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

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Lymphatic filariasis is a vector-borne neglected tropical disease prioritized for global elimination. The filarial nematodes that cause the disease host a symbiotic bacterium, *Wolbachia*, which has been targeted using antibiotics, leading to cessation of parasite embryogenesis, waning of circulating larvae (microfilariae [mf]), and gradual cure of adult infection. One of the benefits of the anti-*Wolbachia* mode of action is that it avoids the rapid killing of mf, which can drive inflammatory adverse events. However, mf depleted of *Wolbachia* persist for several months in circulation, and thus patients treated with antibiotics are assumed to remain at risk for transmitting infections. Here, we show that *Wolbachia*-depleted mf rapidly lose the capacity to develop in the mosquito vector through a defect in exsheathment and inability to migrate through the gut wall. Transcriptomic and Western blotting analyses demonstrate that chitinase, an enzyme essential for mf exsheathment, is down-regulated in *Wolbachia*-depleted mf and correlates with their inability to exsheath and escape the mosquito midgut. Supplementation of in vitro cultures of *Wolbachia*-depleted mf with chitinase enzymes restores their ability to exsheath to a similar level to that observed in untreated mf. Our findings elucidate a mechanism of rapid transmission-blocking activity of filariasis after depletion of *Wolbachia* and adds to the broad range of biological processes of filarial nematodes that are dependent on *Wolbachia* symbiosis.

Wolbachia | lymphatic filariasis | antibiotic treatment | transmission blocking

Lymphatic filariasis is a neglected tropical disease caused by the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, which is estimated to affect ~51 million people (1, 2). These and other medically and veterinary-important filarial nematodes have evolved a mutualistic relationship with the bacterial endosymbiont *Wolbachia* (3). Depletion of *Wolbachia* with antibiotics has significant effects on the nematode host, including a block in growth, development, and adult female embryogenesis, all biological processes with high metabolic demands supported with resources derived from the endosymbiont (4). Importantly, depletion of *Wolbachia* from adult worms is known to dramatically reduce the long lifespan of these nematodes, which has been exploited as a safe curative (macrofilaricidal) therapy (5–9). While most research on the relationship between *Wolbachia* and nematode host has focused on larval and adult nematode life cycle stages within the mammalian host, there has been comparatively less focus on the early nematode life cycle stages and their development in the insect vector.

Microfilariae (mf) are responsible for transmission of the disease to insect vectors and contain the lowest density of *Wolbachia* of the nematode life cycle (10). Lymphatic filarial nematodes release their mf encased within a chitinous sheath, the remains of the nematode eggshell. Shedding of the microfilarial sheath is known to occur both in the mosquito midgut and hemocoel and is essential for further larval development (11, 12). Successful colonization of a permissible vector requires these nematodes to penetrate the mosquito gut wall (12, 13) and migrate to flight muscle tissues, where they undergo two molts to develop into infective third-stage larvae (L3) (14). Throughout this process, *Wolbachia* populations remain at low densities within the mf until the L3 nematode stages (10). This low *Wolbachia* density in mf, as well as a lack of overt deleterious effects after antibiotic treatment in terms of viability and motility (15, 16), has resulted in limited studies being performed on these early life cycle stages. These studies have shown a variable degree of inhibition on development of larvae within insect vectors that are competent for certain filarial parasites after antibiotic depletion of *Wolbachia* from mf. These include *Brugia pahangi* within *Aedes togoi* mosquitoes (17), *B. malayi* within *Aedes* sp. (18), *Litomosoides sigmodontis* within *Ornithonyssus bacoti* mites (19), and *Onchocerca volvulus* within *Simulium damnosum* blackfly (20). These

Significance

Lymphatic filariasis caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* affects 51 million people, leading to severe physical and mental disabilities. A mutualistic symbiosis between these filarial nematodes and *Wolbachia* bacteria has been exploited as a new curative treatment. Epidemiological modeling of anti-*Wolbachia* treatment assumes that transmission persists due to the lag phase before microfilariae (mf) become removed from circulation. Here, we show that *Wolbachia*-depleted mf cannot develop within the mosquito vector—a phenotype associated with down-regulation of *B. malayi* mf-specific chitinase, an enzyme essential for exsheathment. Our findings add to the broad range of host biological processes dependent on *Wolbachia* and suggest that anti-*Wolbachia* treatment mediates a more accelerated impact on elimination of lymphatic filariasis than currently predicted.

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effects on nematode development within the insect vector consequently lead to a reduced number and/or capacity of L3s to infect the final host. This is a critical, yet understudied, aspect of the nematode–*Wolbachia* relationship from the perspective of anti-*Wolbachia* therapy. Specifically, while antibiotic therapy can interrupt nematode transmission via the block in adult embryogenesis, existing circulating mf may take several months after treatment to decline significantly, raising concerns that treated individuals could still contribute to disease transmission. The observation that symbiont depletion may also inhibit the development of mf to the infective L3 stage within the arthropod vector suggests that anti-*Wolbachia* therapies may provide transmission-blocking activity within weeks of administration while avoiding the risk of microfilaricidal side effects common to rapid-acting antifilarial drugs (21–24). Here, we have investigated this phenomenon to determine the underlying mechanism responsible for *Wolbachia* depletion curtailing *B. malayi* mf development in the mosquito vector *Aedes aegypti*.

Results

Depletion of *Wolbachia* Leads to a Block of mf Development to L3. To investigate the impact of *Wolbachia* of *B. malayi* (*w*Bm) depletion on *B. malayi* mf development in mosquitoes, Mongolian gerbils (*Meriones unguiculatus*, jirds) were treated for 2, 4, and 6 wk with tetracycline. Reduction in the *Wolbachia* burden of these mf was 6.0% after 2 wk, 39.4% after 4 wk, and 89.0% after 6 wk of treatment (based on qPCR gene copy ratios of the *Wolbachia wsp* gene versus nematode host *GST*). When fed to *A. aegypti* mosquitoes, it was noted that the number of mf ingested was not significantly different at 4 wk (Mann–Whitney *U* test, $P > 0.3$) but was significantly higher in the tetracycline-treated group than in the untreated controls at 2 and 6 wk ($P < 0.001$ in both cases; *SI Appendix*, Fig. S1).

After 12 d to allow for nematode development, mosquitoes were dissected, and L3s were counted. For each time point of treatment, we noted that statistically significantly fewer L3s were recovered from mosquitoes fed on treated mf than from mosquitoes fed on control mf (Fig. 1*A*). Mosquitoes fed mf from the 2-wk treatment group showed a reduction of recovered L3s by 31.4% compared to matched controls (Fig. 1*A*). Similarly, after 4 wk of treatment, the reduction was 36.4%. However, the greatest difference was noted after 6 wk of treatment at 100% reduction (Fig. 1*A*). This significant impact on L3 development after 6 wk of tetracycline treatment was consistently reproducible, with repeat experiments showing a statistically significant reduction in the number of L3s recovered from mosquitoes (Fig. 1*B* at 72.9% and Fig. 1*C* at 90.5% reduction).

To show that this reduction in L3 development within the mosquito was not due specifically to the effects of the tetracycline class of antibiotics, we treated infected jirds for 2 wk with the antibiotic rifampicin at a known suboptimal concentration. This treatment showed a 35% reduction of *Wolbachia* loads that yielded a 67.4% reduction in L3 recovery from mosquitoes (Fig. 1*D*). Taken together, a dose-dependent reduction in *Wolbachia* loads with 2-, 4-, and 6-wk courses of tetracycline and suboptimal rifampicin treatment shows a statistically significant correlation with the degree of transmission blocking and resultant reduction in L3 recovery rate (*SI Appendix*, Fig. S2). Due to 6 wk of tetracycline treatment causing the most significant inhibition of mf to L3 development in *A. aegypti*, further experiments using transcriptomics, Western blotting, and exsheathment analyses were conducted using this regimen.

Transcriptomic Analysis of Control and 6–Wk-Treated mf. To further elucidate why depletion of *w*Bm results in transmission-blocking activity, RNA-sequencing studies were performed on three biological replicates from treated and untreated mf, leading to a minimum of 137 million trimmed, unaligned reads per replicate. Depletion of the *w*Bm population in this sample was confirmed via qPCR of the single-copy gene *wsp* normalized to nematode *gst*, which showed a 95.7% *w*Bm population depletion. After aligning these reads to the *B. malayi* genome, differential expression analysis identified a total of 1,079 genes (out of a total of 11,535) that showed statistically significant differential expression (determined by a false discovery rate [FDR] of <0.05), with 518 showing up-regulation and 592 showing down-regulation. The magnitude of fold change ranged from −2.6 to 5.0 on a $\log_2$ scale.

It was noted that a cluster of the most statistically significant differentially expressed genes were down-regulated (*SI Appendix*, Fig. S3). A selection of these included the serine–threonine never-in-mitosis protein kinase 8 (NEK8; XM_001902572.1) as well as genes encoding cysteine proteases (XM_001891887.1, XM_001892670.1, and XM_001892669.1) and a serpin-containing protein (XM_001896612.1). The NEK8 protein is believed to play a role in the initiation of mitosis and control of the cell cycle (25), with related proteins in *Caenorhabditis elegans* believed to play a role in successful molting (26). Meanwhile, the cysteine proteases are believed to play a variety of roles, including, but not limited to, digestion, immune response evasion, nematode molting, and embryo development (27–29).

To better summarize the functions of these statistically significant up- and down-regulated genes, Gene Ontology (GO) term enrichment analysis was performed using the TopGO package in R. Within the down-regulated set of genes, this analysis identified 26 GO terms associated with biological processes, 16 with cellular component, and 9 with molecular function identified as being statistically significantly enriched (cutoff *P* value of 0.01; Fig. 2*A*). By contrast, this analysis identified five GO terms associated with biological processes, three with cellular component, and two with molecular function within the up-regulated set of genes (cutoff *P* value of 0.01; Fig. 2*B*).

Of the 51 enriched GO terms within the down-regulated gene set, it was noted that the term proton transmembrane transport showed high statistical significance ($P < 0.001$). Several other related terms, such as proton transporting ATP synthase complex, terms related to the proton-transporting V-type ATPase complex, and proton-transporting ATPase activity were also identified across all three main GO domains (Fig. 2*A*). This indicates that transcripts that make up components of the V-type ATPase complex are significantly down-regulated. In addition to these terms related to proton membrane transport, GO term enrichment also identified two terms that were related to chitinase activity and chitin catabolic processes (Fig. 2*A*). Previous transcriptomics work had identified the GO term chitin catabolic process that was enriched during mf stages specifically (30). The authors identified four genes annotated with this term, and these same four genes were down-regulated in this study after *Wolbachia* depletion. Finally, two additional GO terms that were statistically significantly enriched within the set of down-regulated genes were associated with nematode larval development and multicellular organism development. Of the 10 enriched GO terms within the up-regulated gene set, it was noted that there were several terms related to transport across membranes, such as organic hydroxy compound transport, protein import into mitochondrial matrix, cation channel complex, and channel activity. The latter two terms may indicate that genes responsible for cation transport

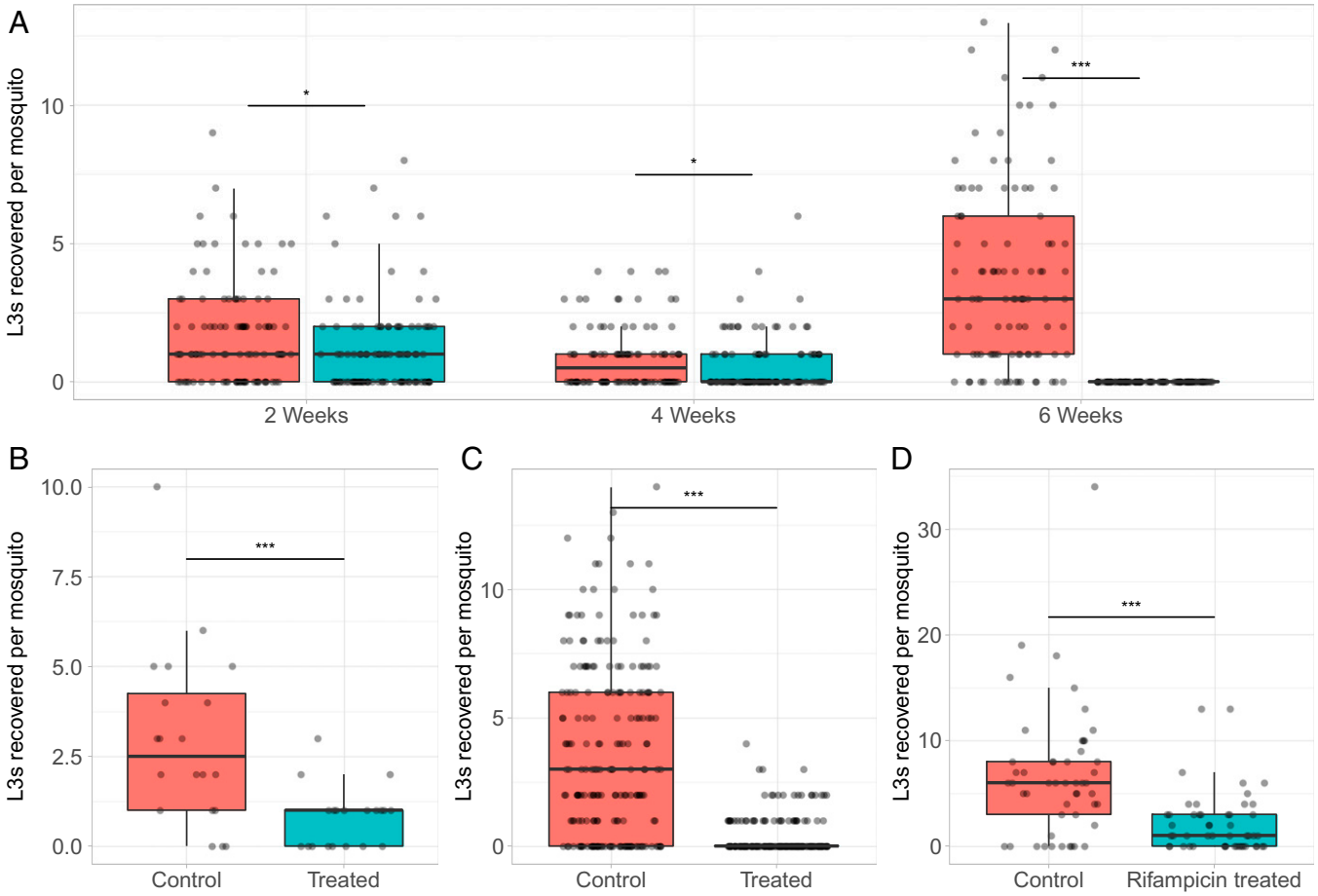

Fig. 1. Box and whisker plots illustrating L3 recovery rates 12 d after blood feeding the mosquito vector using a variety of treatment regimens. (*A*) Recovery rates of L3s from mosquitoes after mf were treated for 2 (n = 100 for control and treated groups), 4 (n = 108 and 100), or 6 (n = 100 for both groups) wk with tetracycline in vivo. Statistically significant reductions were observed for all three treatment durations compared to matched controls (*P < 0.05 for 2 and 4 wk, ***P < 0.001 for 6 wk). (*B* and *C*) Recovery rates of L3s from mosquitoes after in vivo treatment with tetracycline for 6 wk, with both repeats showing a statistically significant reduction in L3 recovery rate (***P < 0.001). For *B*, n = 20 for both control and treated groups, and *C* shows n = 200 for both control and treated groups. Due to the large number of mosquitoes to process, results for Fig. 1*C* were processed over 2 d, with no statistically significant differences observed between like-for-like treatments on the different days. (*D*) Recovery rate of L3s from mosquitoes after in vivo treatment with a suboptimal dose of rifampicin, a member of a different antibiotic class to tetracycline, again showing statistically significant reduction in L3 recovery rates (***P < 0.001); n = 50 for both groups. All statistical significance tests here were performed using the Mann–Whitney U test.

across membranes are enriched within the up-regulated set, possibly as a compensatory mechanism for down-regulation of the V-type ATPase.

Validation of results via qRT-PCR was performed on multiple targets, including four genes identified as differentially expressed that encode two kinases, one PAN domain–containing protein and one organic anion transporter. This is in addition to three genes encoding putative chitinases (*SI Appendix*, Fig. S4). Confirmation of the reduction in chitinase protein was further confirmed by Western blotting using antibodies raised against the chitinase enzymes of *O. volvulus*, which is known to cross-react with *B. malayi* mf chitinase (31). This showed a clear reduction in the amount of chitinase in 6–wk-treated *B. malayi* mf compared to controls after controlling for protein abundance with actin (Fig. 3 and *SI Appendix*, Fig. S5).

Exsheathment in mf Is Significantly Inhibited by *w*Bm Depletion. Following identification that chitinase transcripts were significantly down-regulated in *w*Bm-depleted mf, experiments were performed to assess their exsheathment rates. To identify mf that had successfully exsheathed, we used the lectin wheat germ agglutinin, which will bind to lectins on mf sheaths and fluoresce but will not bind to the exposed cuticle of exsheathed mf (32). These mf can then be counted, and the presence/absence of a sheath can be scored. We first validated this method via chemically inducing exsheathment of either control or treated mf (31, 33). Exsheathment rates of mf were quantified using a combination of fluorescent and brightfield microscopy (*SI Appendix*, Fig. S6). This showed statistically significant differences between the treated and control groups, with control mf showing a median exsheathment rate of 42.0%, while treated mf showed a median exsheathment rate of 2.0% (Fig. 4*A*).

Further experiments ex vivo were carried out using excised midguts of mosquitoes after feeding on blood infected with control or treated nematodes. Midguts of mosquitoes that had been fed with control mf showed a median exsheathment rate of 44.0%, while midguts fed with treated mf showed a median exsheathment rate of 6.3% (Fig. 4*B*). In addition to this reduced rate of exsheathment, mosquitoes fed with treated mf were noted to contain significantly higher numbers of mf than mosquitoes fed control mf (Fig. 4*C*). This observation indicates that a higher proportion of control mf was able to rapidly escape from the midgut. To confirm this observation, mosquitoes were again fed mf treated either with a 6-wk course of tetracycline or a suboptimal dose of rifampicin with matched controls for each. With tetracycline treatment, a statistically significant reduction in mf escape rate was observed (Fig. 4*D*). Suboptimal rifampicin treatment also showed a statistically significant reduction in mf escape rate (Fig. 4*E*).

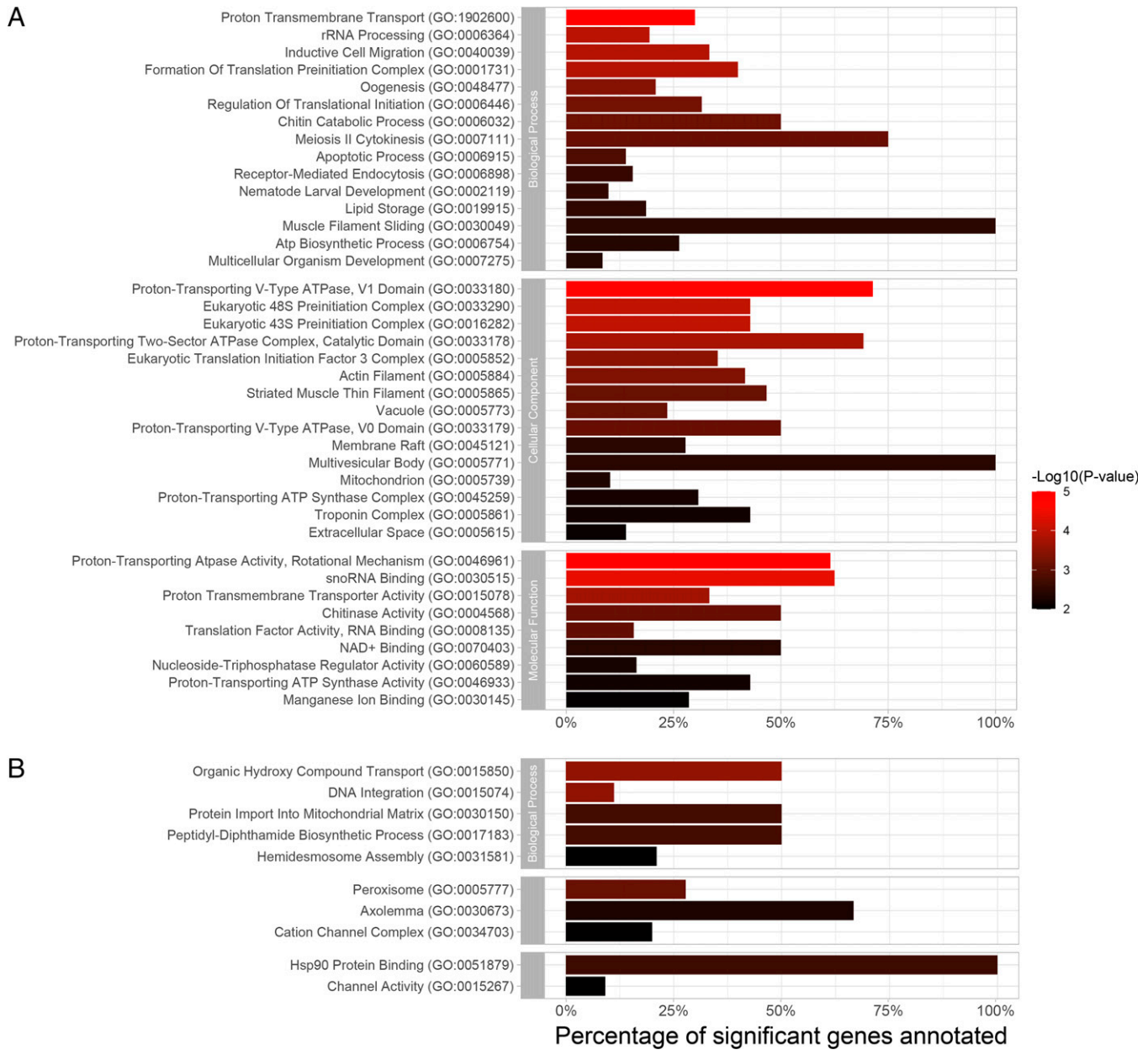

Fig. 2. Bar graphs of GO enrichment analysis of transcriptomics data. The graphs are separated into three segments based on the three main GO categories. Bars are colored on an increasing intensity of statistical significance, with intense reds indicating high significance. (*A*) Top 15 statistically significant GO terms enriched within the set of down-regulated *B. malayi* genes. (*B*) All top statistically significant GO terms enriched within the set of up-regulated *B. malayi* genes.

Supplementation of *Wolbachia*-Depleted mf with Recombinant Chitinase Restores Ability to Exsheath. To further establish whether the reduced rate of exsheathment in *Wolbachia*-depleted mf was due to a loss of chitinase activity, we supplemented cultures of treated and control mf with two commercially available recombinant chitinases derived from *Streptomyces griseus* and *Trichoderma viride*. These were first assayed for appropriate concentrations that could emulate the natural activity of mf chitinases, with an optimal concentration of 10 μg/mL being determined for both chitinases.

B. malayi mf were treated in vivo for 1 wk with the fast-acting anti-*Wolbachia* compound AWZ1066S, which is highly specific for *Wolbachia* (34), or matched controls collected from jirds. Subsequently, mf were then incubated in vitro with one of the two chitinases or a vehicle control. AWZ1066S treatment was shown to reduce *Wolbachia* load by 80.4%. As expected, treated mf that were not supplemented with chitinases showed a statistically significant reduction in exsheathment rates compared to untreated mf (Fig. 5). Chitinase supplementation was observed to effectively restore the exsheathment capability of treated mf, with no statistically significant differences compared to untreated mf regardless of the type of chitinase used.

Discussion

Anti-*Wolbachia* therapy has been previously shown to be effective at blocking nematode development in the mammalian host (3, 4) as well as inducing permanent sterility in adult female nematodes (35) through a block in embryogenesis and a subsequent natural attrition of circulating mf prior to macrofilaricidal efficacy (23, 36, 37). While existing circulating mf may persist for up to 1 y (36, 37), this study shows that anti-*Wolbachia* therapy has a much more immediate effect in blocking transmission of *B.*

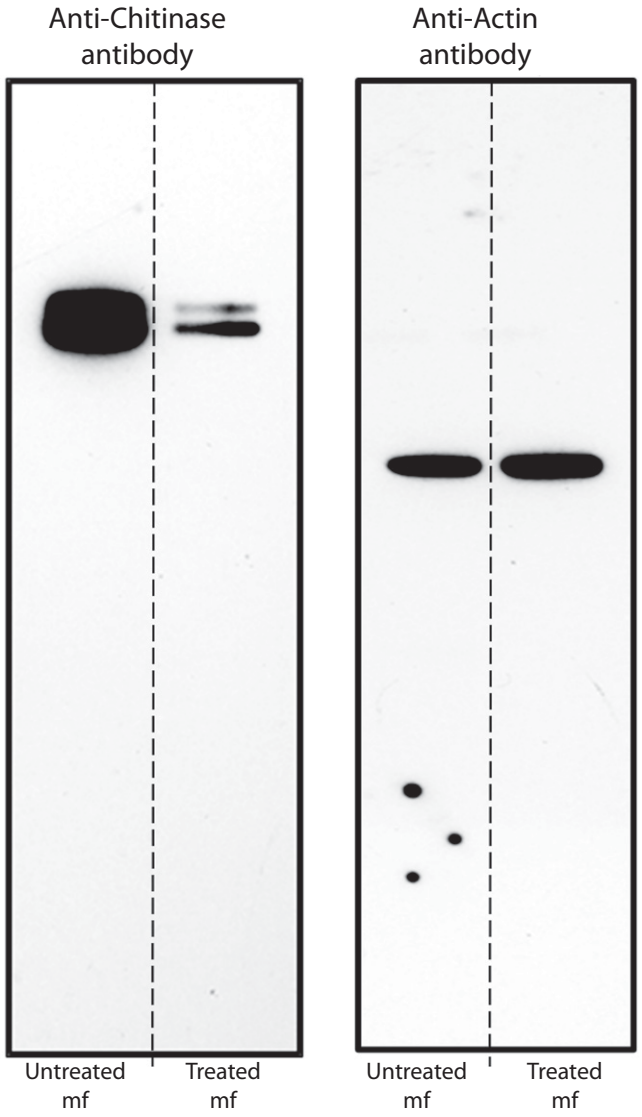

Fig. 3. Western blots of chitinase from mf either treated or untreated with antibiotics. Note how untreated mf showed a greater concentration of chitinase protein than treated mf. Samples were normalized for protein amounts using actin as a control.

malayi. Transmission blocking is observed within weeks of the start of anti-*Wolbachia* oral treatments in our lymphatic filariasis infection model. *Wolbachia*-depleted mf have a significantly reduced rate of exsheathment, which inhibits their ability to infect the mosquito vector, while retaining their motility and viability (16, 19, 38). The evidence for a dose-dependent reduction in *Wolbachia* loads with 2-, 4-, and 6-wk courses of tetracycline treatment correlates with the degree of transmission blocking. The same phenomenon was observed following partial *Wolbachia* depletion with rifampicin, a member of a different class of antibiotic to tetracycline, at a known suboptimal dose that reduced *Wolbachia* by 35% and development to L3 by 67%. This strongly supports *Wolbachia* depletion in mf as a key prelude to transmission-blocking effects, which are most pronounced (79 to 100% reduction of L3 recovery rates) following depletion of *Wolbachia* by >90%, in line with the empirical threshold for all anti-*Wolbachia* therapeutic outcomes (8).

Elucidating the reasons behind this block in transmission via transcriptomics identified a relatively restricted number of genes that showed differential expression. Of particular interest were genes related to chitinase activity, identified as part of the set of down-regulated genes. Several filarial nematodes maintain chitinase enzymes that are critical for nematode development (39) and are expressed at specific life cycle stages that vary based on the nematode species. Microfilarial chitinase is found only in sheathed filarial species and is critical for exsheathment in the mosquito vector (31). In *B. malayi*, this enzyme is highly expressed specifically during the mf stage of development (30) and is stored in the "inner body" organ and secreted shortly after uptake into the mosquito to facilitate exsheathment (31, 40, 41). This is in contrast to the filarial nematodes *O. volvulus* and *Dirofilaria immitis*, which express their chitinase genes during the L3 developmental stages, and with mf who exit the egg shell in utero (42, 43). Because of this, further investigations were performed to look at the effects of this reduced abundance of transcripts. Western blot analysis confirmed the reduction in chitinase abundance, which correlated with an inability to exsheath and escape from the mosquito midgut. Previous studies have indicated that the mf of *B. malayi* begin to escape the mosquito midgut 2 h after uptake, with exsheathment being a prerequisite biological process that occurs just before or during the escape process (12). In the absence of a recombinant chitinase from *B. malayi*, we sourced two commercial chitinase enzymes and used these to supplement cultures of *Wolbachia*-depleted and untreated mf, which restored the ability of *Wolbachia*-depleted mf to exsheath.

While our observations could apply to previous studies on sheathed filarial species (*B. malayi*, *B. pahangi*, and *L. sigmodontis* that showed reduced rates of L3 recovery [17–19]), they do not account for the different developmental impacts observed in species without a sheath, such as *O. volvulus* (20) and *D. immitis* (44, 45). Albers et al. (20) observed a different dynamic in the onset of larval development inhibition, which began 3 mo after the end of a 6-wk course of doxycycline and continued at 4 to 5 mo after treatment, with a reduced proportion of L3 larvae and an increasing proportion of L1 and L2 larvae. In the veterinary heartworm *D. immitis*, McCall et al. (44, 45) showed that mf from doxycycline-treated dogs are able to develop to the infective L3 stage within their mosquito vector, but, importantly, these L3 are unable to develop to adult infections in the dog host. These nematodes are known to develop within the Malpighian tubules of the mosquito vector, and do not penetrate through the midgut. Such differences in tissue tropism that different filarial species have within the insect vector could be a contributing factor to the differences in how nematode development is affected after *Wolbachia* depletion. As such, these past findings imply that *Wolbachia* depletion may have a significant effect on nematode development within, or after, the vector stages, regardless of the presence of a sheath or not. Indeed, it was observed that some of the most statistically significantly down-regulated genes were associated with genes encoding cysteine proteases. These proteases have previously been implicated in nematode development (27, 28), particularly with embryogenesis, where RNA interference studies resulted in structural abnormalities in embryos and altered intrauterine contents (29). These proteases may play roles in impacting mf development to the infective L3 stages and may also be important for unsheathed filarial nematodes in invading the insect vector. Additionally, these cysteine proteases have been linked to the maintenance of *Wolbachia* density in the host, with RNA interference of *B. malayi* cysteine proteases resulting in reduced *Wolbachia* levels (46), although in our study it is the depletion of *Wolbachia* that results in down-regulation of these genes. Furthermore, transcriptomic analysis

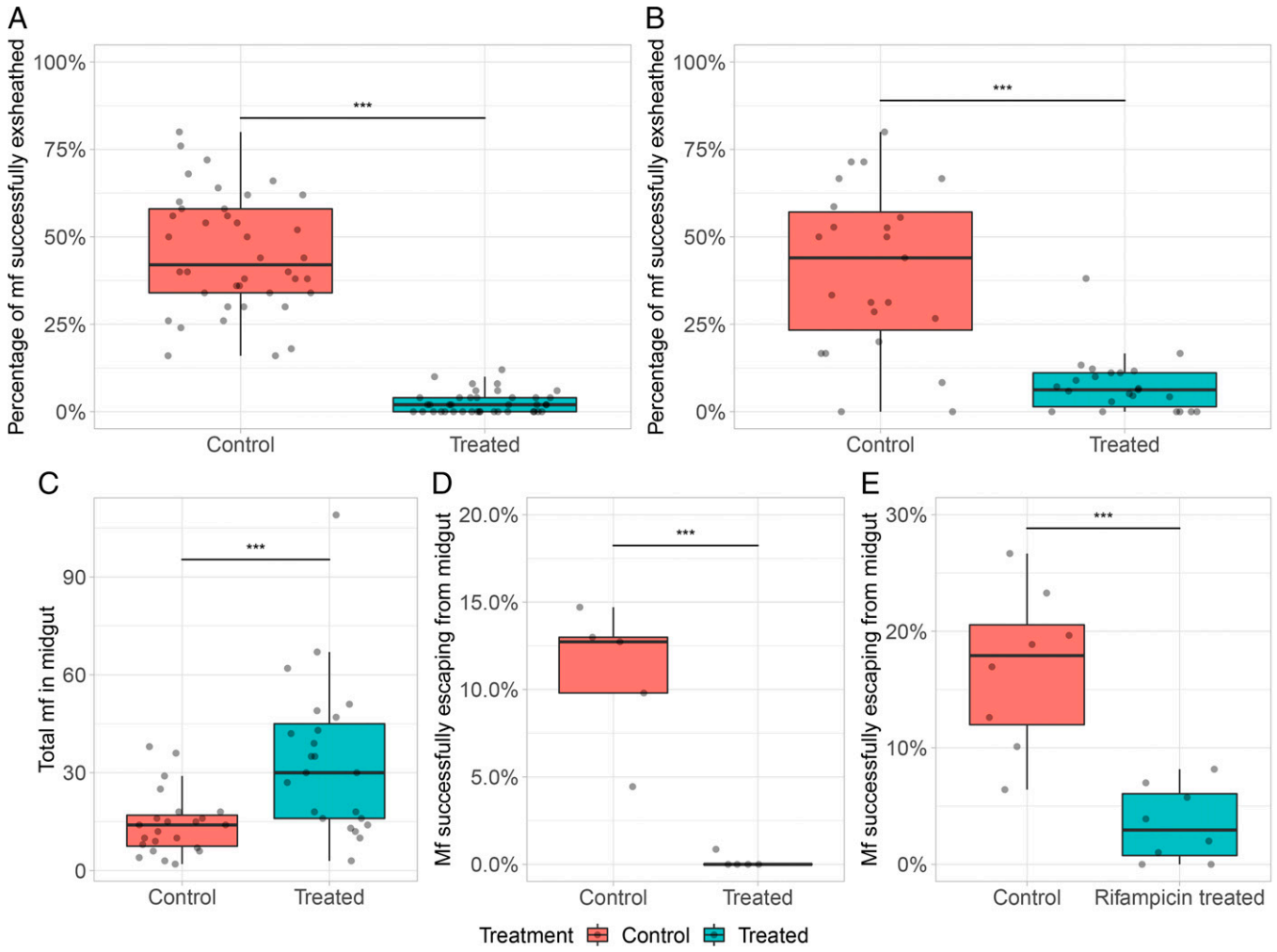

Fig. 4. Box and whisker plots comparing mf exsheathment, midgut tallies, and midgut escape rates based on different treatments and within different conditions. (*A*) Percentage of mf that have successfully exsheathed in vitro after chemical inducement, with control mf showing a statistically significantly higher exsheathment rate ($n = 40$ replicates, 50 mf per replicate, ***$P < 0.001$). (*B*) Percentage of mf that have successfully exsheathed 2 h after uptake into the mosquito vector via a blood meal, with median exsheathment rates of 44.0% and 6.3% for control and treated groups, respectively ($n = 23$ replicates, ***$P < 0.001$). (*C*) Total counts of mf that remained within the mosquito midgut, showing a statistically significant increase in number of treated mf present compared to control ($n = 23$, ***$P < 0.001$), with medians of 14 and 30 mf per midgut for control and treated groups, respectively (***$P < 0.001$,). (*D*) Percentage of mf that successfully escape from the mosquito midgut after tetracycline treatment showing a statistically significant reduction ($n = 5$, ***$P < 0.001$), with median escape rates of 12.7% and 0.2% for control and treated groups, respectively. (*E*) Percentage of mf that successfully escape the mosquito midgut after suboptimal treatment with rifampicin showing a statistically significant reduction ($n = 8$, ***$P < 0.001$), with median escape rates of 17.9% and 2.9% for control and treated groups respectively. All statistical tests were performed used a negative binomial generalized linear model.

as performed during this study had identified several GO terms related to nematode development as enriched within the down-regulated gene set. Further transcriptomic analysis on *Wolbachia*-depleted unsheathed mf, such as *O. volvulus* and *D. immitis*, would be useful to compare with the analysis on *B. malayi* presented here.

In addition to terms related to chitinase activity, multiple terms related to proton transport, and more specifically the V-type ATPase complex, were identified within the down-regulated gene set. This complex is known to be localized in the membrane of vesicle compartments and utilizes ATP to pump protons across membranes and against proton gradients (47, 48). One primary use of this functionality is to generate acidic environments within vacuoles, which in turn allows for the processing and/or optimal function of enzymes (49) as well as for protein degradation and pathogen clearance (50, 51). Considering *Wolbachia*'s localization within host-derived membrane-bound vacuoles (52) and their low population levels during this developmental stage, it may be possible that the nematode host actively controls *Wolbachia*'s population level via this mechanism. Following this, antibiotic depletion of *Wolbachia* may contribute to a reduced rate of transcription for components of the V-type ATPase. In a similar vein, chitinase enzymes in other organisms, such as bacteria (53), unicellular parasites (54), and eukaryotes (55), have been described as operating at highest efficiency at low pH levels. Others have been described as being synthesized as an inactive zymogen form that requires proteolytic cleavage to be activated (56), in turn typically performed by enzymes that require low pH levels (49). It is possible that the chitinase of *B. malayi* may share similar properties, and, as such, reduced transcription of V-type ATPases may have further knock-on effects on the ability to mature filarial nematode chitinases. Investigation into the role of these V-type ATPases in either regulating *Wolbachia* populations or their role in chitinase activity in filarial nematodes will be an interesting avenue for future research.

Overall, the results presented here illustrate a previously unexpected role in *Wolbachia*'s relationship with the nematode host. Specifically, this is through the down-regulation of chitinase in mf-depleted *Wolbachia*, leading to an inability to exsheath and escape from the mosquito midgut and thus

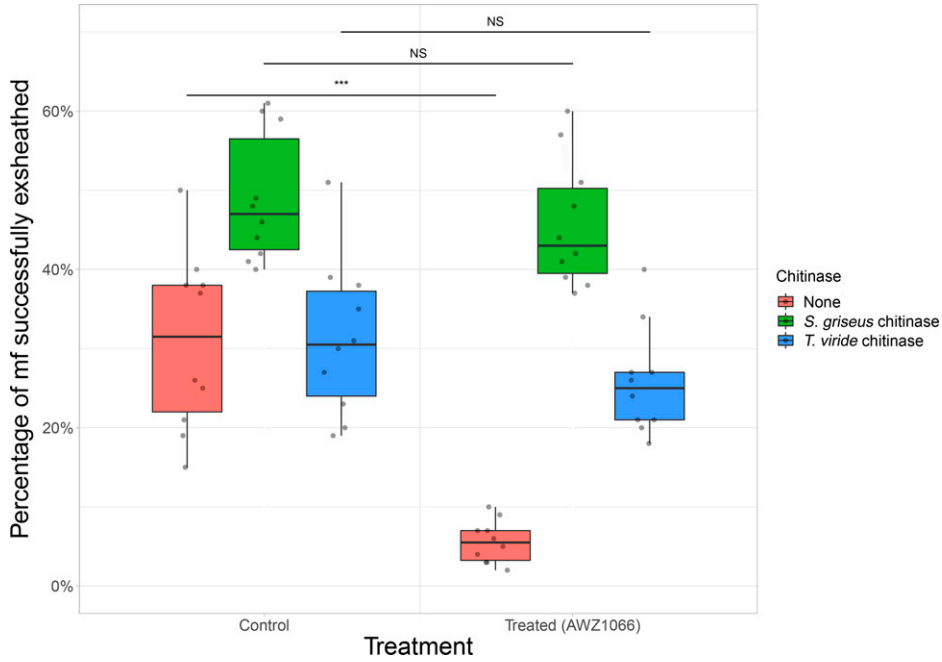

Fig. 5. Box and whisker plots comparing mf exsheathment rates after chemical inducement in vitro after in vivo treatment with the anti-*Wolbachia* compound AWZ1066 with or without supplementation with recombinant chitinases. Supplementation of untreated mf with recombinant chitinase showed either a statistically significant increase (with *S. griseus* chitinase) or no significant changes in exsheathment rate (*T. viride* chitinase). Treated mf were noted to have significantly reduced exsheathment rates when no chitinases were supplemented (***$P < 0.001$). However, treated mf exsheathment rates were effectively recovered after supplementation with chitinases compared to untreated mf, with no significant differences in exsheathment rate between control and treated mf after like-for-like chitinase supplementation ($P > 0.5$). The experiment used $n = 10$ replicates per condition, with 100 mf per replicate; NS, not significant.

blocking further larval development. Knowledge of such transmission-blocking activity is an important addition to the broad range of biological processes dependent on *Wolbachia* symbiosis and further emphasizes the broad range of benefits that anti-*Wolbachia* therapy can deliver (8). Furthermore, such results have significant implications for future modeling studies as well as deployment of future resources to combat these parasitic nematodes. Specifically, epidemiological models typically categorize future treatments as either "macrofilaricide only" or "macrofilaricide + microfilaricide" (57, 58). These models are used to predict the impact of microfilaricide implementation in the context of population-wide effects and the number of delivery rounds required to reach transmission break points. To date, most anti-*Wolbachia* treatments have been considered more aligned with macrofilaricide-only pharmacological agents (57, 58). The reasoning behind this is that the delayed impact on removing mf via disruption of embryogenesis is predicted to be most similar to drugs that kill adult worms without directly affecting circulating mf, giving the latter an opportunity to develop and infect new patients. With the results presented from this work, we would contend that anti-*Wolbachia* drugs should be reappraised as having a macrofilaricide + microfilaricide pharmacological profile. Our reasoning is that *Wolbachia*-depleted mf empirically demonstrate a profound 79 to 100% block in transmission compared to undepleted controls. This should also apply to unsheathed species, as L3 derived from *Wolbachia*-depleted mf are unable to develop to adult infections in the definitive host (20, 45). Thus, *Wolbachia* depletion from mf shows an ability to exert both immediate and long-term suppression of transmission, with the added benefit of avoiding the side effects of more traditional microfilaricide treatment (21, 24).

Methods

See *SI Appendix, Supplementary Methods* for full details on all experimental procedures used.

Parasite Lifecycle Maintenance and Study Design. Male Mongolian jirds obtained from Charles River were infected by intraperitoneal injection with 400 *B. malayi* L3-stage larvae and maintained at the Liverpool School of Tropical Medicine for experimental use. Patently infected jirds were administered the antibiotic of choice (tetracycline hydrochloride, rifampicin, or AWZ1066) for between 1 and 6 wk depending on the experimental protocol. Mf were recovered by peritoneal lavage of jirds at the end of each treatment period. Mf were then purified using a PD10 column and size-exclusion chromatography (Amersham), and concentrations were quantified via microscopy. Mf that were to be used for RNA sequencing were immediately stored in RNAlater (Ambion) after purification.

Mosquito Maintenance and Infection. *A. aegypti* female mosquitoes were reared from eggs obtained from the NIH Filariasis Research Reagent Resource Center. After sucrose starvation, mosquitoes were fed on human blood (blood bank) inoculated with mf (maximum concentration of 20,000 mf/mL of blood) from either treated or control jirds using the Hemotek feeding apparatus (Discovery Workshops) covered with parafilm. Blood-fed females were transferred to a fresh cage, and 13 d after feeding, up to 200 were then dissected in phosphate-buffered saline, with the number of infective stage larvae (L3) counted.

Assaying mf uptake into the mosquito was done using 50 females fixed in Carnoy's fixative (two parts ethanol and one part glacial acetic acid) immediately after feeding. These samples were processed as described by Arzube and Shelley (59) with slight modifications.

Quantification of *Wolbachia* Depletion in mf and L3 Nematodes. DNA was extracted from mf (8,000 per replicate) and used to quantify *Wolbachia* loads using the *Wolbachia* surface protein (*wsp*) gene copy number and normalized to the nematode glutathione *S*-transferase (GST) gene by qPCR (10). Primer sequences for all amplified genes are included in *SI Appendix*, Table S1, with *SI Appendix, Supplementary Methods* containing full thermocycling details.

Exsheathment Assays of *B. malayi*. The in vitro mf exsheathment assay was adapted from that of Devaney and Howells (33). For chitinase replacement experiments, we first measured the chitinase activity of mf using the protocol described by Amick et al. (60) and used this to construct a standard curve to measure commercial chitinase activity (Sigma-Aldrich, C6137 and C8241, respectively) against mf chitinase activity.

Nucleic Acid Extraction for RNA Sequencing. Mf in RNAlater (Ambion) were first diluted via adding an equal volume of phosphate-buffered saline to the sample, and centrifuged at 2,000 × *g* at 4 °C for 10 min to pellet mf. The pelleted mf were then processed using the TriZol–chloroform isolation procedure after homogenization using a bead beater (Minilys, Bertin Instruments). The remaining suspension was reserved for Western blotting assays. Isolated RNA was then purified using Exiqon's miRCURY RNA isolation kit, cell & plant, in accordance with the manufacturer's instructions. Extracted RNA was then assayed for quality and rRNA depleted using Terminator 5′-phosphate–dependent exonuclease (Epicentre), in accordance with the manufacturer's instructions, before being processed using a commercial purification kit (Zymo, RNA Clean & Concentrator-5) following manufacturer's instructions (*SI Appendix, Supplementary Methods*). These samples were then assayed for RNA concentration before being sent to the University of Liverpool's Centre for Genomic Resources for RNA sequencing using an Illumina HiSeq 4000. Raw fastq files were trimmed for adapters and quality, with a minimum quality window score of 20, and reads of less than 20 bases were discarded.

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. Protein extracts were fractionated using Invitrogen Bolt 12% Bis-Tris, 1.0-mm mini protein gels. Separated proteins were electrophoretically transferred to nitrocellulose, and the membranes were blocked for 1 h at room temperature by incubation with rabbit antisera to *O. volvulus* Chitinase-1 (Ov-CHI-1) (42) at a 1:5,000 dilution in blocking buffer. Goat anti-rabbit IgG (heavy and light chain) horseradish peroxidase conjugate (Cell Signaling, 1:5,000 dilution) was used to localize antibody/antigen complexes. Blots were developed by incubation with an enhanced chemiluminescence (ECL) SuperSignal chemiluminescent substrate horseradish peroxidase system (Pierce, Thermo Fisher Scientific) for 5 min and then exposed to X-ray CL Xposure films (Thermo Fisher Scientific); signals were revealed using a Photons Developer Instrument. For Western blots of actin loading control, the nitrocellulose membranes used above were stripped using Restore Plus Western blot stripping buffer (Thermo Fisher Scientific) for 15 min at room temperature and washed three times with Tri-saline Tween buffer, blocked again, and reprobed with anti-β-actin (Santa Cruz, 1:3,000 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) (61). The aligned RNA sequence expression data were quantified using the program FeatureCounts (v1.5.0-p3) (62) and used as input into the program edgeR (v3.30.3) (63) for differential gene expression analysis. Differential expression used a pairwise comparison, with untreated nematodes acting as the reference point against nematodes treated with tetracycline for 6 wk. GO analysis was conducted via separating the list of significantly differentially expressed genes into up- or down-regulated genes and comparing against the entirety of *B. malayi*'s genome as a "background" set using the program TopGO (v2.40.0) (64).

Data Availability. RNA-sequencing datasets have been deposited in NCBI under BioProject ID number PRJNA772674. Supplementary data tables (Excel format) have been deposited at Figshare under DOI numbers 10.6084/m9.figshare.16847671, 10.6084/m9.figshare.16847707, and 10.6084/m9.figshare.16847719. All other study data are included in the article and/or *SI Appendix*.

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