Wolbachia Stimulates Immune Gene Expression and Inhibits Plasmodium Development in Anopheles gambiae

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Abstract

The over-replicating wMelPop strain of the endosymbiont Wolbachia pipientis has recently been shown to be capable of inducing immune upregulation and inhibition of pathogen transmission in Aedes aegypti mosquitoes. In order to examine whether comparable effects would be seen in the malaria vector Anopheles gambiae, transient somatic infections of wMelPop were created by intrathoracic inoculation. Upregulation of six selected immune genes was observed compared to controls, at least two of which (LRIM1 and TEP1) influence the development of malaria parasites. A stably infected An. gambiae cell line also showed increased expression of malaria-related immune genes. Highly significant reductions in Plasmodium infection intensity were observed in the wMelPop-infected cohort, and using gene knockdown, evidence for the role of TEP1 in this phenotype was obtained. Comparing the levels of upregulation in somatic and stably inherited wMelPop infections in A.e. aegypti revealed that levels of upregulation were lower in the somatic infections than in the stably transfected line; inhibition of development of Brugia filarial nematodes was nevertheless observed in the somatic wMelPop infected females. Thus we consider that the effects observed in An. gambiae are also likely to be more pronounced if stably inherited wMelPop transfections can be created, and that somatic infections of Wolbachia provide a useful model for examining effects on pathogen development or dissemination. The data are discussed with respect to the comparative effects on malaria vectorial capacity of life shortening and direct inhibition of Plasmodium development that can be produced by Wolbachia.

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Introduction

Wolbachia pipientis is an intracellular maternally inherited bacterial symbiont of invertebrates that is very common in insects, including a number of mosquito species [1,2]. It can manipulate host reproduction in several ways, including cytoplasmic incompatibility (CI), whereby certain crosses are rendered effectivly sterile. Females that are uninfected produce infertile eggs when they mate with males that carry Wolbachia, while there is a ‘rescue’ effect in Wolbachia-infected embryos such that infected females can reproduce successfully with any males. Therefore uninfected females suffer a frequency-dependent reproductive disadvantage. Wolbachia is able to rapidly invade populations using this powerful mechanism [3–5].

A strain of Wolbachia called A MelPop has been identified that over-replicates in somatic tissues and roughly halves the lifespan of laboratory Drosophila melanogaster [6]. A transfection of A MelPop from Drosophila into the mosquito Aedes aegypti also leads to a similarly shortened lifespan in the lab, as well as inducing strong CI, which has made it a very promising candidate for the development of new strategies for controlling mosquito-borne diseases [7]. All mosquito-borne pathogens require an extrinsic incubation period before they can be transmitted that is relatively long (~9 days for malaria) compared to mean mosquito lifespan in the field; therefore, a reduction in the number of old individuals in the population will reduce disease transmission [8–11].

We recently found that the presence of aMelPop also produces a major upregulation of a large number of immune genes in A. aegypti and inhibits the development of filarial nematode worm parasites [12]. We hypothesized that the two effects are functionally related – higher levels of immune effectors in aMelPop-infected mosquitoes render them better able to kill parasites [12]. Homologs of some of the A. aegypti genes that are upregulated in the presence of aMelPop have been previously shown to have the ability to regulate development of Plasmodium parasites in Anophelines, for example a transgene encoding cecropin-A/a synthetic cecropin-B of Hyalomma axyridis; the NF-kB-like transcription factor Rel2 controlling the Imd pathway; and TEP (Thioester containing) opsonization proteins [13–20]. It has recently been shown that the aMelPop-infected A. aegypti line
Author Summary

Malaria is one of the world's most devastating diseases, particularly in Africa, and new control strategies are desperately needed. Here we show that the presence of Wolbachia bacteria inhibits the development of a malaria parasite in the most important Anopheles mosquito species of Africa. In addition we show that the presence of Wolbachia results in the switching on of immune genes that are known to affect development of many species of malaria parasite. When added to the lifespan-shortening effects of this particular strain of Wolbachia, and the general ability of Wolbachia to spread through insect populations, our study provides a stimulus for the development of Wolbachia-based malaria control methods. It also provides new insights into the wide range of effects of Wolbachia in insects.

has impaired ability to transmit an avian malaria, Plasmodium gallinaceum [21]. It is possible that these effects of wMelPop could be particular to the Ae. aegypti transinfection; however, if comparable upregulation of orthologous immune genes, and inhibition of Plasmodium development are also seen in the important Anopheles vectors of human malaria, it may provide a stimulus to the development of new Wolbachia-based malaria control strategies.

To address this question we used Anopheles gambiae, the most important vector of malaria in Africa, which like Ae. aegypti is not naturally infected with Wolbachia. The creation of stably inherited lines of An. gambiae is likely to require a long period of microinjection and selection, as had to be performed for Ae. aegypti [7]. However, in advance of the successful creation of an An. gambiae stable transinfection, the effects of the presence of wMelPop on immunity and malaria transmission can be tested using an established wMelPop-infected An. gambiae cell line [22] and the ability to create somatic lifetime infections of Wolbachia in adult female mosquitoes by intrathoracic inoculation [23,24]. The wMelPop strain forms disseminated somatic infections in its natural Drosophila host [6], in common with some but not all Wolbachia strains [25]. Given that a) Plasmodium parasites will travel solely through somatic tissues on their journey to the salivary glands, and b) that many of the known antimalarial immune effectors are humoral/systemic, we consider that the creation of somatic infections of Wolbachia via adult inoculation represents a useful model for stably inherited germline-associated infections. To examine this hypothesis further, we also created somatic wMelPop infections in Ae. aegypti, in order to compare the magnitude of the effects on mosquito immunity and filarial nematode parasite development with those observed in the stably wMelPop-transinfected line.

Results

Immune gene expression in An. gambiae

Given that a stable wMelPop infection of An. gambiae does not yet exist, it was necessary to create transient somatic infections by intrathoracic inoculation with purified Wolbachia. RNA from these transinfected females was then tested for expression levels of six immune genes, and upregulation of all these genes was observed compared to buffer injected and E. coli - injected controls (Figure 1). Of these genes, LRIM1 and TEP1 (whose products have been shown to interact in the opsonisation response) have previously been shown to have an important inhibitory or antagonistic effect on Plasmodium development [18–20]. Importantly, injected mosquitoes were left for eight days before Plasmodium challenge or qRT-PCR, and therefore the pulse of immune gene upregulation caused by the injury itself or by the E. coli challenge would be expected to have already passed [15].

The wMelPop infected cell line MOS55 [22] showed upregulation of all six selected immune genes compared to an uninfected cell line created by tetracycline curing of infected MOS55 (Figure 2). These data add confidence to the hypothesis that it is the presence of wMelPop itself that is inducing immune gene upregulation, and by extension Plasmodium inhibition, and that these effects are not artefacts of the intrathoracic injection process. The degree of upregulation was different for some genes in the cell line than observed for the somatic in vivo transinfection. However these differences would be expected given that many immune genes are primarily expressed in particular cell types/organs in adult mosquitoes, such as the fat body cells or in the case of TEP1,
the haemocytes [18], and the cellular composition of this larval-derived cell line is unknown.

Effects on the development of *Plasmodium berghei*

Three *Plasmodium berghei* challenge experiments were conducted on transiently *Wolbachia*-infected *A. gambiae* females compared to buffer injected, uninjected, and in one case *E. coli*-injected controls (Figure 3a–c). In all three experiments highly significant reductions in intensity of oocyst infection in the wMelPop transinfected females were observed compared to the other treatments, while there were no significant differences between any of the control treatments within each experiment. Mean *P. berghei* intensities were reduced in the wMelPop-infected mosquitoes by between 75% and 84% compared to the corresponding buffer injected control groups. A further experiment confirmed the lack of any significant differences in intensity between the *E. coli*-injected, buffer injected and uninjected controls (data not shown).

In order to obtain evidence for a causal link between the immune upregulation and the *Plasmodium* inhibition phenotypes, TEP1 knockdown was undertaken by injection of dsRNA at the same time as *Wolbachia* injection. Significantly higher oocyst numbers were observed compared to the control where dsLacZ was injected at the same time as *Wolbachia* (Figure 3d). This experiment provides evidence for a significant contribution of *Wolbachia*-induced TEP1 upregulation to the *Plasmodium* inhibition phenotype.

Utility of transient somatic wMelPop infections

We assessed the utility of the transient wMelPop somatic infection model by comparing the effects on host immunity and pathogen development with those observed in stable inherited infections of wMelPop. To do this we utilized a filarial nematode-resistant line of another mosquito species, *Ae. aegypti*, in which we have previously carried out *Brugia pahangi* challenges on a stable wMelPop-infected transinfected line [7,12]. We created somatic wMelPop infections using exactly the same methodology as carried out for *An. gambiae*, and after eight days challenged them with *B. pahangi* or carried out qRT-PCR.

The somatic *Wolbachia* infection also induced upregulation of selected immune genes (*PGP51, CECD, CLIPB37, CTI*) (Figure 4a). The scale of upregulation was considerably lower than observed in the comparable *Ae. aegypti* stable transinfection as previously reported [12]. Likewise, challenge of the somatically wMelPop infected females with *B. pahangi* did produce a significant reduction in the numbers developing to the L3 (infectious) stage compared to the controls (Figure 4b), as was previously observed in the stable inherited wMelPop infected line, which showed >50% reduction in mean numbers of L3 compared to the *Wolbachia*-uninfected control at the same microfilarial challenge density [12]. Using quantitative PCR comparing three groups of two mosquitoes with the single copy genes *ftsZ* (*Wolbachia*) and *Actin5C* (*Ae. aegypti*) for normalization, we estimated that there were approximately 176±70 times more wMelPop cells in the stably infected line compared to the somatic infections. This may explain this reduced effect on gene upregulation. Therefore we conclude that intrathoracic inoculation can be a valuable way to test the effects of *Wolbachia* on host immunity and pathogen transmission. Although extrapolations to different mosquito species and parasites must be made with care, it does seem likely that the effects observed for somatic *Wolbachia* infections using the methodology reported here are likely to be smaller than for a stable inherited infection, and thus that the estimations made may be conservative.

An experiment to test whether the immune upregulation observed in wMelPop-infected mosquitoes affects the density of the *Wolbachia* itself was conducted using the stable inherited infection of wMelPop in an *Ae. aegypti* Ref*®* background [7,12]. *Wolbachia* *ftsZ* gene expression (used as a proxy for *Wolbachia* density) was found to be higher in dsRel2-injected than in dsLacZ-injected mosquitoes at both day six and day ten post-injection (Figure 4c). These data suggest that the immune effectors controlled by the Imd (*Rel2*-controlled) pathway can influence *Wolbachia* densities. The very high rate of maternal transmission observed in wMelPop-infected mosquitoes affects the density of the *Wolbachia* itself was conducted using the stable inherited infection of wMelPop in an *Ae. aegypti* Ref*®* background [7,12]. *Wolbachia* *ftsZ* gene expression (used as a proxy for *Wolbachia* density) was found to be higher in dsRel2-injected than in dsLacZ-injected mosquitoes at both day six and day ten post-injection (Figure 4c). These data suggest that the immune effectors controlled by the Imd (*Rel2*-controlled) pathway can influence *Wolbachia* densities. The very high rate of maternal transmission observed in wMelPop-infected mosquitoes affects the density of the *Wolbachia* itself was conducted using the stable inherited infection of wMelPop in an *Ae. aegypti* Ref*®* background [7,12].

**Discussion**

The data reported strongly support the hypothesis that wMelPop can inhibit the development of *Plasmodium in Anopheles*
malaria vector mosquitoes. The *An. gambiae/P. berghei* combination, although not one that occurs in nature, does represent a tractable and well studied model for which considerable information is already available about *Plasmodium* killing mechanisms; however we recognize the challenge experiments will ultimately need to be repeated with the far less tractable human parasite *P. falciparum* once a stably inherited *Wolbachia* transinfected line of *An. gambiae* has been created. The densities of *P. berghei* used in laboratory challenges such as these can be high compared to those of *P. falciparum* that would occur in nature, although the mean intensities recorded in these studies lie within the range recorded for *P. falciparum* in the field. The significant reductions in intensity we recorded in laboratory experiments are considered likely to translate to significant reductions in oocyst prevalence/transmission in a real-life setting.

The knockdown experiment provided evidence for a major role of TEP1, and by extension LRIM1 whose products interact as part of the same opsonization pathway [20], in the inhibition of *P. berghei* development. This is the first time a direct link between the *Wolbachia* pathogen inhibition and immune upregulation phenotypes has been made. A more detailed and exhaustive investigation of the relative contributions of different components of the *Anopheles* immune system to *Plasmodium* killing can be made once stable inherited *Wolbachia* infections have been established.

Taken together with the recent report of reduction in *P. gallinaceum* development in *wMelPop*-infected *Ae. aegypti* [21], the

**Figure 3. An. gambiae somatically infected with wMelPop: challenges with Plasmodium berghei.** Each panel represents an independent experiment showing mean numbers of oocysts per midgut (parasite intensities), comparing *An. gambiae* challenged with *P. berghei* eight (A–C) or five (D) days after intrathoracic inoculation with, in A–C, *Wolbachia* wMelPop compared to buffer (BI) and non-injected (NI) controls plus in C, E. coli (EI); and in (D) *Wolbachia*+dsLacZ (WLI), *Wolbachia*+dsTEP1 (WTI) and NI. Parasite survival was determined by oocyst counting on day 10 post infection. In A–C significant reductions in intensity were observed in WI females compared to the NI, BI and EI controls: ***P<0.001; ** P<0.01. *P. berghei* prevalence was also significantly reduced (P<0.05) in WI compared to one or more of the controls: expt. A, NI = 78.5% (33/42); BI = 81.8% (27/33), WI = 60.0% (27/45); expt. B, NI = 88.4% (23/26), BI = 92.3% (12/13), WI = 57.1% (12/21); expt. C, NI = 90.3% (28/31), BI = 96.0% (24/25), WI = 63.1% (12/19), EI = 81.2% (13/16). In experiment D intensity was significantly lower in the WLI group compared to WTI and NI, *P<0.05. Prevalence was 81% (39/48) for NI, 81% (13/16) for WTI and 50% (6/12) for WLI.

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Figure 4. Immune gene expression and challenges with *Brugia pahangi* in *Ae. aegypti* somatically infected with *wMelPop*, and effects of immune knockdown on *Wolbachia* density. A) The expression of four immune genes were analyzed by qRT-PCR: a peptidoglycan recognition
data increase the desirability of creating stably inherited \( \alpha \text{MelPop} \) infections in important malaria vectors. The potential combination of lifespan shortening and direct inhibition of \textit{Plasmodium} development in the mosquito would represent a very attractive control strategy, since both of these phenotypes are critical components of malaria vectorial capacity. A simple model exploring relative contributions of these two parameters to vectorial capacity is shown in Figure 5. Though lifespan reduction and \textit{Plasmodium} inhibition can each substantially reduce the vectorial capacity of a mosquito population, together they act synergistically to reduce transmission. Depending on the scale of lifespan reduction that would be observed under field conditions, which is as yet unknown, the \textit{Plasmodium} inhibition effect could dramatically increase the efficacy of the \( \alpha \text{MelPop} \) infection in reducing malaria transmission.

Other \textit{Wolbachia} strains might also show malaria inhibition effects, particularly if they reach high somatic densities and/or induce large-scale immune stimulation. Here we show that the use of transient somatic infections of \textit{Wolbachia} by adult female inoculation followed by pathogen challenge is a valuable means to test likely effects on immunity and transmission. This is significant as it allows comparison and selection of strains for the most desirable properties prior to the lengthy, and technically very challenging, process of creating stably inherited \textit{Anopheles} transfections. If other \textit{Wolbachia} strains can be identified which also inhibit \textit{Plasmodium} transmission, they would represent an attractive alternative to \( \alpha \text{MelPop} \) if they do not shorten lifespan to the same extent, since they are therefore likely to have much lower fitness costs. Only the \( \alpha \text{MelPop} \) strain has to date been found to produce a strong life-shortening phenotype.

Laboratory estimates suggest that transfection of \( \alpha \text{MelPop} \) in \textit{Aedes aegypti} can reduce fitness by around 50\% [7]. This would appear to make it difficult for this strain of \textit{Wolbachia} to spread by means of CI through natural populations [26], particularly where populations are fragmented. However, fitness estimates made in relatively benign laboratory conditions, where a comparatively large fraction of the population become old, can overestimate the relative costs of infection. In the field most mosquitoes die early and few live long enough to experience higher \textit{Wolbachia}-induced mortality (although those that do are significant to disease control, if they would otherwise have lived long enough to transmit the infection). As shown in Figure 5 reductions in longevity and \textit{Plasmodium} inhibition together determine vectorial capacity and it will also be important to understand the joint effects of the two phenotypes on mosquito fitness in the field. Detailed knowledge of the demographics of the target species is also important [27]. Selective pressures acting on the host would likely modulate the life-shortening phenotype over time, but this may not occur rapidly enough to prevent a sustained period of disease control.

\textit{Wolbachia} is now known to inhibit the dissemination or development of a variety of insect pathogens and insect-borne pathogens – various \textit{Drosophila} pathogenic viruses, dengue and chikungunya viruses of humans, and filarial nematode parasites in addition to \textit{Plasmodium} [12,21,28–31]. Some of these pathogen-inhibition phenotypes have been reported in \textit{Drosophila} species that naturally harbour \textit{Wolbachia}, in other words they are not restricted to species such as \textit{Ae. aegypti} or \textit{An. gambiae} in which \textit{Wolbachia} forms a novel transfection. On a broader level these \textit{Wolbachia} cases can be added to various other examples where bacterial symbionts have been shown to provide protective effects against one or more pathogens [32,33], although the mechanisms involved are likely to be diverse. Parallels can also be drawn with the effects of entomopathogenic fungi, which can both reduce \textit{Anopheles} lifespan and directly inhibit \textit{Plasmodium} development [34–36]. Pathogen inhibition represents a new and increasingly significant component of our understanding of the effects of \textit{Wolbachia} in insects, and provides excellent prospects for the development of novel malaria control strategies.

**Materials and Methods**

**Ethics statement**

All procedures involving animals were approved by the ethical review committee of Imperial College and by the United Kingdom
Government (Home Office), and were performed in accordance with United Kingdom Government (Home Office) and EC regulations.

Somatic wMelPop infections

Wolbachia wMelPop was purified from the infected An. gambiae cell line MOS55 [22,37] as previously described [23,24]. This protocol has previously been shown to allow Wolbachia replication in the recipient An. gambiae [24]. Cells obtained from one 75 CM2 flask were re-suspended in 100 μL of Schneider medium without antibiotics (optical density, OD = 0.09). 69 nL of this Wolbachia suspension (or 69 nL Schneider for the controls) were microinjected into the thorax of young An. gambiae females of the G3 strain or An. aegypti females of the Refm strain [38] using an Nanocjet microinjector (Drummond). The mosquitoes were supplied with 10% sucrose ad libitum and left to recover for at least eight days prior to qRT-PCR or challenge experiments. A similar OD of 0.1 for E. coli was used to inject another set of controls.

qRT-PCR and qPCR

Gene expression levels were monitored using qRT-PCR. Total RNA was extracted with Trizol reagent from groups of ten An. gambiae or An. aegypti females maintained at 26°C and 70% relative humidity, and cDNAs were synthesised from 1 μg of total RNA using SuperScript II enzyme (Invitrogen). qRT-PCR was performed on a 1 to 20 dilution of the cDNAs using dsDNA dye SYBR Green I. Reactions were run on a DNA Engine thermocycler (MJ Research) with Chromo4 real-time PCR equipment and protocols were the same as those described above. The single copy genes Actin5C and Rs7 for An. aegypti were used to estimate relative numbers of Wolbachia normalized against the mosquito genome.

Plasmodium berghei challenge experiments

General parasite maintenance was carried out as previously described [41]. P. berghei ANKA 2.34 parasites were maintained in 4–10-week-old female Théler’s Original (TO) mice by serial mechanical passage (up to a maximum of eight passages). Hypertetocytosis was induced 2–3 days before infection by treating mice with 200μl i.p. phenylhydrazinium chloride (6mg/ml in PBS; ProLabo UK). Mice were infected by intraperitoneal (i.p.) injection and infections were monitored on Giemsa-stained tail blood smears.

Table 1. Oligonucleotide primers used in quantitative PCR experiments and dsRNA synthesis.

| Gene Name | Accession no. | Forward Primer | Reverse Primer |
|-----------|---------------|----------------|----------------|
| An. gambiae |               |                |                |
| CEC1      | AGAP000693    | CCAGAGCAACAAACACACCAA | GCAGCTCCACTACGACAGAAGA |
| DEF1      | AGAP011294    | CATTGCGCAGTGGAACTA  | GATACCAGACAGCACAGTGA |
| LRM1      | AGAP006348    | CATTCCGGTAGTGGATG   | CTTCTTACGGCTTCCATTTT |
| TEP1      | AGAP010815    | CGCCCGAAGGCTAATGTTG  | CTTGCCAAGCAAGCCAGCTG |
| CTL4      | AGAP005335    | ATCCGAAATCTGACCTGAC  | CTGCGAGCGAATCAATAT |
| CLIPB3    | AGAP003249    | CAGAATGTCTCCACACTG   | GCTCAGGGGCGCAGATAAG |
| RS7R      | AGAP010592    | AGAAACGACGACACACCATC  | GCTGCAAACTTCCGATAT |
| dsRNA-Tep1 [17] | AGAP010815 | TAATACGACTCAAAATGTGGTACAG | TAATACGACTCAATAGGGACACCTACGAG |
| An. aegypti |               |                |                |
| PGRPS1    | AAE0009474    | TGGAGCAGAATGGTACA    | GGCTATGCAATCGTTACT |
| CECD      | AAEL005958    | GCTGACTCAAACCGAAGAG  | TCTTACAAACACGGAGAG |
| CLIPB3    | AAE005035     | TTGGGAGAAAAAGAAAGAC  | GATCGCTCCACAGAGAAG |
| Galactose-specific CTL | AAE005561 | GCTGGGGGTGAAATACACT  | CACCATGTTCTACATC |
| Actin5C   | AAEL011197    | ATCGTACGAAATCCCGATG  | ACAGACATCTTCTCGTCA |
| RpS7      | AAEL004175    | CAGTACGCTGTTACTTCCAT  | CAGGACACTCATCGAGTCGA |
| Rel2 [43] | AAE007624    | GGACGAGGACGCGCGAGTTGAGC  | TCCAGGGCCGGAGATAGTC |
| dsRNA-Rel2 [43] | AAE007624 | TAATACGACTCAATAGGGACACCTACGAGTTTACT  | TAATACGACTCAATAGGGACACCTACGAGTTTACT |
| Wolbachia wMel |               |                |                |
| ftsZ      | WD_0723       | TGATGCTGCAAGCAATAGAG  | TCAATGCCGTTGCCCAAC |
| E. coli   |               |                |                |
| dsRNA-LacZ | EG10527       | TAATACGACTCAATAGGGACACCTACGAGTTTACT  | TAATACGACTCAATAGGGACACCTACGAGTTTACT |

Previously published oligonucleotides are indicated by the reference number following the gene name. doi:10.1371/journal.ppat.1001143.t001
In four independent experiments, individual 4–10 week old Thaler’s Original (TO) mice were treated with 200μL i.p. phenylhydrazinium chloride (PH; 6mg/ml in PBS; ProLabo UK) to induce hyper-reticulocytosis. Three days later mice were injected by intraperitoneal (i.p.) injection with 10^6 parasites of *P. berghei ANKA* 2.24 as described previously [41]. Three days post mouse infection, batches of 100 starved *Anopheles gambiae* strain G3 females, eight days post infection with *Wolbachia*, buffer, *E. coli* or uninjected controls, were allowed to feed on the infected mice. 24h after feeding, mosquitoes were brieﬂy anesthetized with CO2, and unfeds removed. Mosquitoes were then maintained on fructose (3%/w/v) fructose, 0.05%/w/v p-aminobenzoic acid) at 19–22°C and 50–80% relative humidity. At day 10 post-feeding, mosquito midguts were dissected, and oocyst numbers (intensity) and prevalence recorded. The Kruskal-Wallis test was used to compare oocyst counts (intensity of infection) and Fisher’s exact test for prevalence (percentage of mosquitoes containing at least one oocyst).

**Gene knockdown experiments**

T7-tailed primers (see Table 1) were used to amplify fragments of the TEPI and REL2 gene from female cDNA template or the *LacZ* gene from *E. coli* total DNA. dsRNA was synthesized using the T7 Megascript kit (Ambion) and adjusted to a concentration of 3 or 4 μg/μl in RNase free water for dsREL2 and dsTEPI respectively. For REL2 KD 69μl of dsRNA were injected per female mosquito, for TEPI-*wolbachia* KD 69 μl of a mix of 2 parts dsRNA to 1 part of purified αMelPop in Schneider’s medium (OD 0.3) were injected into the thorax of CO2 anesthetized female *An. gambiae* mosquitoes (total ~200 per group). Five days after injection (in order to still fall within the gene knockdown period), mosquitoes were fed on a *Plasmodium* infected mouse.

### Brugia pahangi filarial nematode challenge

*Ac. aegypti* mosquitoes of the filaria-susceptible Ref^a_1 strain were fed on sheep blood containing 25 *B. pahangi* microfilaria per μL eight days post *Wolbachia* innoculation, plus buffer-injected controls of the same age; any females that did not feed properly were removed. Dissections were carried out 10 days after the infective blood meal under a dissecting stereomicroscope. Kruskal-Wallis tests were used to compare counts of *B. pahangi* L3 (infective stage larve).

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### Author Contributions

Conceived and designed the experiments: ZK SPS. Performed the experiments: ZK AMB SBP MSCB. Analyzed the data: ZK SPS MSCB. Contributed reagents/materials/analysis tools: HCJG RES. Wrote the paper: ZK SPS.

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### Author Contributions

Conceived and designed the experiments: ZK SPS. Performed the experiments: ZK AMB SBP MSCB. Analyzed the data: ZK SPS MSCB. Contributed reagents/materials/analysis tools: HCJG RES. Wrote the paper: ZK SPS.
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