Supplementary Information

Wolbachia depletion blocks transmission of lymphatic filariasis by preventing chitinase-dependent parasite exsheathment

Short title: Nematode transmission blocked by Wolbachia removal

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Male Mongolian jirds (*Meriones unguiculatus*, jirds) obtained from Charles River were infected by intraperitoneal injection with 400 *Brugia malayi* (*B. malayi*) L3 stage larvae and maintained at the Liverpool School of Tropical Medicine for experimental use. All jirds were aged between three to six months and weighed between 80 to 100g at the start of infections. These were maintained under specific pathogen-free (SPF) conditions at the biomedical services unit (BSU), University of Liverpool, UK, with free access to food and water. All experiments were approved by the ethical committees of the University of Liverpool and Liverpool School of Tropical Medicine (LSTM) and conducted under Home Office Animals (Scientific Procedures) Act 1986 (UK) requirements. After infection, jirds were left for minimum 12 weeks to reach patency before any treatment began. For experiments comparing transmission and development of mf within mosquitoes after tetracycline treatment, patently infected jirds were administered the antibiotic of choice in either drinking water (2.5mg/ml tetracycline hydrochloride (Sigma-Aldrich, UK)) for two, four or six weeks, or a solution of polyethylene glycol 300, propylene glycol and distilled water at a volume ratio of 11:5:4 respectively (rifampicin, weight-corrected volume of 5mg/kg) once daily via oral gavage for a week. Treatment of jirds with AWZ1066 was given at weight-corrected volumes of 100mg/kg dissolved in a solution of PEG300, propylene glycol and distilled water at a volume ratio of 11:5:4 respectively. Treatment with AWZ1066 was administered to infected jirds via oral gavage twice-daily for seven days, followed by a seven day wash-out period with no treatment. All jird treatments were performed alongside matched untreated controls. Microfilariae were recovered from the peritoneal cavity of jirds by peritoneal lavage at the end of each treatment period. Mf were then purified using a PD10 column size exclusion chromatography (Amersham), and concentrations quantified via microscopy. Mf that were to be used for RNA-sequencing were immediately stored in RNA-later (Ambion) after purification.

Mosquito maintenance and infection

*Aedes aegypti* female mosquitoes were reared from eggs obtained from the NIH Filariasis Research Reagent Resource Center (FR3). After sucrose-starvation (for 18-20 hours), three- to five-day-old mosquitoes were fed on human blood (blood bank) inoculated with...
mf (maximum concentration 20,000 mf/ml of blood) from either treated or control jirds using the Hemotek® feeding apparatus (Discovery Workshops, UK) covered with parafilm®. After feeding, only those females that had bloodfed were transferred to a fresh cage. Thirteen days post-exposure, up to 200 mosquitoes were dissected in phosphate-buffered saline and numbers of infective stage larvae (L3) counted.

For assaying mf uptake into the mosquito, 50 mosquitoes per group were fixed in Carnoy’s fixative (2-parts ethanol and 1-part glacial acetic acid) immediately after feeding to allow assessment of mf uptake at a later stage. These samples were processed as described by Arzube and Shelley [1] with slight modification. Briefly, individual mosquito abdomens were separated from the other body parts and the semi-hard blood meal removed and transferred to a clean slide. The blood meal was softened in a drop of water before being macerated with fine needles. A drop of lacto-propionic orcein was added and slides were left in a humidity chamber for 30 minutes, before a cover slip was added and numbers of mf present in the blood meal was assessed by light microscopy.

**Quantification of Wolbachia depletion in microfilariae and L3 nematodes**

DNA was extracted from mf (8,000 per replicate) or infective L3 nematodes (single nematodes) using the QIAamp DNA Mini kit (Qiagen) according to manufacturer’s instructions after an initial overnight incubation at 56°C with 35µl proteinase K and 100µl ATL buffer, with DNA eluted in water. The number of Wolbachia present in adult worms and mf was assessed by quantification of the Wolbachia surface protein (wsp) gene copy number and normalised to the nematode glutathione S-transferase (GST) gene by qPCR [2]. Primer sequences for all amplified genes are included in Table S1.

DNA were amplified in duplicates in 20µl reactions containing 1x QuantiTect SYBR Green PCR master mix (Qiagen). Amplification of the wsp gene utilised a final concentration of 0.3 µM each for forward and reverse primers, 3mM MgCl₂ and 2µl of DNA. Amplification of the GST gene utilised a final concentration of 0.35 µM of forward and reverse primers, 1.5mM MgCl₂ and 1µl of DNA. Primer sequences are included in Table S1. qPCR was performed using the Bio-Rad CFX384 C1000 thermal cycler (Bio-Rad laboratories LTD) with a denaturation step of 95°C for 15min then 40 cycles at 95°C for 15s, 57°C (GST) or 60°C (wsp) for 30s, and 72°C for 30s. Quantification was
determined by Bio-Rad CFX manager software by comparing the DNA samples to that of a standard curve generated from serial dilution of plasmid DNA of the appropriate gene.

**Exsheathment assays of *B. malayi***

The *in-vitro* mf exsheathment assay was adapted from that of Devaney and Howells [3]. Briefly, 100 purified *B. malayi* mf were suspended in 100µl of phosphate-free buffer in 96 well plate in five replicates, incubated at 27°C for two hours of incubation. Subsequently, 1.0µl of each of 2M CaCl$_2$ and 1M MgSO$_4$ were added to make a final concentration of 20mM CaCl$_2$ and 10mM MgSO$_4$. The plates were incubated at 27°C for 1h, and the level of mf exsheathment was viewed and recorded. The plate then was incubated for a further 15h before assessment of the level of exsheathment. Counts of sheathed and exsheathed mf were carried out using a fluorescent wheat germ agglutinin (WGA) assay as described by Rao *et al.* [4] with an Echo Revolve fluorescent microscope (s/n:12452, SEMPREX.COM, USA). The *in-vivo* mf exsheathment assay utilised the midguts of 24 mosquitoes fed on blood infected with either untreated, or six-week tetracycline treated mf. Mosquitoes were removed two hours after feeding to repletion, where individual engorged midguts were excised, washed into separate wells of a 96-well plate with RPMI media supplemented with 10% FBS, 1% penicillin streptomycin, and 1% amphotericin B (full RPMI media), and then macerated. The contents of each well were then divided into five wells, with a total volume of 100µl per well. Counts of sheathed and exsheathed mf were then carried out using the WGA assay as described over two days due to logistical constraints. Mosquitoes that were mf-negative in their midguts were excluded from further analysis.

For chitinase replacement experiments, we first measured the chitinase activity of mf, using the protocol described by Amick *et al.* [5]. Briefly, enzymatic hydrolysis of non-fluorescent 4-methylumbelliferyl-N,N,N-trisaccharide (4-MU-(GlcNAc)$_3$) via chitinase releases fluorescent 4-methylumbelliferone (4MU). This fluorescence can be measured in alkaline pH using a fluorimeter with excitation at 360nm, emissions at 450nm. Using this information, 10,000 mf of *B. malayi* were first homogenised in 100µl of 1x McIlvaine buffer (50mM citrate, 0.1M phosphate at a pH of 5.2). This homogenate was then centrifuged at 14,000x g for 10 minutes to remove insoluble matter, and chitinase activity was measured in 96 well plates, with individual wells containing 95µl of substrate buffer.
with 5µl soluble MF extracts. In this case, the substrate buffer is 100µl of 4-MU-(GlcNAc)_3 in 2.17ml of 1x McIlvaine buffer. This was incubated for 15 minutes at 37°C, with fluorescence subsequently measured at 350 nm excitation, 440 emission in a Varioskan™ LUX multimode microplate reader (Thermo Fisher).

Once this was done, a standard curve was constructed to compare the activity of two commercial chitinases from *Streptomyces griseus* and *Trichoderma viride* (Sigma-Aldrich, catalogue C6137 and C8241 respectively) against mf chitinase activity. The standard curve was constructed using 4- methylumbelliferone in glycine buffer. A final stock solution for both commercial recombinant chitinases was created at 1mg/ml, dissolved in water. From this stock solution, a 1 in 100 dilution (final concentration 10µg/mL) was made with full RPMI media containing 100 µl in 96-well plates (Corning). The assay was incubated for a minimum of 15 hours before assessment of exsheathment using the WGA assay as described previously.

**Statistical analyses used**

All statistical analyses were performed using the R programming language [6], with the rstatix package (v0.7.0) [7] used for conducting Mann Whitney-U tests, with the MASS (v7.3-54) [8] and emmeans (v1.6.2-1) [9] packages used for creating (negative) binomial generalized linear models for statistical tests, followed by post-hoc tests. Suitability of these statistical tests were evaluated using the DHARMa package [10]. Plots were generated using the packages ggplot2 (v3.3.5), ggpubr (v0.4.0) and gridExtra (v2.3) [11–13].

**Nucleic acid extraction for RNA sequencing**

Mf in RNA-Later (Ambion) were first diluted via adding an equal volume of phosphate-buffered saline to the sample, then centrifuged at 2,000x g at 4°C for 10 minutes to pellet mf. The supernatant was removed, before 1ml of TriZol Plus reagent (Invitrogen) was added. Samples were then transferred to separate sterile 2ml screw cap tubes containing ceramic beads of different sizes (1.4 and 2.8mm) (CKMix, Bertin). Batches were homogenized at 6,000rpm (Minilys, Bertin Instruments) for 4 x 30 seconds, cooling on wet ice for 30 seconds in between. Molecular grade chloroform (Invitrogen) was then added at a rate of 200µl for every 1ml of TRIzol reagent, and mixed by vigorously shaking the tube
for 15 seconds, followed by incubation at room temperature for 5 minutes. The sample was then centrifuged at 12,000x g for 15 minutes at 4°C. The upper aqueous phase was removed and transferred to a nuclease-free 1.5ml tube, and the remaining phases were stored for subsequent proteomics experiments. An equal volume of ice cold 70% ethanol was added to each sample and vortexed for 2 - 3 seconds. The tube was inverted several times to disperse any precipitate.

Subsequent binding, washing and elution of RNA from the sample was then conducted using Exiqon’s miRCURY™ RNA Isolation Kit – Cell & Plant, in accordance with manufacturer’s instructions. Any remaining gDNA was removed via an on-column treatment using Baseline-ZERO™ DNase (Epicentre). Final RNA was eluted in 2 x 50μl molecular grade water (Invitrogen). RNA samples were assayed for total RNA concentration and purity via the use of a Nanodrop spectrophotometer (ThermoFisher Scientific), and subsequent quality analysis using a 2100 Agilent Bioanalyzer machine system (Agilent, Total RNA Nano chip), with all samples having a minimum RNA-Integrity score of 8, or having clearly defined peaks for both 18S and 28S rRNA. In order to remove excess ribosomal RNA species from both Wolbachia and B. malayi, total RNA was treated with Terminator 5’Phosphate-Dependent Exonuclease (Epicentre), in accordance with the manufacturer’s instructions. The samples were then cleaned for chemical and protein impurities via use of a commercial purification kit (Zymo, RNA Clean & Concentrator-5), following manufacturer’s instructions.

RNA concentration of these rRNA-depleted samples were then quantified via use of a Qbit 3.0 machine (Invitrogen), before being sent to the University of Liverpool’s Centre for Genomic Resources for RNA sequencing via use of an Illumina HiSeq 4000, and the NEBNext Ultra Directional RNA library preparation kit (New England Biolabs). Two separate paired-end sequencing runs (2x 150 bp) were performed for each sample. Files for all data were then converted from raw basecall to fastq using CASAVA 1.8 (Illumina). The raw Fastq data files were trimmed for the presence of Illumina-specific adapter sequences using Cutadapt (v1.2.1)[14] with the option “–O 3” and further trimmed for quality using Sickle (v1.200)[15], with a minimum quality window score of 20, and reads less than 20 bases were discarded. Sequencing quality scores were assessed using FASTQC
Trimmed RNA-sequencing datasets are available under NCBI BioProject ID number PRJNA772674.

**Gel Electrophoresis and Western blotting**

Parasite protein material was recovered from the phenol-ethanol supernatant after RNA extraction using a protein precipitation method following the manufacturer’s protocol. Briefly, 1.5 ml isopropanol per 1 ml of TRIzol reagent was added to the phenol-ethanol supernatant. This was then incubated for 10 min, then centrifuged for 10 min at 12,000 x g at 4°C to pellet proteins. The pellet was washed twice with 0.3 M guanidine hydrochloride in 95% ethanol, followed by one wash with 100% ethanol. The pellet was air dried and resuspended in 100 µl 1% SDS. Protein solutions were then diluted with 2 X electrophoresis sample buffer to make a final extraction (3% (w/v) SDS, 62 mM Tris-HCl, pH 6.8, 15% (v/v) glycerol) containing 5% β-mercaptoethanol. Insoluble material was removed by centrifugation for 5 min at 16,000 x g. Extracts were then fractionated using Invitrogen™ Bolt™ 12%, Bis-Tris, 1.0 mm, Mini Protein Gel. Separated proteins were electrophoretically transferred to nitrocellulose, and the membranes were blocked for 1 h at room temperature by incubation in a blocking buffer containing 5% skim milk (Sigma) in Tris/saline/Tween (TST: 0.01 M Tris, pH 8.5, 0.15 M sodium chloride, 0.1% Tween 20). Blots were incubated with rabbit antisera to Ov-CHI-1 [17] at 1:5000 dilution in blocking buffer. Goat anti-rabbit IgG (heavy and light chain) horseradish peroxidase conjugate (Cell signalling, 1:5000 dilution) was used to localize antibody/antigen complexes. Blots were developed by incubation with ECL SuperSignal Chemilluminiscnt substrate HRP system (Peirce, Thermo Fisher Scientific) for 5 minutes, and then exposed to x-ray CL Xposure films (Thermo Fisher Scientific), signals were revealed using Photons Developer Instrument. For Western blots of actin loading control, the nitrocellulose membrane used above were stripped off using Restore Plus Western Blot Stripping Buffer (Thermo Fisher Scientific) for 15 minutes at room temperature and washed 3 times with TST buffer, blocked again and re-probed with anti β-actin (Santa Cruz, 1:3000 dilution). Images were then developed by ECL autoradiograph.
Sequencing alignment and differential expression analysis

RNA-sequence reads from processed fastq files were aligned to the genome of *B. malayi* (GenBank ID GCF_000002995.3) using Subread-Aligner’s Subjunc program (v1.5.0)[18] and the options “--multiMapping, -B 2”, giving a Binary Alignment/Map (BAM) output file. The BAM files were then separated into aligned and unaligned reads using Samtools (v0.1.19)[19], with one BAM file containing aligned RNA-sequence reads mapped to *B. malayi*.

The aligned RNA-sequence data from both *B. malayi* and wBm were quantified against their respective genome annotations held on the NCBI database. This quantification used the program FeatureCounts (v1.5.0-p3)[20] to obtain read counts that were then subsequently used in differential expression analysis. This was used as input into the program EdgeR (v3.30.3)[21] for differential expression analysis, using the filterByExpr command to filter out lowly expressed genes and the QLF test for analysis. Differential expression utilised a pairwise comparison, with untreated nematodes acting as the reference point against nematodes treated with tetracycline for 6 weeks. Gene Ontology analysis was conducted via separating the list of significantly differentially expressed genes into up- or downregulated genes. These two sets were then used as a ‘test’ set, and were analysed against the entirety of *B. malayi*’s genome as a ‘background’ set using the program TopGO (v2.40.0)[22], using a minimum node size of 5, the elim algorithm and Fisher statistic, with a p-value cutoff of 0.05 for statistical significance. The top 15 GO terms based on statistical significance, was then used as input for plotting using the R programming language [6] and ggplot2 (v3.3.5)[11].

**Validation of RNA-sequencing by RT-qPCR**

To confirm results of differential expression analysis, the DNAse-treated, rRNA-depleted samples that were prepared for sequencing were used as template for cDNA synthesis, using the SuperScript IV First Strand Synthesis System (Invitrogen) following manufacturer’s instructions. A total of seven genes were selected for analysis by RT-qPCR (three genes predicted to encode for chitinases that showed statistically significant downregulation, and an additional four genes that showed statistically significant up- or down-regulation), as well as two additional housekeeping genes. These four genes selected
for analysis were based on a minimum raw read count > 1,000, a minimum absolute log2 FC ≥ 1, and an FDR < 0.05. The 2 genes selected to act as calibrator genes were selected based on their predicted housekeeping functions as Actin-related protein 3 (Bm1_17980) and DNA-dependent RNA polymerase subunit 3 (Bm1_49670) genes. These calibrator genes are associated with typical ‘housekeeping’ genes, showed close to zero log2-fold change from differential expression analysis, as well as an FDR value of greater than 0.9, as determined by differential expression analysis. Primer sequences for all amplified genes are included in Table S1.

Amplification of all genes utilised a final concentration of 0.3μM for each primer. All tests were carried out in triplicate, within a total volume of 20μl and included 2μl of DNA from each sample, and 10μl of QuantiTect SYBR Green PCR kit master mix (Qiagen). A standard curve was also generated for all genes in triplicate. All qPCR reactions were conducted using a CFX 384 Real Time PCR detection system (BioRad), with all reactions heated for an initial 15 minutes at 95°C, and run for 40 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C.
Supplementary Figures

Figure S1: Box and whisker plots illustrating mf counts recovered from mosquito bloodmeals immediately after feeding, comparing between mf that have or have not been treated with tetracycline for between two to six weeks. Note how mosquitoes fed on blood infected with treated mf have statistically significantly higher mf uptake rates after two and six weeks of treatment with tetracycline as compared to their matched controls. While four weeks of treatment did not see statistically significant differences in mf uptake rate, there is a slight trend towards higher uptake. This higher mf uptake rate is despite a reduced L3 recovery rate, as described in the main text.
Figure S2: Dotplot comparing L3 recovery against Wolbachia depletion of the different experiments conducted as part of this study, with an additional trend-line and $R^2$ value. L3 recovery rates were calculated as a percentage of the treated group when compared to their respective matched controls, whilst Wolbachia reduction is a percentage of the WSP:GST ratios of the same comparison. Trendline was drawn using ggpubr [12], and significance p-value calculated using a linear model in the R programming language [6].
Figure S3: Volcano plot of statistically significantly differentiated genes in *B. malayi* mf, with respect to untreated controls. Statistically significant genes are coloured either red (for downregulation) or green (for upregulation), with genes that are annotated as having roles in chitinase or proton-transmembrane transport highlighted. Figure was generated using ggplot2 [11].
Figure S4: Comparison of fold-change for genes of interest, between qPCR and RNA-seq differential expression results. (A) – Chitinase genes, (B) – Highly up- or down-regulated genes with statistical significance. Genes were compared using two housekeeping genes, actin-related protein 3 (XM_001895019.1), and DNA-directed RNA polymerase III subunit RPC7 (XM_001901366.1). Figure was generated using ggplot2 [11].
Figure S5: Complete blots (left) from Figure 3 of the main manuscript (right).
Figure S6: Brightfield and fluorescent microscopy of live mf in mosquito midguts that have, or have not successfully exsheathed, after staining with wheat germ agglutinin. 
(A) Mf that still retain their sheath are visible in both brightfield (left) and fluorescence (right) microscopy. (B) Mf that have successfully exsheathed are still visible under brightfield microscopy, but no longer visible under fluorescence microscopy.
## Supplementary Tables

**Table S1**, containing information on primer sequences used in this manuscript.

| PRIMER TARGET | PRIMER SEQUENCE |
|---------------|-----------------|
| WSP FORWARD   | TGT TGG T(AG)T TGG T(GC)T TGG TG |
| WSP REVERSE   | AAC CAA A(AG)T AGC GAG C(C)T C CA |
| GST FORWARD   | GAG ACA TCT TGC TCG CAA AC |
| GST REVERSE   | ATC ACG GAC GCC TTC ACA G |
| XM_001902572.1 FORWARD (NIMA-RELATED KINASE 8) | CTG TGA AGG CAG AGC GTA CA |
| XM_001902572.1 REVERSE (NIMA-RELATED KINASE 8) | AAC ACA CAG CCT AAA GCC CA |
| XM_001895501.1 FORWARD (PAN-DOMAIN CONTAINING PROTEIN) | GCG CAG CAG TGC TTT TAT GT |
| XM_001895501.1 REVERSE (PAN-DOMAIN CONTAINING PROTEIN) | AAG CCG AAA GAC AGC CAA GT |
| XM_001901839.1 FORWARD (PROTEIN KINASE DOMAIN CONTAINING PROTEIN) | CGC AAA TGA AGC ATG GCG TA |
| XM_001901839.1 REVERSE (PROTEIN KINASE DOMAIN CONTAINING PROTEIN) | TGT TGG TGG TTG TGC TCC TT |
| XM_001897528.1 FORWARD (SODIUM-INDEPENDENT ANION TRANSPORTER) | TTTT GAG GTC AGT ACC GCC G |
| XM_001897528.1 REVERSE (SODIUM-INDEPENDENT ANION TRANSPORTER) | GGG CAG GCT AGC AAA CAA AC |
| XM_001895019.1 (HOUSEKEEPING ACTIN-RELATED PROTEIN 3) | TGG CAG ATA CGC CTG AGT TC |
| XM_001895019.1 (HOUSEKEEPING ACTIN-RELATED PROTEIN 3) | TGT GAC GAC AGA TGC TTG GT |
| XM_001901366.1 (HOUSEKEEPING DNA-DIRECTED RNA POLYMERASE III SUBUNIT RPC7) | TCG ATG TAG AGG AGC GGG AT |
| XM_001901366.1 (HOUSEKEEPING DNA-DIRECTED RNA POLYMERASE III SUBUNIT RPC7) | TGG AAG TCG TGA CCA ATC CG |
| XM_001894829.1 FORWARD | TGA TGA AAC TGA AGA AAC TTC CGA |
| XM_001894829.1 REVERSE | GTC GCT GGA CAT TGC ATC AC |
| XM_001897137.1 FORWARD | CAA TGC GCA AAT AAC ATT GCA T |
| XM_001897137.1 REVERSE | TAT CCG GCG CAT TCG TCA T |
| XM_001897135.1 FORWARD | GGA TGG ACA TTA GAT AAT CCT TCG G |
| XM_001897135.1 REVERSE | GTT TTC GAT GCT GAC GAC GG |
Table S2 (separate file), containing information on all counts of microfilariae or L3's collected as part of the associated manuscript. Available at: https://doi.org/10.6084/m9.figshare.16847671
Full details on data generation are available in the related manuscript.

Table S3 (separate file), containing full results from edgeR RNA-sequencing differential expression analysis of B. malayi microfilariae controls compared to treatment with tetracyclines, used in the associated manuscript for drawing conclusions. Available at: https://doi.org/10.6084/m9.figshare.16847707
Full details on data generation are available in the related manuscript.

Table S4 (separate file), containing the full results of Gene Ontology enrichment analysis using statistically significantly up- and down-regulated genes from Supplementary table 2. Available at: https://doi.org/10.6084/m9.figshare.16847719
This data comes from B. malayi microfilariae before and after treatment with tetracycline to remove the Wolbachia endosymbiont. Full details on data generation are available in the related manuscript.
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