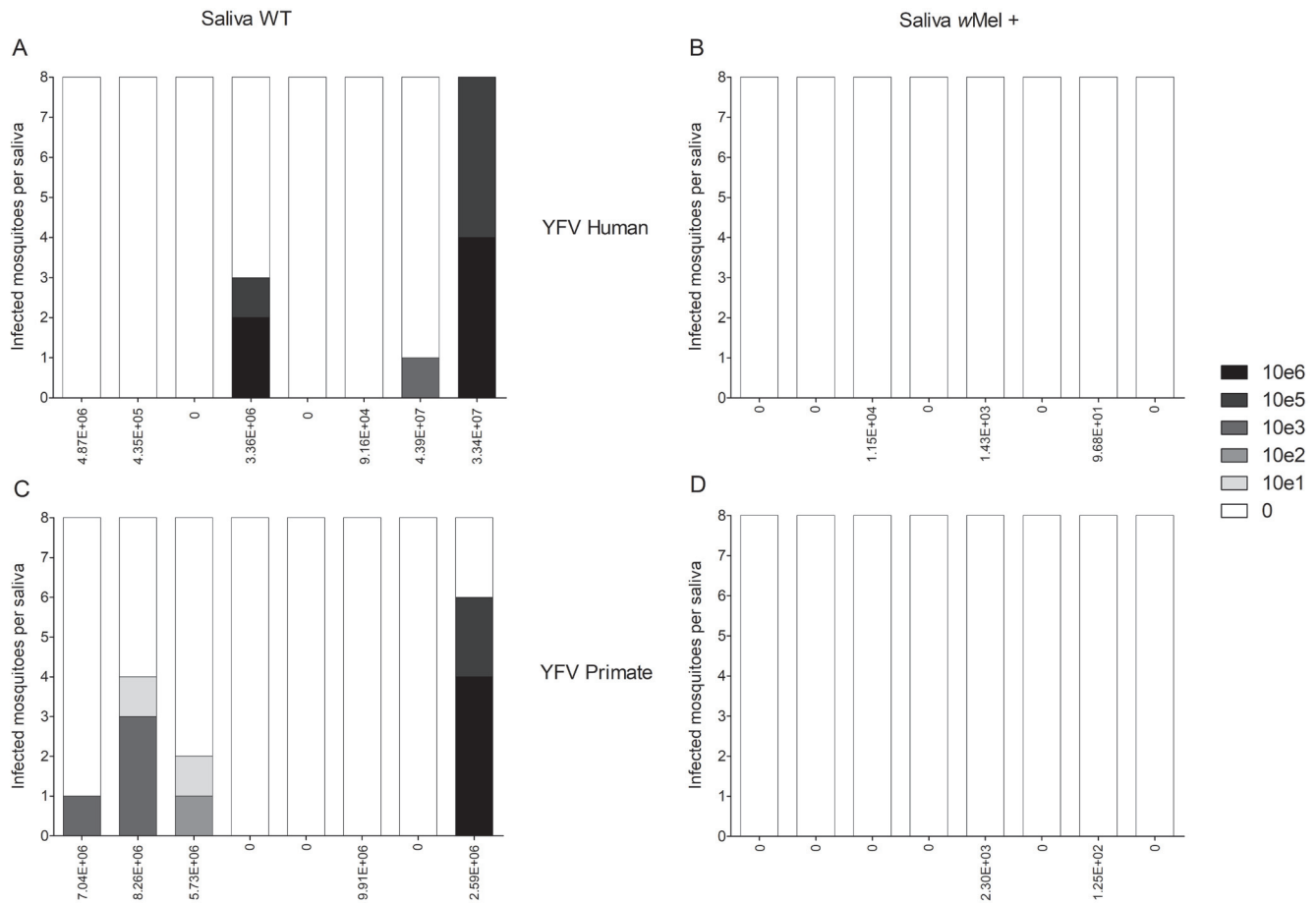


Figure 4. Indirect evaluation of yellow fever virus (YFV) transmission through mosquito saliva. Saliva from both groups of infected mosquitoes were collected at 14 dpf. Individual saliva samples (WT or wMel +) were analyzed into eight naïve (WT) mosquitoes (bars) and, after five days, these injected mosquitoes were analyzed. (A) Mosquitoes injected with mosquito saliva or (B) wMel+ mosquitoes, challenged with human virus. (C) Mosquitoes injected with WT mosquito saliva or (D) wMel+ mosquitoes, challenged with primate virus. Values below each bar depicts the viral load of each mosquito head and thorax which donated that saliva. Positive mosquitoes were quantified through RT-qPCR and the grey-scale represents the number of YFV copies (0 to 10^6 copies), per mosquito.

towards reduction of arbovirus has been analyzed^{39,40} and early indication of positive effect has been recently reported⁴¹. In Brazil, WMP is expanding its coverage into Rio de Janeiro and Niterói municipalities and epidemiological studies in order to determine arbovirus reduction is underway.

The blocking ability conferred by *Wolbachia* has been directly related to the density of the bacterium within main mosquito tissues such as midgut and/or salivary glands^{15,42}, where viruses replicate to further produce infectious particles⁴³. In our study, and as observed by Pereira *et al.*, 2018²⁷, the density of *Wolbachia* was constant at 7 or 14 days after virus exposure. However, there was a reduction of wMel + density at 21dpf, which did not impact the blocking ability towards the virus (Figure 3). The variation on the density (or titer) of *Wolbachia* within the host has been previously observed, which could be related to the aging of the host⁴².

In the present study, the presence of *Wolbachia* in mosquitoes greatly reduced YFV infection, except for 7dpf, when the

infection rate was low in all groups. Further effect of *Wolbachia* towards YFV was verified when individually collected mosquito saliva was injected into naïve mosquitoes or into a susceptible mice strain and their infectivity was analyzed. This first technique has been widely used by our group and others^{16,27,44}, and it is a robust proxy of the potential of individual saliva towards virus transmission. When the source of saliva came from *Wolbachia*-positive mosquitoes, there was no infection in any injected mosquito. Through projection of these results into natural conditions, the YFV transmission could be greatly reduced, as previously modeled for *Dengue virus*³⁹.

Another interesting fact of this work was the increase in viral load observed after the second blood feeding in WT mosquitoes. This same fact was not observed in wMel + mosquitoes. This shows that the blocking ability of *Wolbachia* persists even after the addition of extra blood nutrients (through a second blood meal) and that its blocking effect occurs within 7 days after infection. The reason to include the second blood meal was that antibodies to YFV could be present in the blood and

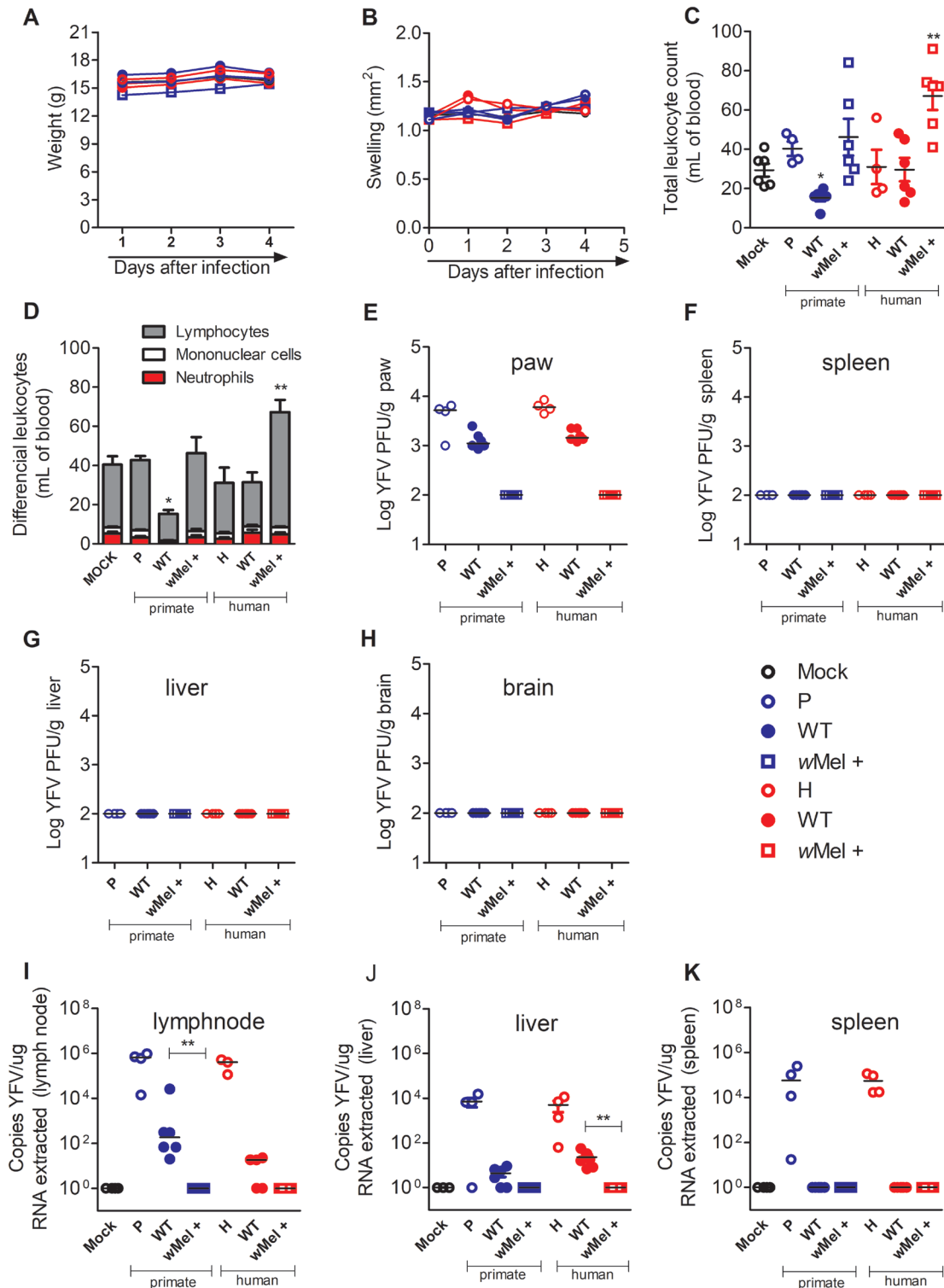


Figure 5. Saliva from *Wolbachia*-positive mosquitoes lose its capacity to transmit yellow fever virus *in vivo*. A129^{-/-} mice were inoculated with 1×10^4 PFU of YFV primate (empty blue circles) and human YFV (empty red circles) or with a pool of saliva from wild type (WT) YFV primate (full blue circles), WT YFV human (full red circles), *Wolbachia*-positive (wMel +) YFV primate (empty blue squares) and *Wolbachia*-positive YFV human (empty red squares) previously infected with YFV via intraplantar route (50 μ l/paw). Control mice (MOCK group) received 50 μ l of PBS solution (empty black circle). **(A)** Body weight analysis shown as body weight (g) of mice. **(B)** Paw volume measured daily and shown as swelling (mm²). On day 4 post-infection mice were euthanized and the following analysis performed. **(C–D)** Total and differential leukocyte counts in the blood. **(E–H)** Viable viral loads recovered from paw **(E)**, spleen **(F)**, liver **(G)** and brain **(H)** by plaque assay in Vero cells. Results are shown as Log PFU/g of tissue. **(I–K)** Viral RNA copies recovered from popliteal lymph node **(I)**, liver **(J)** and spleen **(K)** by RT-qPCR. Data was presented as mean \pm SEM or median (n=4 mice for MOCK, n=6 mice for WT P, wMel + P, WT H and wMel + H groups and n=4 for YFV P and YFV H, one-way anova).

therefore, promoting negative effect towards the virus in WT mosquitoes, but this was not the case. Caragata *et al.* (2013)⁴⁵ studied the effect of cholesterol towards the *Drosophila C virus*. This mechanism could be present in our experimental mosquitoes, but further studies on this aspect should be developed.

Interestingly, in our experiments, the overall infectivity in mosquitoes was not high, even in control (no *Wolbachia*) mosquitoes. This shows the reduced vector competence of natural Brazilian *Ae. aegypti* populations, which could explain why most of the cases reported on the recent outbreaks in Brazil were in proximity to green areas of parks and forests, where natural YFV mosquito vectors such as *Haemagogus* and *Sabethes* are easily found^{11,12,46}.

Our results show that the presence of wMel strain of *Wolbachia* in mosquitoes has the potential to greatly reduce the transmission potential of *Ae. aegypti* for YFV. It is important for public health agencies of arbovirus endemic countries to have constant awareness of the potential of *Ae. aegypti* to become an urban vector for *Yellow fever virus* once again^{6,47}. If that becomes reality, *Wolbachia*-infected mosquitoes could be a powerful tool for YFV control, along with the currently applied vaccination program^{10,48}.

Lastly, it is important to consider the possible vector competence of other mosquito species and the possibility of *Wolbachia* virus evolution and, therefore possible lack of interference in this system. If that is the case, other strategies should be consider, as the use of other strains of *Wolbachia* to try to block virus transmission by that particular mosquito species. Integration of complementary strategies are the best solution for arbovirus control.

Data availability

Underlying data

The data underlying Figure 3, Figure 4 and Figure 5, as well as viral sequencing data is available from Open Science Framework, <https://doi.org/10.17605/OSF.IO/PUZ69>⁴⁹.

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Genome sequences generated in this study are publicly available in GenBank database: M377_IV[Human]MinasGerais_PadreParaiso|2017-02-04: accession number, [MK249065](#); M127_IV[Primate]MinasGerais_NovaLima|2018-01-15: accession number, [MK249066](#).

Extended data

Table S1. YFV reference strains information, <https://doi.org/10.17605/OSF.IO/PUZ69>

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

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Current Peer Review Status:  

Version 2

Reviewer Report 23 April 2019

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Andrew van den Hurk

School of Chemistry and Molecular Biosciences, University of Queensland, St. Lucia, Queensland, Australia

The authors have done a good job of addressing the reviewer's comments. I have no further comments to make.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I am a public health entomologist who researches mosquito-borne pathogens, with a focus on arboviruses. In particular, the research integrates field and laboratory based studies to understand arbovirus transmission cycles, and assesses novel surveillance and control strategies with view to limiting their impact on human health.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 17 April 2019

<https://doi.org/10.21956/gatesopenres.14075.r27068>

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Bianca C. Carlos

School of Agricultural Sciences, Department of Bioprocesses and Biotechnology, Multiuser Central Laboratory, São Paulo State University (UNESP), Botucatu, Brazil

In this revised version the authors have fully addressed the points and concerns raised on Version 1.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vector-pathogen interactions; vector competence; mosquito immunity; functional genomics; microbiota;

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 25 March 2019

<https://doi.org/10.21956/gatesopenres.13999.r26932>

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Bianca C. Carlos

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This is a very elegant manuscript provided by Rocha and colleagues reporting the effect of Wolbachia on yellow fever virus (YFV) transmission by *Aedes aegypti*. For testing this, the authors use an already well-known and established *Ae. aegypti* line carrying Wolbachia (wMel mosquitoes) to evaluate its competence for two closely related YFV isolates, one from humans and another from primates. Finally, the efficacy of the wMel *Ae. aegypti* strain to block YFV transmission is tested by assessing the number of infectious viral particles in the mosquito saliva by two independent methods: 1) intrathoracic inoculation of mosquitoes or 2) paw inoculation of immune-deficient mice with the saliva of either WT or wMel YFV-infected mosquitoes. Clearly, Wolbachia has a potent effect against YFV, reducing both the infection level of YFV in the mosquito body and the rate of YFV-infected mosquitoes when comparing wMel mosquitoes orally exposed to YFV to their respective WT control mosquitoes. More strikingly, the wMel strain is shown to be unable to transmit YFV by the two indirect transmission assays carried out by the authors. Suggestions and comments are presented below with the intention to improve the manuscript, especially the clarity and accuracy of the methods/design and data presentation.

1. Methods/design description: some elements related to this part of the manuscript should be further expanded and detailed for a better understanding and accuracy of the manuscript. Especially, the authors should consider:

- Improving the description of the YFV infection assays in mosquitoes. For example, it is not clear the number of mosquitoes and replicates used to determine the viral load in the body of WT or wMel mosquitoes.
- Similarly, it is important to clarify the design and data presentation of the transmission assays in mosquitoes. For example, it is not clear if each bar on Figure 4 represents the data combination of 8 mosquitoes injected with the same saliva of a given mosquito (1 x 8 x 8) or if it is the data collected from an individual mosquito injected with the saliva of a given individual mosquito (1 x 1 x 8). This may also cause confusion when interpreting this figure as the Y-axis labels refer to "infected mosquitoes per saliva" while the subtitles refer to "the number of YFV copies...per mosquito". Additionally, the authors should check the position of the graphs on the right (A and C) and left (B and D) panels as they seem not to match their respective description in the subtitles.
- An important question that was raised is why the authors have chosen to inject mosquito saliva into mice instead of feeding such mosquitoes on mice in order to test transmission directly.

2. Results/hypothesis/conclusions:

- The leukocyte counts are significantly high in mice inoculated with the saliva of wMel mosquitoes orally exposed to YFV (Figures 5C and 5D). Do the authors have any hypothesis to explain why this is happening?
- Because the WT mosquito population used in this work presented a relatively low vector competence to YFV, on page 10 (Discussion, second column) the authors infer this phenotype as a representation of Brazilian *Ae. aegypti* populations. While this is a plausible hypothesis, I would suggest the authors to be more cautious with this statement as vector competence to YFV of many other *Ae. aegypti* populations must be tested before one assumes this fact.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Vector-pathogen interactions; vector competence; mosquito immunity; functional genomics; microbiota;

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 08 Apr 2019

Luciano Moreira, Oswaldo Cruz Foundation, Belo Horizonte, Brazil

Reviewer – Dr. Jaime A. Souza-Neto and Bianca C. Carlos

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1. Methods/design description:some elements related to this part of the manuscript should be further expanded and detailed for a better understanding and accuracy of the manuscript. Especially, the authors should consider:
 - Improving the description of the YFV infection assays in mosquitoes. For example, it is not clear the number of mosquitoes and replicates used to determine the viral load in the body of WT or wMel mosquitoes.
 - >> *In the section on methods in the item mosquitoes and infection, the requested information was included:*
- Viral load was analyzed at 7, 14 and 21 days post feeding (dpf), via RT-qPCR and the number

of mosquitoes analyzed per group are presented in figures 3B, C and D, ranging from 17 to 20. Additionally, a subset of mosquitoes (at 7dpf) received an extra blood meal and were collected at 14dpf, when *Wolbachia* density and viral load was determined. *Wolbachia* density was analyzed on mosquitoes from the three time-points as follows: 40 mosquitoes on 7 dpf, 39 mosquitoes on 14 dpf and 38 mosquitoes after 21 dpf.

- Similarly, it is important to clarify the design and data presentation of the transmission assays in mosquitoes. For example, it is not clear if each bar on Figure 4 represents the data combination of 8 mosquitoes injected with the same saliva of a given mosquito (1 x 8 x 8) or if it is the data collected from an individual mosquito injected with the saliva of a given individual mosquito (1 x 1 x 8). This may also cause confusion when interpreting this figure as the Y-axis labels refer to “infected mosquitoes per saliva” while the subtitles refer to “the number of YFV copies...per mosquito”. Additionally, the authors should check the position of the graphs on the right (A and C) and left (B and D) panels as they seem not to match their respective description in the subtitles.

>> *The information requested on the number of nanoinjected mosquitoes was introduced in the methods section (Mosquito saliva transmission assay) as shown below:*

Each mosquito received 276 nL and were kept for 5 days before whole body RNA extraction, followed by RT-qPCR. Usually, with one saliva sample it is possible to inject 15 mosquitoes, but due to mortality, 8 mosquitoes were analyzed from each nanoinjected saliva sample.

>> *The panels were indeed misplaced. Thank you for pointing this out. Please see below:*

Saliva from both groups of infected mosquitoes were collected at 14 dpf. Individual saliva samples (WT or wMel +) were analyzed into eight naïve (WT) mosquitoes (bars) and, after five days, these injected mosquitoes were analyzed. (**A**) Mosquitoes injected with WT mosquito saliva or (**B**) wMel+ mosquitoes, challenged with human virus. (**C**) Mosquitoes injected with WT mosquito saliva or (**D**) wMel+ mosquitoes, challenged with primate virus. Values below each bar depicts the viral load of each mosquito head and thorax which donated that saliva. Positive mosquitoes were quantified through RT-qPCR and the grey-scale represents the number of YFV copies (0 to 10^6 copies), per mosquito.

- An important question that was raised is why the authors have chosen to inject mosquito saliva into mice instead of feeding such mosquitoes on mice in order to test transmission directly.

>> *The reason why we have not performed the experiment by feeding infected mosquitoes directly on the mice was because we had no biosafety approval to perform these experiments. Therefore, the saliva samples had to be transported to another institution (UFMG), where the mice were located, and then used there.*

2. Results/hypothesis/conclusions:- The leukocyte counts are significantly high in mice inoculated with the saliva of wMel mosquitoes orally exposed to YFV (Figures 5C and 5D). Do the authors have any hypothesis to explain why this is happening?

>> *Mosquito saliva is a very complex concoction of mixture of proteins (>100 proteins), which exerts several functions in the host by circumventing, for example, vasoconstriction, platelet aggregation, coagulation, and inflammation or host hemostasis. Several works in literature have shown that mosquito saliva by itself exerts profound effects on mouse and human immune*

systems. For example, Vogt and colleagues (2018), using a humanized mice model, have shown that mosquito saliva alters several human blood leukocytes populations such as hematopoietic, NK, NKT, B and myeloid cells. However, the isolate and specific effect of mosquito saliva in modulating blood leukocyte counts was not observed in our work. This finding leads us to believe that wolbachia infection could be associated with such blood leukocyte counts increase. However, when we look closely at the results, we observe that the increase in blood leukocyte counts in mice that received saliva of wMel mosquitoes occurred especially (statistically significant) only upon exposure to human YFV isolate but not after the primate YFV strain inoculation (Fig 5C, 5D). These results suggest that the leukocyte increase observed in the wMel hYFV group was probably due to an interaction between the wolbachia-infected saliva and the human viral isolate in comparison to mice that received the YFV isolate from primates. However, the mechanisms underlying these findings require further investigation.

Vogt MB, Lahon A, Arya RP, Kneubehl AR, Spencer Clinton JL, Paust S, Rico-Hesse R. [Mosquito saliva alone has profound effects on the human immune system](#). *PLoS Negl Trop Dis*. 2018 May 17;12(5):e0006439. doi: 10.1371/journal.pntd.0006439.

- Because the WT mosquito population used in this work presented a relatively low vector competence to YFV, on page 10 (Discussion, second column) the authors infer this phenotype as a representation of Brazilian *Ae. aegypti* populations. While this is a plausible hypothesis, I would suggest the authors to be more cautious with this statement as vector competence to YFV of many other *Ae. aegypti* populations must be tested before one assumes this fact.

>> Thanks for this point. It was included with the information that the results were obtained in this particular population.

Besides working with recently isolated virus from human and primate sources, the difference in the present study refers to the way in which this particular population of mosquitoes have been infected.

Competing Interests: No competing interests were disclosed.

Reviewer Report 01 March 2019

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Andrew van den Hurk

School of Chemistry and Molecular Biosciences, University of Queensland, St. Lucia, Queensland, Australia

The deployment of *Aedes aegypti* transinfected with the *Wolbachia* is a highly promising strategy for suppressing the transmission of a number of globally important arboviruses, such as dengue,

Zika, chikungunya and yellow fever viruses. In the current manuscript, the authors conducted laboratory-based experiments to assess the ability of the *wMel* strain of *Wolbachia* to inhibit the transmission potential of two outbreak strains of yellow fever virus by *Aedes aegypti*. As has been demonstrated with other flaviviruses in numerous similar studies, *wMel* inhibited replication and subsequent transmission when compared with *Wolbachia*-negative mosquitoes. This is a relatively well written manuscript describing experiments with outcomes of interest to readers of Gates Open Research. However, there are some components of the manuscript that need to be clarified and/or justified.

Below are specific comments as they relate to the reported study:

- It is difficult to understand the justification for including the mouse component of the work. The final sentence of the introduction is misleading. As it reads, it indicates that mice were used to demonstrate transmission (i.e. that mosquitoes actually fed on mice which were then monitored for evidence of infection). However, mice (along with mosquitoes) were used to indicate whether saliva collected using an in vitro method contained infectious virus and not to demonstrate transmission directly. If mice had been used to demonstrate transmission directly, then this would have added greatly to the novelty and significance of the study by demonstrating that transmission is affected in an animal model and not just using in vitro assessment of transmission (which is what almost all other studies do).
- The actual number of saliva samples tested was relatively low, so interpretation of the findings with regards to transmission blocking should be undertaken with caution, especially when extrapolating to the field.
- “Pluripotency” is not really an appropriate descriptive term in this context.
- The background in the abstract needs to mention that there is an efficacious vaccine and then state issues with its widespread roll-out (i.e. supply, logistics etc.). This provides a segue into why *Wolbachia* may be appropriate for YFV control.
- Also in the abstract (5th line): *Aedes aegypti* is still the main (urban) vector, not “used to be”.
- Introduction: In lines 3 and 4 of paragraph 2, it is important to emphasise that *Haemagogus* and *Sabethes* are important sylvan vectors in South America. Africa has a different suite of sylvan vectors. Also, insert a comma after *Sabethes*, as the way it currently reads, it sounds as though they are all urban vectors.
- Also in this paragraph (lines 9-12), state that there was (thankfully) no evidence of urban transmission during these outbreaks.
- Line 10 of paragraph 3: Need a segue into the *Wolbachia*-based control. Something like “*Wolbachia* is being deployed to limit arbovirus transmission...”. Then describe what *Wolbachia* actually is.
- Methods: Need to be consistent on whether it is PCR or RT PCR.
- Methods: Please provide references for the IFA and qRT-PCR in regard to the virus isolation.

- Methods: In the first paragraph of the mosquito saliva transmission assays (line 9), state whether saliva was from individual mosquitoes or from pools of saliva.
- Methods: Viral detection on (in) infected mosquitoes and mice:
 - Remove “infected” from this heading, as it is not known whether mosquitoes are infected until they are tested.
 - Also, line 1 of first paragraph: qPCR detects viral RNA.
 - What was the justification for using inoculation of mosquitoes and mice to demonstrate transmission? Why not a cell culture based system?
 - In terms of the mice, is there evidence that the strain used was highly susceptible to YFV infection? Why was this strain of mice used?
- In terms of the multiplex assay, can some data be supplied regarding the relative efficiencies of each of the components of the assay? How does sensitivity compare to singleplex assays? What were the limits of detection, especially for detection of YFV?
- More information on the quantification of *Wolbachia* and YFV would be beneficial, especially with respect to the RPS 17 sequence and why it was included.
- Results: The authors state that there “was no significant difference between infection rates resulting from the human or primate virus isolates.”. Please provide the statistical test used to compare rates and provide significance levels. Please provide statistical tests to show that infection rates were indeed significantly different between *Wolbachia*-positive and - negative mosquitoes.
- Results: Paragraph 2, lines 9-11 – this is speculation or if there is previously published work on this it should be provided – in the discussion.
- Figure 3:
 - Were only the head and thorax of YFV-infected mosquitoes tested for *Wolbachia* density? One would think not, given they are *Wolbachia* infected. Clarify.
 - Line 4, the sentence starting with (B) needs to state that these were the YFV copy numbers from the start.
 - In the last sentence, what does the significance level refer to?
 - Was this absolute or relative quantification of YFV RNA? Clarify.
- Results: The number of saliva samples from WT and wMel+ was 8 for each of the viruses (primate and human). Why was such a relatively low number (especially of the WT) of saliva samples tested? In Figure 3, there was a much larger number of mosquitoes tested. Was transmission not attempted with these mosquitoes? The number of saliva samples tested really was very low compared to what could and should have been tested (especially by mosquito injection, where there shouldn't really be any limitation to the number of samples processed).
- Figure 4: The legend and figures are the wrong way around. Do Figures 4A and 4C actually refer to wMel+ infected saliva?

- The discussion needs to consider some important factors:
 - What are the possible mechanisms causing the *Wolbachia*-mediated virus inhibition? This is pertinent in this case, because the effect of subsequent blood meals was examined.
 - *Wolbachia*-based control strategies are undoubtedly promising tools for control of *Aedes aegypti* transmitted viruses. However, the authors should discuss any potential issues that could arise with *Wolbachia*-based approaches in the future.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I am a public health entomologist who researches mosquito-borne pathogens, with a focus on arboviruses. In particular, the research integrates field and laboratory based studies to understand arbovirus transmission cycles, and assesses novel surveillance and control strategies with view to limiting their impact on human health.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 08 Apr 2019

Luciano Moreira, Oswaldo Cruz Foundation, Belo Horizonte, Brazil

Reviewer – Dr. Andrew van den Hurk

The deployment of *Aedes aegypti* transinfected with the *Wolbachia* is a highly promising strategy for suppressing the transmission of a number of globally important arboviruses, such as dengue, Zika, chikungunya and yellow fever viruses. In the current manuscript, the authors conducted laboratory-based experiments to assess the ability of the wMel strain of

Wolbachia to inhibit the transmission potential of two outbreak strains of yellow fever virus by *Aedes aegypti*. As has been demonstrated with other flaviviruses in numerous similar studies, *wMel* inhibited replication and subsequent transmission when compared with *Wolbachia*-negative mosquitoes. This is a relatively well written manuscript describing experiments with outcomes of interest to readers of Gates Open Research. However, there are some components of the manuscript that need to be clarified and/or justified.

Below are specific comments as they relate to the reported study:

- It is difficult to understand the justification for including the mouse component of the work. The final sentence of the introduction is misleading. As it reads, it indicates that mice were used to demonstrate transmission (i.e. that mosquitoes actually fed on mice which were then monitored for evidence of infection). However, mice (along with mosquitoes) were used to indicate whether saliva collected using an in vitro method contained infectious virus and not to demonstrate transmission directly. If mice had been used to demonstrate transmission directly, then this would have added greatly to the novelty and significance of the study by demonstrating that transmission is affected in an animal model and not just using in vitro assessment of transmission (which is what almost all other studies do).

>> *Thank you for pointing this out. We agree with this and we have edited the text accordingly. Now the end of the introduction reads: "We found that Wolbachia had a major impact on virus replication in mosquitoes, as well as reduced the potential of YFV transmission via saliva, as indirectly determined via mosquitoes or a mouse model".*

The reason why we have not performed the experiment by feeding infected mosquitoes directly on the mice was because we had no biosafety approval to perform these experiments. Therefore, the saliva samples had to be transported to another institution (UFMG), where the mice were located, and then used there.

- The actual number of saliva samples tested was relatively low, so interpretation of the findings with regards to transmission blocking should be undertaken with caution, especially when extrapolating to the field.

>> *We agree with the reviewer, but we think we were able to have good sampling numbers if we consider that 2 isolates (primate and human) were used and the results are quite similar.*

- "Pluripotency" is not really an appropriate descriptive term in this context.

>> *The idea of using the word "Pluripotency" was to describe the ability of Wolbachia to reduce transmission of several different pathogens. If there is no big issue on this, we would prefer to keep it and change Arbovirus to Arboviruses to better illustrate what we wanted.*

- The background in the abstract needs to mention that there is an efficacious vaccine and then state issues with its widespread roll-out (i.e. supply, logistics etc.). This provides a segue into why *Wolbachia* may be appropriate for YFV control.

>> *Thank you for pointing this out. We agree with the reviewer and edited the text accordingly to include this suggestion:*

Background: Yellow fever outbreaks have re-emerged in Brazil during 2016-18, with mortality rates up to 30%. Although urban transmission has not been reported since 1942, the risk of re-urbanization of yellow fever is significant, as *Aedes aegypti* is present in most tropical and sub-tropical cities in the World and used to be the main vector in the past.

Although the YFV vaccine is safe and effective, it does not always reach populations at greatest risk of infection and there is an acknowledged global shortage of vaccine supply. The introgression of *Wolbachia* bacteria into *Ae. aegypti* mosquito populations is being trialed in several countries (www.worldmosquito.org) as a biocontrol method against dengue, Zika and chikungunya. Here, we studied the ability of *Wolbachia* to reduce the transmission potential of *Ae. aegypti* mosquitoes for yellow fever virus (YFV).

- Also in the abstract (5th line): *Aedes aegypti* is still the main (urban) vector, not “used to be”.

>> OK. This has been corrected:

Although urban transmission has not been reported since 1942, the risk of re-urbanization of yellow fever is significant, as *Aedes aegypti* is present in most tropical and sub-tropical cities in the World and still remains the main vector of urban YFV.

- Introduction: In lines 3 and 4 of paragraph 2, it is important to emphasise that *Haemagogus* and *Sabethes* are important sylvan vectors in South America. Africa has a different suite of sylvan vectors. Also, insert a comma after *Sabethes*, as the way it currently reads, it sounds as though they are all urban vectors.

>> The text has been modified to accommodate the reviewer’s suggestion.

The yellow fever virus (YFV) is a member of the Flaviviridae family and transmitted by sylvan mosquitoes of the genus *Haemagogus* and *Sabethes*, in South America and *Aedes aegypti* in urban settings^{5–8}.

- Also in this paragraph (lines 9-12), state that there was (thankfully) no evidence of urban transmission during these outbreaks.

>> The text has been modified:

“Although the last reported cases of urban transmission in Brazil occurred in 1942, in 2016–2017, the country faced major outbreaks of the disease mainly in the states of Minas Gerais, Espírito Santo and Rio de Janeiro. In 2018, the epidemic also extended to São Paulo State⁹. According to the Brazilian Ministry of Health, from July 2017 to April 2018, there were 1,127 YFV cases with 328 deaths, with no evidence of urban transmission.”

- Line 10 of paragraph 3: Need a segue into the “Wolbachia-based control. Something like “Wolbachia is being deployed to limit arbovirus transmission...”. Then describe what Wolbachia actually is.

>> The text has been modified to accommodate the reviewer’s suggestion:

“Population control of *Ae. aegypti* mosquitoes using insecticides has been a mainstay of vector-borne disease control methods for decades but is undermined by widespread insecticide resistance. A promising innovative strategy, based on a bacterium called *Wolbachia pipientis*, has been trialed in many countries. *Wolbachia* is a maternally transmitted bacterial endosymbiont and is naturally present in at least 40% of all insect species¹⁴.”

- Methods: Need to be consistent on whether it is PCR or RT PCR.

>> For all the experiments related to the virus we used RT-qPCR. Regular PCR was used for the sequencing only. Some corrections were made on RT-qPCR throughout the text.

- Methods: Please provide references for the IFA and qRT-PCR in regard to the virus isolation.

>> *Ok. Two references have been added.*

IFA was performed with a monoclonal YFV antibody donated by Evandro Chagas Institute (Arbovirology and Hemorrhagic Fevers Section) and conjugated goat anti-mouse IgG labeled with fluorescein FITC (MP Biomedicals) according to Adungo *et al.* 2016¹⁸ with modifications. Images were obtained using an Olympus microscope model BX51 with DP72 camera and DP-2BSW software. Viral molecular confirmation was performed using RNA extracted from the culture supernatant of each isolate, followed by amplification of the genetic material as described below in the viral detection section according to Domingo *et al.* 2012¹⁹. For mosquito infections, the YFV isolates were replicated in C636 cells (*Ae. albopictus*) cultured in Leibovitz 15 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) for 5 days at 28°C. Viral load was confirmed by RT-qPCR and later through plaque assays (PFU) in VERO cells (CCL81) grown in DMEM medium (Gibco) and 3% Carboxymethylcellulose (Sigma) supplemented with 2% FBS (Gibco) at 37°C and 5% CO₂²⁰.

- Methods: In the first paragraph of the mosquito saliva transmission assays (line 9), state whether saliva was from individual mosquitoes or from pools of saliva.

>> *Saliva samples came from individual mosquitoes. The text has been modified for clarity.*

"Individual saliva samples were injected into WT mosquitoes, after 2 to 4 days of emergence."

- Methods: Viral detection on (in) infected mosquitoes and mice:
 - Remove "infected" from this heading, as it is not known whether mosquitoes are infected until they are tested.

>> *Ok, done:*

"Viral detection in mosquitoes and mice"

- Also, line 1 of first paragraph: qPCR detects viral RNA.

>> *Ok, done:*

"Detection of viral RNA on infected mosquitoes and mice samples were performed through quantitative real-time PCR (RT-qPCR) using LightCycler® Multiplex RNA Virus Master (Roche), according to the previously published protocol."

- What was the justification for using inoculation of mosquitoes and mice to demonstrate transmission? Why not a cell culture based system?

>> *We have tested, in the past, individual saliva samples into cell culture and we were not able to have successful viral growth. Therefore, we have chosen to use these indirect methods (mosquito or mice) to show that the virus was indeed infectious. If the mosquito or mouse became infected it is a good sign that the saliva contained active and infectious virus.*

- In terms of the mice, is there evidence that the strain used was highly susceptible to YFV infection? Why was this strain of mice used?

>> *In this study A129-/-SV129 strain of mice was used. The A129-/- mice strain was chosen based*

on the fact that they are deficient in important innate immune components, more specifically the type I interferons α/β receptor. Type I Interferons (IFN- α/β) plays a significant role in preventing viral replication and protecting against arboviral infections such as Zika, Dengue and Yellow fever viruses (1-5). They are the gold standard models to evaluate virus replication and therapeutical drugs due their elevated susceptibility to infection.

Lazear HM, Govero J, Smith AM, Platt DJ, Fernandez E, Miner JJ, Diamond MS. (2016). A mouse model of Zika virus pathogenesis. *Cell Host Microbe*, 19, 1-11. Published online April 5, 2016. <http://dx.doi.org/10.1016/j.chom.2016.03.010>

Rossi SL, Tesh RB, Azar SR, Muruato AE, Hanley KA, Auguste AJ, Langsjoen RM, Paessler S, Basilaski N, Weaver SC. (2016). Characterization of a novel murine model to study zika virus. *Am J Trop Med Hyg*, 16-0111; Published online March 28, 2016. <http://dx.doi.org/10.4269/ajtmh.16-0111>

Sarathy VV, Milligan GN, Bourne N, Barrett ADT. (2015). Mouse models of dengue virus infection for vaccine testing. *Vaccine*. 2015 Dec 10; 33(50): 7051-7060. [10.1016/j.vaccine.2015.09.112](http://dx.doi.org/10.1016/j.vaccine.2015.09.112).

Yauch LE, Shresta S. (2008). Mouse models of dengue virus infection and disease. *Antiviral Res*. 2008 Nov;80(2):87-93. doi: 10.1016/j.antiviral.2008.06.010.

Meier KC1, Gardner CL, Khoretchenko MV, Klimstra WB, Ryman KD. (2009). A mouse model for studying viscerotropic disease caused by yellow fever virus infection. *PLoS Pathog*. 2009 Oct;5(10):e1000614. doi: 10.1371/journal.ppat.1000614

- In terms of the multiplex assay, can some data be supplied regarding the relative efficiencies of each of the components of the assay? How does sensitivity compare to singleplex assays? What were the limits of detection, especially for detection of YFV?

>> We have done, please see the [graph](#) and [table](#) linked here, a comparison of the single or multiplex assay with a plasmid standard curve and the results are quite similar.

- More information on the quantification of *Wolbachia* and YFV would be beneficial, especially with respect to the RPS 17 sequence and why it was included.

>> Ok, done:

A fraction (1/20) of the total isolated RNA was used in the reactions. Head and thorax samples from YFV-challenged mosquitoes were analyzed in duplicate through RT-qPCR and viral and *Wolbachia* quantification were performed in comparison with serial dilution of a standard curve of the respective genes cloned into the pGEMT plasmid (Promega) ^{16,27}. Therefore, it was possible to calculate the number of copies per tissue. As a mosquito control gene we used the RPS 17S sequence of *Ae. aegypti* (Moreira 2009)¹⁵.

- Results: The authors state that there “was no significant difference between infection rates resulting from the human or primate virus isolates.”. Please provide the statistical test used to compare rates and provide significance levels. Please provide statistical tests to show that infection rates were indeed significantly different between *Wolbachia*-positive and -negative mosquitoes.

>> We have included the following statement: There was no significant difference between infection rates resulting from the human or primate virus isolates (Mann-Whitney U test $p > 0.05$). As for the comparisons between different groups we have added the corresponding statistical analyses values in the legend of Fig 3.

Red lines indicate the median wMel copies (Mann-Whitney U test, ** $p = 0.0062$). (**B**) Analysis

of copies of viral RNA on 7dpf -WT x wMel+ (** $p=0.0028$) and YFV Human x Primate WT ($p = 0.43$), (**C**) 14dpf YFV Human and Primate WT x wMel + (**** $p<0.0001$), YFV Human x Primate WT ($p=0.75$), YFV Human x Primate extra blood meal WT ($p=0.78$), YFV Human WT x WT extra blood meal (** $p=0.0061$) and YFV Primate WT x WT extra blood meal (** $p=0.0056$) and (**D**) 21 dpf -WT x wMel+ (**** $p=0.0001$) and YFV Human x Primate ($p= 0.51$). Empty black circles and triangles are WT mosquitoes, whereas empty green circles and triangles depict mosquitoes with wMel +. Black filled circles and triangles are mosquitoes that received a second blood meal. The red line indicates the median YFV copies. Wild type (WT) or positive (wMel +) were orally infected with two YFV isolates and virus dissemination in mosquitoes was analyzed at different times post infection. (**A**) YFV infected mosquitoes' heads and thoraces were analyzed for *Wolbachia* density at different times post-infection through real time RT-qPCR, based on a *Wolbachia* standard curve. Red lines indicate the median wMel copies (Mann-Whitney U test, ** $p=0.0062$). (**B**) Analysis of copies of viral RNA on 7dpf -WT x wMel+ (** $p=0.0028$) and YFV Human x Primate WT ($p = 0.43$), (**C**) 14dpf YFV Human and Primate WT x wMel + (**** $p<0.0001$), YFV Human x Primate WT ($p = 0.75$), YFV Human x Primate extra blood meal WT ($p=0.78$), YFV Human WT x WT extra blood meal (** $p=0.0061$) and YFV Primate WT x WT extra blood meal (** $p= 0.0056$) and (**D**) 21 dpf -WT x wMel+ (**** $p=0.0001$) and YFV Human x Primate ($p= 0.51$). Empty black circles and triangles are WT mosquitoes, whereas empty green circles and triangles depict mosquitoes with wMel +. Black filled circles and triangles are mosquitoes that received a second blood meal. The red line indicates the median YFV copies.

- Results: Paragraph 2, lines 9-11 – this is speculation or if there is previously published work on this it should be provided – in the discussion.

>> *We apologize but we are not sure where this is on the text. Could you please add more information on where you affirming there is a speculation? We could not find where you mentioned about Paragraph 2, lines 9-11.*

Figure 3:

- Were only the head and thorax of YFV-infected mosquitoes tested for *Wolbachia* density? One would think not, given they are *Wolbachia* infected. Clarify.

>> *The same samples that were used to evaluate the infection were also tested for Wolbachia quantification in the multiplex assay. The analysis of the results was done by targeting the gene of interest. In this way, it generated the graph 3A for the quantification of Wolbachia in the analyzed tissue that was head and thorax. As Wolbachia is present in practically all mosquito tissues, these analyses are possible.*

- Line 4, the sentence starting with (B) needs to state that these were the YFV copy numbers from the start.

>> *Ok, done:*

(B) *Analysis of copies of viral RNA on 7dpf -WT x wMel+ (** $p=0.0028$) and YFV Human x Primate WT ($p = 0.43$)(...)*

- In the last sentence, what does the significance level refer to?

>> *the level of significance was added for all pairwise comparisons in the legend text.*

- Was this absolute or relative quantification of YFV RNA? Clarify.

>> *as stated on the legend title both the Wolbachia as well as the YFV were done via absolute*

quantification.

- Results: The number of saliva samples from WT and wMel+ was 8 for each of the viruses (primate and human). Why was such a relatively low number (especially of the WT) of saliva samples tested? In Figure 3, there was a much larger number of mosquitoes tested. Was transmission not attempted with these mosquitoes? The number of saliva samples tested really was very low compared to what could and should have been tested (especially by mosquito injection, where there shouldn't really be any limitation to the number of samples processed).

>> *Each saliva sample was collected and processed individually. Each saliva sample was injected into up to 8 naïve mosquitoes. This clearly show how sample numbers are multiplied (up to 64 for each group). It was merely the question of numbers and trying to lower the costs.*

We have collected 20 saliva samples from each group. Eight were used to inject the mosquitoes (as explained above) and the other 12 were used in two (pooled) to inject mice (6 mice per group).

- Figure 4: The legend and figures are the wrong way around. Do Figures 4A and 4C actually refer to wMel+ infected saliva?

>> *Thank you for pointing this out! The text has been corrected:*

Saliva from both groups of infected mosquitoes were collected at 14 dpf. Individual saliva samples (WT or wMel +) were injected into eight naïve (WT) mosquitoes (bars) and, after five days, these injected mosquitoes were analyzed. (A) Mosquitoes injected with WT mosquito saliva or (B) wMel+ mosquitoes, challenged with human virus. (C) Mosquitoes injected with WT mosquito saliva or (D) wMel+ mosquitoes, challenged with primate virus. Values below each bar depicts the viral load of each mosquito head and thorax which donated that saliva. Positive mosquitoes were quantified through RT-qPCR and the grey-scale represents the number of YFV copies (0 to 10^6 copies), per mosquito.

- The discussion needs to consider some important factors:
 - What are the possible mechanisms causing the *Wolbachia*-mediated virus inhibition? This is pertinent in this case, because the effect of subsequent blood meals was examined.

>> *In paragraph 5 of the discussion we include this information:*

Another interesting fact of this work was the increase in viral load observed after the second blood feeding in WT mosquitoes. This same fact was not observed in wMel + mosquitoes. This shows that the blocking ability of *Wolbachia* persists even after the addition of extra blood nutrients (through a second blood meal) and that its blocking effect occurs within 7 days after infection. The reason to include the second blood meal was that antibodies to YFV could be present in the blood and therefore, promote negative effect towards the virus in WT mosquitoes, but this was not the case. Caragata *et al.* (2013)⁴⁵ studied the effect of cholesterol towards the Drosophila C virus. This mechanism could be present in our experimental mosquitoes, but further studies on this aspect should be developed.

- Wolbachia*-based control strategies are undoubtedly promising tools for control of *Aedes aegypti* transmitted viruses. However, the authors should discuss any potential issues that could arise with *Wolbachia*-based approaches in the future.

>> *It is important to consider the possible vector competence of other mosquito species and the possibility of Wolbachia/ virus evolution and lack of interference in this system. If that is the case, other strategies should be consider, as the use of other strains of Wolbachia to try to block virus*

transmission by that particular mosquito species. We have added this to the end of the discussion. "Lastly, it is important to consider the possible vector competence of other mosquito species and the possibility of Wolbachia/ virus evolution and lack of interference in this system. If that is the case, other strategies should be consider, as the use of other strains of Wolbachia to try to block virus transmission by that particular mosquito species. Integration of complementary strategies are the best solution for arbovirus control."

Competing Interests: No competing interests were disclosed.