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The development and utilization of an in vivo RNA interference protocol to elucidate gene functions and identify potential drug targets in the filarial nematode *Brugia malayi*

Chuanzhe Song
Iowa State University

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The development and utilization of an *in vivo* RNA interference protocol to elucidate gene functions and identify potential drug targets in the filarial nematode *Brugia malayi*

By

Chuanzhe Song

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Program of Study Committee:

Michael J. Kimber, Major Professor

Timothy A. Day

Steve Carlson

Lyric Bartholomay

Thomas J. Baum

Iowa State University

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To my family, wife Hannah, and daught Kathryn for their support and encouragement

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ABSTRACT

Since its first characterization in 1998 in the free-living nematode *Caenorhabditis elegans*, RNA interference has been considered a powerful reverse genetics tool to investigate nematode biology. But to date, current RNAi protocols for parasitic nematodes have proven unreliable and inconsistent.

We established an alternative RNAi protocol targeting the filarial nematode *Brugia malayi* in-host whereby the parasites are exposed to RNAi triggers as they develop within the intermediate host, the mosquito *Aedes aegypti*. Using this *in vivo* RNAi protocol, we successfully quantified the suppression of five *B. malayi* genes associated with known or putative drug targets. Administration of a random exogenous dsRNA resulted in no phenotypic abnormalities, demonstrating the specificity of the *in vivo* RNAi protocol.

In vivo RNAi experiments revealed that the cathepsin L-like cysteine protease *Bm-cpl-1* plays a role in worm migration, survival and overall health. Suppression of *Bm-cpl-1* resulted in inhibited worm motility and capacity to properly navigate to the head for transmission thus abolishing the transmission potential of the worm. *Bm-cpl-1* suppression also affected worm development as evident by a reduction in worm length post *Bm-cpl-1* suppression.

The potential of the *in vivo* RNAi protocol to aid drug development was further validated using four known or putative drug targets of interest: β -tubulin (*Bm-tub-1*), a glutamate-gated chloride channel alpha subunit (*Bm-GluCl- α 3A*), a G protein-coupled acetylcholine receptor (*Bm-gar-2*), and a FMRamide-like peptide (*Bm-flp-21*). Suppression of these genes resulted in a combination of decreased motility, worm survival, migration, and worm physiological

abnormalities verifying or validating each of the four drug targets potential for anthelmintic drug development.

CHAPTER 1. INTRODUCTION

I. BRIEF OVERVIEW OF THE CURRENT SITUATION IN PARASITIC NEMATODES

Parasitic nematodes pose major economic and health problems to humans, animals, and plants globally. It is estimated that over 2 billion people, mainly in the developing regions, are infected with one or more species of parasitic nematode [1]. Human parasitic infection leads to varying degrees of morbidity for the infected individual. In the case of the filarial nematode *Onchocerca volvulus*, the circulating microfilariae beneath an infected individual's skin can cause debilitating adverse effects ranging from itching to severe dermatitis and vision loss [2]. Persistent infection with gastrointestinal parasitic nematodes in humans is associated with chronic and long term health problems such as anemia, stunted growth, nutritional deficiency and cognitive deficiencies [3, 4] which can lead to lifelong adverse effects. Pre- and school aged children tend to harbor the greatest parasite burden than any age group [1]. As a result, the negative health impact for these children can be chronic and long-lasting [1]. For example, hookworm infection in children can lead to reduced memory and cognition [4] resulting in diminished educational performance, school attendance [5], and ultimately a reduction in the future wage-earning capacity [6].

Aside from the detrimental effects parasitic nematodes have on human health and quality of life, they also inflict a heavy toll on economics. For example, the parasitic roundworm *Ascaris suum*, which inhabits the pig intestine, results in an estimated \$155 million economic loss annually in the US swine industry alone [7, 8]. In agriculture, crop loss due to plant parasitic nematodes is estimated to be over \$100 billion annually [9].

The health implications associated with parasitic nematodes are significant. In the absence of effective vaccines to prevent and guard against infection in both human and animals, the burden of controlling parasite infection and alleviating symptoms falls on chemotherapeutic drugs [10]. Although a variety of anthelmintic drugs are currently available such as ivermectin, benzimidazoles, and levamisole [11], in comparison to the number and diversity of parasitic nematodes, the available drugs are few.

To further complicate the matter, the development of new anthelmintic drugs is slow in coming. Of the 1,556 new chemotherapeutic compounds brought to market between 1975 and 2004, only two, albendazole and ivermectin, were developed to treat human parasitic nematodes [12, 13].

The limitation mentioned above is evident in lymphatic filariasis for which available chemotherapeutic options are limited to two drugs, ivermectin and diethylcarbamazine (DEC) [14]. While both drugs are efficient in eliminating the microfilariae from the host body, neither can eliminate the adult parasite from the host [15 - 17].

Intensive use of the limited drug types in veterinary health have led to instances of drug resistance with resistance reported for broad spectrum anthelmintics such as albendazole, levamisole, and ivermectin as well as to narrow spectrum anthelmintics such as closantel [18]. Although to date no anthelmintic drug resistance has conclusively been documented in human health, the potential is a continual concern. For example, the nicotinic acetylcholine receptor targeted drug, levamisole, was introduced in 1970 and resistance was reported in 1979 in *Trichostrongylus colubriformis* and *Ostertagia circumcincta* [10, 301]. Similarly, one of two

available drugs for filarial worm control, ivermectin, was introduced in 1981 and resistance was reported seven years later in 1988 in *Haemonchus contortus* [10, 302].

Similar situations have been documented for the various other wide spectrum anthelmintics such as benzimidazole and a relatively recently introduced macrocyclic lactone, moxidectin [10].

The limited number and effectiveness of available anthelmintic drugs against parasitic nematodes, along with the potential for drug resistance, brings to light the urgent need for new and more effective anthelmintic drugs. But the development of new and more efficient anthelmintic drugs requires a thorough understanding of both parasitic nematode biology and the mode of action of currently available drugs.

The paucity of understanding of the mechanism of action for anthelmintic drugs is evident in the case of the filarial worms where the pharmacological mode of action for ivermectin and DEC is not fully understood [19]. Although ivermectin has been found to act as an agonist at invertebrate glutamate-gated chloride channels [20], paradoxically the *in vitro* treatment of cultured microfilariae with ivermectin at pharmacologically relevant concentrations did not produce an effect consistent with our understanding of this channel [21]. Thus the effect of ivermectin on filarial worms may not be primarily a result of action mediated through the glutamate-gated chloride channels as is more likely the case in adult and larvae of other gastrointestinal and lung worms that exhibited inhibition of both body motion and feeding when treated with the drug [19]. Similarly, the mode of action for DEC after over 60 years of use remains a mystery [19]. To date no candidate receptor for DEC has been found [19]. In 2005, McGarry *et al.* found pharmacological and genetic evidence that implicates DEC interaction with

both host arachidonate- and nitric oxide- dependent pathways in eliciting the anti-filarial effect, but how DEC interacts with these pathways remains unknown [22].

In conclusion, parasitic nematodes inflict debilitating and potentially lifelong morbidity and mortality on infected individuals as well as cause global economic losses [8, 9]. With the limited quantity and effectiveness of available drugs, new drugs need to be developed. In order for the efficient development of new and novel anthelmintic drugs to combat parasitic nematode development, better understanding of parasitic nematode biology and pharmacology of current anthelmintic drugs is needed. One family of parasitic nematodes that is in need of more potent and efficient anthelmintic chemotherapeutic agents is filarial nematodes.

II. BRIEF INTRODUCTION TO THE LYMPHATIC FILARIASIS PROBLEM

Lymphatic filariasis (LF) is a disease caused by filarial nematodes, primarily *Wuchereria bancrofti* (90% of cases) and *Brugia malayi* (10% of cases) [23], which are transmitted through the bite of infected mosquitoes. In 1997, the World Health Organization identified lymphatic filariasis as the second leading infectious cause of permanent and long-term disability [24], and the World Health Assembly's International Task Force for Disease Eradication identified lymphatic filariasis as one of six potentially eliminable diseases [25].

It has been estimated that 120 million people, or approximately 2% of the global population, are infected with LF and 1.2 billion at risk of infection in 81 endemic countries [24, 26]. LF is a health problem that has accompanied humans throughout history [27] causing crippling morbidity and debilitating stigmatization that perpetuates socioeconomic instability.

A. Pathogenesis of Lymphatic Filariasis

Filarial worm infection causes damage to the individual's lymph system and induces lymph vessel dilation [28-35]. Damage to the lymphatic system is caused by a combination of mechanical [36, 37] and induced [28-35] damage. The subsequent release of microfilaria larvae further stimulates lymph vessel dilation as the host innate immune response is triggered by phagocytosis of degenerating microfilariae [28, 32, 35]. Furthermore, antigen from the filarial worm endosymbiont *Wolbachia* also contributes to the pathogenesis of lymphatic filariasis through stimulation of host immune responses [38].

Despite damage to the lymph system, most infected individuals do not develop clinical symptoms and in many cases, these individuals are unaware that they are infected [23]. In general, these individuals tend to have high parasitemia (as measured by circulating microfilariae,

the stage of the parasite that is taken up by the vector) and a down-regulated immune response associated with the significant lymph damage, despite a lack of visible clinical symptoms [39-43].

Among infected individuals in endemic regions, approximately 12.5% exhibit clinical symptoms, primarily lymphedema [44], where the lymph vessels become dilated and inefficient at transporting lymph leading to fluid extravasation into the surrounding tissues [38]. Lymphedema can occur in the legs and arms of the infected individual, but it can also occur in the breast as well [45]. In addition to the accumulation of fluid in the arm, leg, breast and/or scrotum resulting from an impaired lymph system, bacterial infection of the skin and lymph system readily takes place resulting in pachyderma, the thickening and hardening of the affected skin [52].

B. Socio-Economic Impact of Lymphatic Filariasis

It is estimated that 5 million Disability Adjusted Life Years (DALY) are lost yearly to lymphatic filariasis [53]. The annual economic loss due to LF disability is estimated to be approximately \$1 billion but these estimates are likely underestimated [53]. A study comparing the productivity of male weavers in rural India determined that a 27.4% productivity loss is attributed to chronic LF [54].

In addition to the economic loss associated with chronic filarial worm infection, the infected individual also faces significant social stigmatization. Social shame, fear, and embarrassment are common psychological traumas endured by infected individuals [55]. Individuals report suffering from shame and suicidal thoughts, and marriages are often devoid of physical intimacy [55].

In conclusion, LF causes significant morbidity as a result of damage to the lymph system of infected individuals, and significant economic losses to the community. In addition, for the 40 million infected individuals that exhibit clinical symptoms of lymphatic filariasis such as elephantiasis and hydrocele, social stigmatization is a daily burden.

C. Biology / Life Cycle of *Brugia malayi*

The development of juvenile microfilaria to the infectious L3 stage takes place within the mosquito intermediate host while maturation and propagation occur in human and a few other mammalian definitive hosts [56]. There are several genera of mosquitoes that are natural vectors for the filarial worms that cause LF, including: *Aedes*, *Anopheles*, *Culex*, and *Mansonia* [56].

Larvae Development within the Mosquito Intermediate Host

Susceptible mosquitoes become infected with filarial worms by ingesting juvenile microfilariae during the blood meal. Once ingested, the microfilariae sense temperature and environmental changes [57-60] and quickly penetrate the midgut epithelium such that they gain access to the hemocoel within hours of ingestion [25]. Once the microfilariae penetrate through the midgut epithelium, they migrate in the hemolymph toward the thoracic musculature [56]. At the thoracic musculature, the microfilariae penetrate and take up harborage within indirect flight muscle cells and differentiate into non-feeding sausage-shaped L1 larvae [56, 62]. Within the infected indirect flight muscle cell, the parasite undergoes the L1 to L2 molt. The L2 stage parasites actively ingest mosquito cellular components and multiply several times in size before undergoing one more molt into the L3 infectious stage and exit the cell (Figure 1) [61]. Filarial worm parasites within the mosquito host grow several times in size from a microfilaria of ~200 μm to the infectious L3 larva of ~ 1,350 μm in length [62]. The development from microfilariae

to L3 infectious parasite within the mosquito takes approximately 10 to 12 days depending on environmental factors such as temperature [56].

Ultrastructural studies of infected *Aedes* flight muscle cells reveal that the cell undergoes nuclear enlargement, indicative of a repair response, while the parasite develops within the cell [61]. But once the developing larvae mature to the L3 stage and exits the cell, the severely compromised cell undergoes degeneration [61]. Upon exiting the indirect flight muscle cell, the L3 infectious parasites migrate to the head and proboscis of the mosquito where they wait until the mosquito takes a blood meal [56]. During the blood meal, the L3 will exit by penetrating through the labellum of the mosquito proboscis onto the host skin residing within a drop of mosquito hemolymph [56]. The infectious L3 parasites then infect the human/mammalian host through entry by the mosquito puncture hole [56, 62].

Filarial Worm Development within the Mammalian Host

Within the human/mammalian host, the infectious L3s migrate to the lymphatic vasculature where they undergo two additional molts into L4 then adult worms [56]. Within the lymphatics, the mature male and female filarial worms, whose life span is 5-15 years, mate. The mated female worms then produce large quantities of immature progeny microfilariae that are released into the blood stream for a new mosquito host to take up and perpetuate the transmission cycle (Figure 1) [63].

In summary, microfilariae released by adult female filarial worms are taken up by mosquitoes during a blood meal. Microfilariae develop into infectious L3 in the course of roughly two weeks within the mosquito intermediate host. Upon blood feeding, the infectious L3 exit through the proboscis of the mosquito and enter the mammalian host using the puncture hole

made by the mosquito. Once inside the mammalian host, worms develop into the adult lymphatic filarial worm and reside within the host lymphatic system for upwards of 15 years. Within the host lymphatic system, mated female filarial worms will produce progeny and perpetuate the cycle.

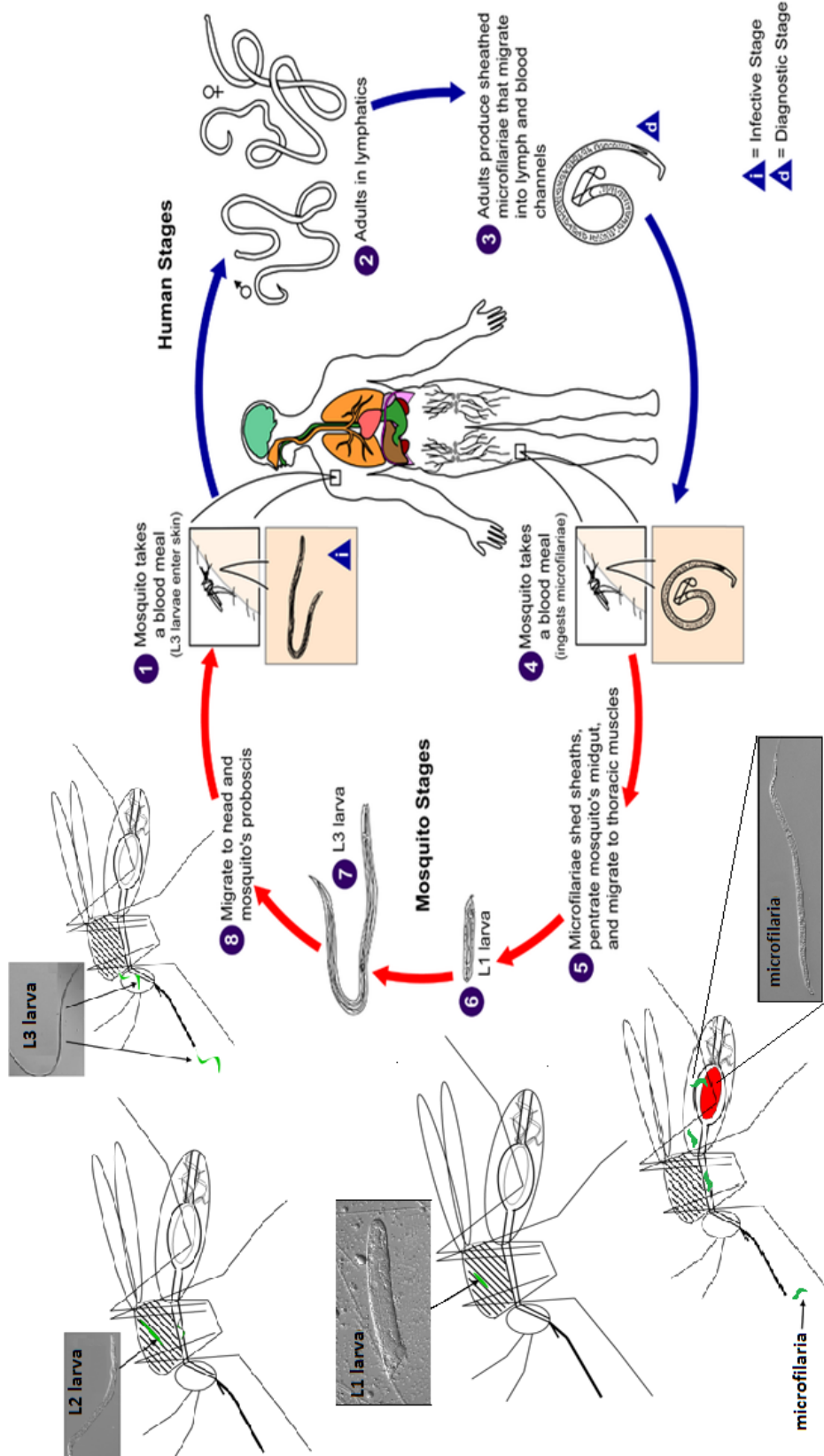


Figure 1: *Brugia malayi* life cycle. (1) The infective mosquito carrying infectious L3 *Brugia malayi* larvae (~1,350 μm in length) takes a blood meal. The L3 larvae burrow out of the mosquito's proboscis onto the host skin and reside in a pool of mosquito hemolymph. The L3s enter the host via the mosquito puncture hole and migrate to the host lymphatic system where they undergo two additional molts into L4 and finally adult parasites. (2) Within the lymphatics, the male and female *Brugia malayi* mate and the female produce microfilariae (mf) of approximately 200 μm in length. (3) The mf migrates into the lymph and blood stream. (4) An uninfected mosquito takes a blood meal from a microfilariaemic individual taking up the mf. (5) The ingested mf rapidly penetrates through the midgut epithelium within 2 hours and migrates toward the thoracic musculature. (6) Microfilariae penetrate and take harborage within the indirect flight muscle cell and differentiate into non-feeding L1 larvae 6 hrs to 3 days post mosquito infection. The L1 larvae molts into the L2 larvae, which actively feed off intracellular component 3 to 5 day post mosquito infection. (7) The L2 larvae molt into infectious L3 larvae. (8) The developmentally arrested L3 larvae exit the indirect flight muscle cell and migrate into the head and proboscis of the host mosquito starting as early as 8 days post infection depending on environmental condition. Images from Erickson, *et al.* (2009) [62] and CDC (2010) [76]. Images reproducible under the Open Access Creative Commons Attribution License.

D. Global Effort in the Control of Lymphatic Filarial Infection

Despite decades of effort and promising research results [64-69], a vaccine against a filarial worm has yet to emerge. Vector controls have been considered and proven effective in certain geographically isolated locations such as the Solomon Islands where both malaria and filarial worm transmission have been reduced, with prevalence of microfilaremia reduced to less than 2% [70]. Unfortunately, vector control is both labour intensive and costly [71]. The rapid development of insecticide resistance further challenges the success of vector control programs [72]. Despite its draw backs, vector control when accompanied with chemotherapeutic drug treatment have been shown to be significantly more effective at interrupting transmission than to vector control alone [300]. Thus it would appear chemotherapy will continue to play an integral role in transmission control [68, 300].

Chemotherapeutic Efforts

Prior to the Global Programme to Eliminate Lymphatic Filariasis (GPELF) and associated Mass Drug Administration (MDA) programs, chemotherapeutic control of lymphatic filarial worm transmission for mass population in endemic regions relied on the use of 0.1 to 0.4% diethylcarbamazine (DEC) fortified salt. This concept was first introduced in 1967 and employed in Brazil when Hawking and Marques found that DEC is stable even after cooking [73]. This method has since been successfully carried out in parts of China, Taiwan and India [74]. In one county in Shandong, China, the use of 0.24% DEC fortified salt reduced the microfilaremia rate from 9% to less than 1% after 6 months of use [74]. The success of DEC fortified salt programs requires considerable effort to ensure the cooperation of salt manufactures and local officials as well as the effective prevention of individuals acquiring non-medicated salt, as in the case of

Papua New Guinea where food is often prepared using seawater, thereby rendering the use of DEC fortified salt ineffective [72].

DEC, which has been used to treat filarial infection since 1947 [75], is widely used in regions not co-endemic with other filarial worms such as *Onchocerca volvulus* where its use is contraindicated as it can lead to severe and fatal post-treatment reactions [72]. DEC administration to patients who also have onchocerciasis have led to the worsening of onchocerciasis related vision-loss [76]. In co-endemic regions, ivermectin is used in place of DEC [45]. The use of ivermectin on patients co-infected with *O. volvulus* is generally safe but in rare incidences serious and sometimes fatal adverse reactions have occurred [19], but the pathogenesis for this toxic syndrome in humans has yet been resolved [77, 78].

The current Global Programme to Eliminate Lymphatic Filariasis (GPELF) effort to alleviate morbidity and eliminate transmission of the lymphatic filarial worms uses annual administration of oral anti-filarial drug combinations to endemic or at risk communities through the MDA program [79]. Depending on the co-endemic situation, either DEC or ivermectin is given in conjunction with the benzimidazole anthelmintic, albendazole [24]. Albendazole administered alone to infected individuals appears to be ineffective as either a microfilaricide or macrofilaricide, but when administered together with ivermectin or DEC, albendazole appears to enhance the effectiveness of the two drugs [80].

GPELF Progress, Success and complications

In the first 8 years of the GPELF program from 2000 to 2007, approximately 570 million infected and at risk individuals in 48 lymphatic filariasis endemic countries were treated through the MDA program. Based on the report an estimate of approximately 6.6 million newborns were

spared filarial worm infection [81]. An added benefit of the MDA program is that ivermectin ingested from a treated individual by mosquitoes resulted in decreased mosquito survival rate and fertility [82, 83, 84] thus reducing the number of vector hosts for filarial worm transmission.

Despite the success of GPELF reported so far, there is no convincing evidence that the GPELF goal of complete lymphatic filarial worm elimination can be achieved [45]. For one, the lack of cooperation or inappropriate use of anti-filarial drugs by at risk or infected individuals poses a significant challenge for the success of the GPELF program. In the case of patients in the Colombo district of Sri Lanka, only 61% reported taking the recommended DEC treatment course [299]. Further challenges to the GPELF program effort arise when socio-economic troubles such as regional conflicts and famine force infected populations to migrate into regions without MDA coverage.

Problem with Current Chemotherapeutic Drugs

To complicate matters further, at present DEC and ivermectin are the two only drugs safe for human use without incurring any serious adverse side effects. Unfortunately both drugs are only effective as microfilaricides. Unlike ivermectin, which appears to have little to no macrofilaricidal activity against adult filarial worms, DEC has been found to kill up to 40% of the adult worms [45, 86, 87], but this macrofilaricidal activity of DEC is insufficient to cure an infected individual.

To date, the mechanism of action for ivermectin and DEC remains uncertain. Ivermectin has been found to act on glutamate-gated chloride channels in free-living nematodes as well as certain gastrointestinal parasitic nematodes, inhibiting the motility and feeding capacity of these worms [19, 20]. In culture, microfilariae of human lymphatic filarial nematodes appear

unaffected by clinically significant concentration of ivermectin [21, 88, 89]. The reason for this has yet to be resolved. Thus the pharmacological basis for the microfilaricidal effect of ivermectin remains poorly understood. Similarly, the pharmacological basis of DEC, which was introduced in 1947, remains a mystery. Recent work by McGarry *et al.* found pharmacological and genetic evidence implicating the host arachidonate- and nitric oxide-dependant pathways in the microfilaricidal and limited macrofilaricidal effect of DEC [22].

In conclusion, lymphatic filariasis causes significant socio-economic losses and morbidity. The GPELF effort to eliminate lymphatic filariasis relies on the mass drug administration of either ivermectin or DEC, the two only anti-filarial drugs available, along with albendazole which have been found to enhance the potency of the two anti-filarial drugs. Due to the effectiveness of ivermectin and DEC only as a microfilaricide, the only current option for eradicating lymphatic filariasis is to prevent worm transmission. This does not do much for the 12.5% of the 120 million infected individuals suffering from lymphadema. Due to the long lifespan of adult filarial worms and problems associated with patient noncompliance, the availability of an efficacious microfilaricide would both simplify control and facilitate eradication. Limited understanding of filarial worm biology and the mode of action for existing drugs challenge our ability to develop more effective anti-filarial drugs.

III.A BRIEF OVERVIEW OF RNA INTERFERENCE

First characterized by Fire *et al.* in 1998 on the free-living nematode *Caenorhabditis elegans*, RNAi allows investigators to suppress specific genes of interest using RNA triggers to mediate target-specific mRNA destruction resulting in transcriptional suppression [94]. This relatively new technique provides a simple and inexpensive tool for reverse genetics on difficult to manipulate organisms such as parasitic nematodes [90-92]. Despite the difficulty experienced so far, RNAi in parasitic nematodes has the potential to assist in elucidating the biology of parasitic nematodes and anthelmintic drug mode of action paving the way for the development of new and more efficient anthelmintic drugs.

A. The RNAi Mechanism

RNAi is elicited either when exogenous long double-stranded RNA (dsRNA) is introduced into the cell or when endogenous dsRNA / short hairpin RNA (shRNA) is produced intracellularly, and then processed into short interfering RNA (siRNA) duplexes of 21~23 base pairs (bps) in length, called primary siRNA, by the RNaseIII endoribonuclease enzyme Dicer. Synthetic siRNA can also be applied exogenously to bypass Dicer and substitute for primary siRNA. Primary siRNA can either be incorporated into the RNAi-induced Silencing Complex (RISC) or be used in RNAi signal amplification through RNA Dependent RNA Polymerases (RdRP) to produce secondary siRNAs [95, 96]. Gene specific suppression is elicited via targeting mRNA cleavage by RISC which consists of the incorporated siRNA guide strand and several other essential components, one of which is a catalytic Argonaute protein (Figure 2) [95, 96].

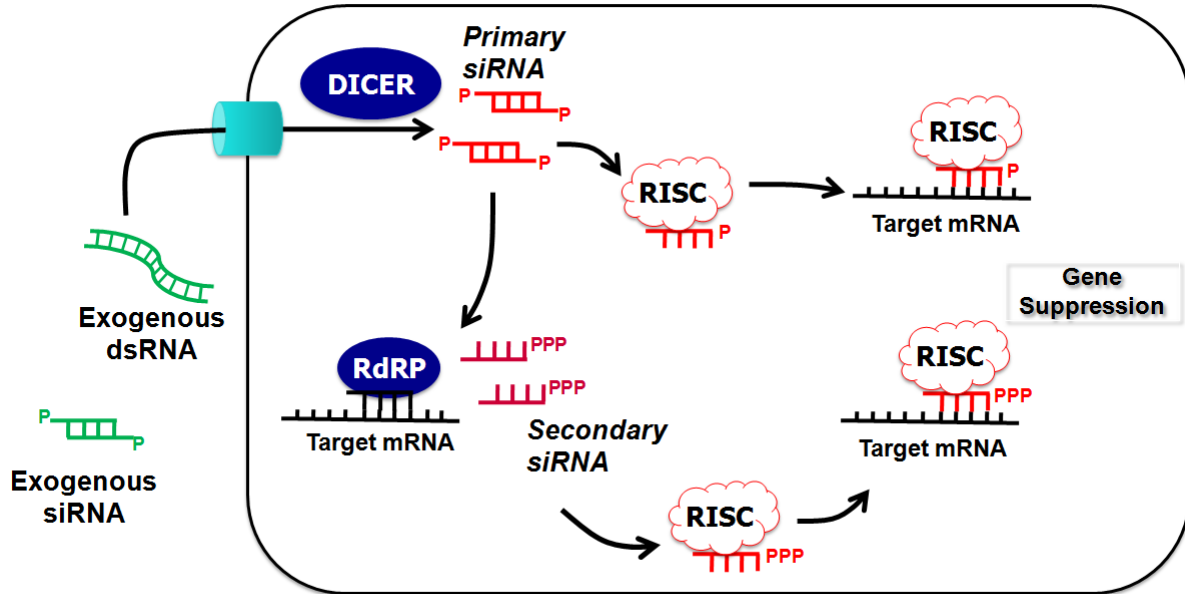


Figure 2: RNAi Pathway. Exogenous siRNA or dsRNA is taken up into the cell whereby DICER processes dsRNA into primary siRNA. RNA Dependent RNA Polymerase (RdRP) utilizes the primary siRNA or exogenous siRNA as template for RNAi signal amplification. The amplified siRNA (Secondary siRNA), Primary siRNA and/or exogenous siRNA is incorporated into the RNA-induced Silencing Complex (RISC) to mediate gene specific suppression through cleavage of the target mRNA.

B. Uptake of RNAi Trigger

The mechanism of dsRNA uptake, or how the exogenous RNAi trigger enters the cell, is most extensively studied in *C. elegans*. Among the currently identified proteins mediating dsRNA uptake is Systemic RNA Interference Deficient (SID)-2 [98, 102]. SID-2 deficient *C. elegans* mutant are resistant to both RNAi via soaking and feeding, but fully susceptible to both the microinjection and transgenic RNAi methods [98]. Expressing *C. elegans* SID-2 in *C. briggsae*, which is refractory to environmentally supplied RNAi triggers, restores RNAi susceptibility, but expression of the *C. briggsae* ortholog of the *C. elegans* SID-2 protein in *C. elegans* SID-2 mutants does not rescue the dsRNA uptake capability [98].

Several other protein families related to the dsRNA uptake mechanism have been found: Feeding Defective (FED) [103] and RNAi Spreading Defective (RSD) [104]. Both FED and RSD family mutants are refractory to RNAi induced by feeding on dsRNA-producing bacteria [103, 104]. Based on the structure and interaction of RSD family proteins it would appear that ingested dsRNA is endocytosed by vesicles through the gut lumen [105, 106].

C. Dissemination of RNAi Signal

Studies done by Feinberg and Hunter using transgenic *C. elegans* expressing GFP in both the pharynx and body wall muscles demonstrated the capacity for an RNAi trigger, introduced by bacteria feeding, to disseminate to the body wall muscles [108]. Further experimentation on SID-1 mutants showed that with SID-1 mutants only GFP expression at the pharynx (site of dsRNA uptake) was silenced indicating an incapability to disseminate the silencing signal [108]. Thus SID-1 is important for the systemic spread of RNAi trigger.

To determine whether SID-1 is responsible for the export or import of RNAi trigger, SID-1 protein was functionally expressed in *Drosophila* cells, which lack both an SID-1 homology as well as efficient dsRNA uptake mechanism [109], enabled rapid uptake of environmental dsRNA [109]. The presence of the RNAi trigger cellular export mechanism has been confirmed [105], but its identity remains elusive.

D. Primary siRNA Biogenesis

Before RNAi can take place, the internalized dsRNA needs to be first processed into short (21 to 30 nucleotide) siRNA duplexes [113-117]. The processing of dsRNA into siRNA is primarily mediated by the Dicer complex which contains the ~200 kDa RNaseIII enzyme, Dicer. [118].

In *C. elegans*, four components form the Dicer complex [204] to process long dsRNA into short siRNA [119, 120, 204]: the dsRNA binding protein (dsRBP) Rde-4, the PAZ (Piwi, Argonaute and Zwiille)-PIWI (P element-induced wimpy testes) Argonaute protein Rde-1, the catalytic enzyme Dicer, and a DexH Box Helicase Drh-1 (Figure 2). Following siRNA processing, the PAZ-PIWI Argonaute protein, Rde-1, binds to the processed siRNA duplex and transports it to appropriate downstream RNAi pathway complex [170] (Figure 3).

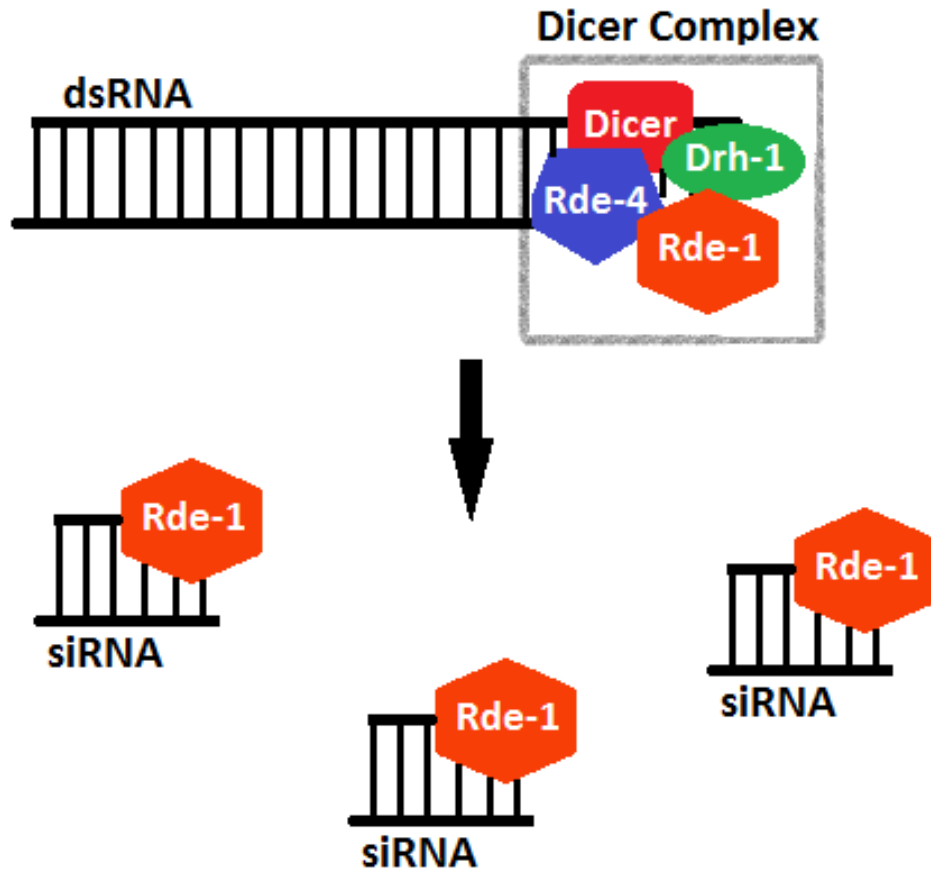


Figure 3: Schematic representation of the siRNA synthesis mechanism. In *C. elegans*, exogenously supplied dsRNA is processed into siRNA by the Dicer Complex consisting of the dsRNA binding protein (dsRBD) Rde-4, the PAZ (Piwi, Argonaute and Zwiile)-PIWI (P element-induced wimpy testes) Argonaute protein Rde-1, the catalytic enzyme Dicer, and a DexH Box Helicase Drh-1. The PAZ-PIWI Argonaute protein, Rde-1 binds to the siRNA products and transports them to downstream elements of the RNAi pathway.

The Catalytic Enzyme Dicer

The endonuclease Dicer protein, which functions as a monomer [116], consists of two conserved RNase III domains, a dsRBD located in the carboxyl terminus, an amino terminal DExH/Helicase-box domain, a small domain DUF283 of unknown function, and a PAZ domain [125].

Incorporating Exogenous dsRNA onto DICER Protein

The exogenous dsRNA, which tends to be blunt on both ends, initially undergoes a nonspecific cleavage to produce 3' dinucleotide overhangs thus allowing it to be anchored in the 3' overhang binding pocket of the PAZ domain [127]. *In vitro* studies on human Dicer indicate that Dicer produces siRNA unidirectionally from one end of the dsRNA substrate to the other through processive dicing [128].

PAZ Domain of Dicer

The PAZ domain, which contains roughly 130 residues, serves to anchor the 3' end of the dsRNA, rendering it stationary and thus making it possible for subsequent cutting of the dsRNA to produce siRNA of a fixed length [129].

DExH/Helicase Box Domain of Dicer Protein

Kinetic analysis on wild type and mutant human Dicer complex indicates that the DExH/Helicase-box domain function to inhibit Dicer catalytic activity since removal of the DExH/Helicase-box domain from Dicer increased Dicer production of siRNAs [127, 130].

Internal Molecular Ruler of Dicer

In Dicer the distance between the PAZ and the two RNaseIII domains acts as a molecular ruler [127]. In *Giardia intestinalis* Dicer, the distance between the PAZ and the two RNaseIII

active sites is 65Å, equivalent to a 25 nucleotide long RNA duplex, which is exactly the same length as *G. intestinalis* Dicer's siRNA product length [127, 205]. This was validated using truncated *G. intestinalis* Dicer complex lacking the PAZ domain, which resulted in the production of siRNA products of varying length [127, 205]. This explains the variation in product size across different organism species and Dicer enzymes.

E. Secondary siRNA / RNAi Signaling Amplification

In *C. elegans*, primary siRNA generated by Dicer is further amplified via a cytoplasmic RNA Dependent RNA Polymerase (RdRP) [131, 132, 135-137]. This amplification step, which occurs in the presence of target mRNA, is essential in eliciting the RNAi effect in *C. elegans* because inhibiting secondary siRNA synthesis resulted in a lack of RNAi [131-133]. Using transgenic *C. elegans* over-expressing *unc-22:GFP* chimeric mRNA at the intestine, Jose and Hunter demonstrated the presence of RNAi signal amplification when *unc-22* mutant phenotype was observed with GFP suppression despite the lack of *unc-22* functions at the site of *unc-22:GFP* expression (*unc-22* function in the body wall muscles) [100].

In *C. elegans*, the Argonaute Protein Rde-1 Transports Primary siRNA from Dicer Complex and Recruit the Formation of RDRP Complex to Make Secondary siRNA

In *C. elegans*, the PAZ-PIWI Argonaute protein Rde-1 selectively binds to the siRNA strand that is thermodynamically less stable at the 5' end called the guide strand [138, 139] and dissociates from the Dicer complex [204]. The dissociated Rde-1 + siRNA duplex complex triggers the formation of the secondary siRNA amplification machinery to produce secondary siRNAs (Figure 4) [140].

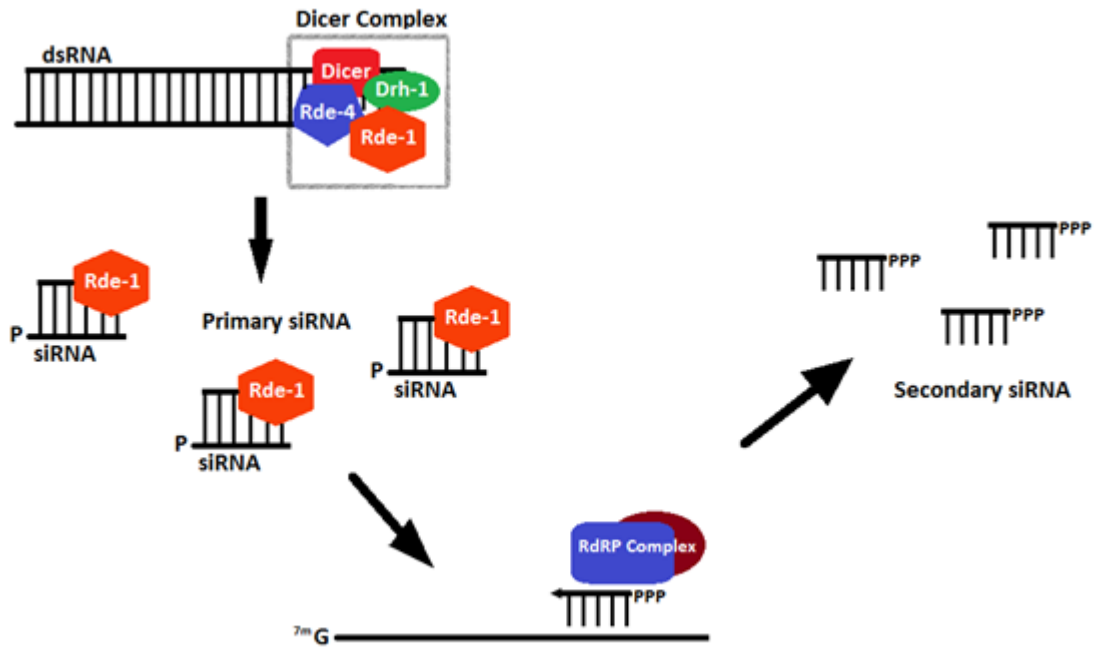


Figure 4: Secondary siRNA Synthesis. The PAZ-PIWI Argonaute Rde-1 binds to Dicer produced primary siRNA and dissociates from the Dicer Complex. Rde-1 triggers the formation of the RNA Dependent RNA Polymerase (RdRP). RdRP attaches onto a target mRNA and produces RNAi signal amplification in the form of secondary siRNAs.

In the Rde-1 assembly, the passenger strand can be viewed as the guide strand's first RNA target [138, 139, 158]. After discarding the passenger siRNA strand, the conformation of the Argonaute protein is one where the seed region (bases 2 to 6 from the 5' end of the guide strand) is arranged so that it is fully exposed and protruding outward toward the intracellular environment [166, 167] allowing for the efficient locating of target mRNA [168].

Upon binding to a target mRNA, bases 2-8 of the guide strand starting from the 5' end form a Watson-Crick paired A-form double helix with the complementary region of the target mRNA [168, 169]. Simultaneously, RDE-1 recruits the amplification machinery RdRP to the target mRNA to perform unprimed RNA synthesis of secondary siRNA [114, 133]. The secondary siRNA is characteristically different from primary siRNA in that it possess a 5' triphosphate as opposed to a 5' monophosphate end [114, 133], it is slightly shorter in length than primary siRNA, and it cleaves target mRNA more efficient than primary siRNA [170]. The secondary siRNA can either re-enter the RdRP associated pathway to further amplify the RNAi signals or it can be incorporated into RISC and facilitate in RNAi mediated mRNA cleavage [170].

Regulation of the Secondary siRNA Synthesis Mechanism

The existence of a secondary siRNA synthesis mechanism appears to be an essential step in the RNAi machinery, but it needs to be carefully controlled to prevent adverse problems such as amplification of off-target silencing signals in the absence of target mRNA. To guard against this, the RdRP amplification mechanism only takes place in the presence of target mRNA [133, 171]. To prevent the exponential growth of RNAi triggers leading to persistent gene suppression, a class of argonaute proteins called Secondary Argonaute Proteins (SAGO), which lack the catalytic residues for cleaving target mRNA, bind to and reduce the number of available siRNA

for further amplification [172]. Other RNAi control mechanisms exist to prevent escalating RNAi signal amplification and they will be discussed later.

F. RNAi – induced Silencing Complex (RISC)

RISC is the heart of the RNAi machinery. RISC identifies and binds to target mRNA using the incorporated siRNA guide strand and carries out endonucleolytic cleavage of target mRNAs. The cleaved mRNA is then released and degraded through exonuclease activity, resulting in the reduction of post-transcriptional gene expression [118].

As with secondary siRNA synthesis, siRNA duplexes are loaded in a selective orientation based on the asymmetry rule [173], allowing the strand with the thermodynamically less stable 5' end to serve as the guide strand [138, 139]. The asymmetry rule in which the guide strand is selected depends on the thermodynamic stability of the first 4 bases of each strand [138, 139]. The incorporation of the siRNA duplex requires the aid of the RISC Loading Complex (RLC) [174, 175].

G. RISC Loading Complex (RLC)

At the heart of RLC is the Dicer + dsRBP heterodimer [160, 161, 176]. In the case of *Drosophila*, the absence of Dicer-2 and dsRBP R2D2 results in a deficient RNAi machinery in both *in vivo* and *in vitro* models [177, 178]. But when Dicer-2 is restored *in vivo* through transgenic expression or supplemented with Dicer-2 and R2D2, the RNAi machinery is restored [177, 178]. In addition to the Dicer-2 + R2D2 heterodimer, the *Drosophila* RLC appears to be comprised of several other factors based on the slower migration speed of extracted *Drosophila* RLC on native gels [161, 162]. However, what these additional factors are remains a mystery.

Sontheimer and colleagues demonstrated that during siRNA loading of the *Drosophila* Argonaute (Ago)-2, the Argonaute protein associates with Dicer-2 and R2D2 in forming the RISC complex [179]. The unique binding feature of the Dicer-2 + R2D2 heterodimer allows for the preferential incorporation of the guide strand onto the Argonaute protein [138, 139, 180]. Dicer-2 holds the 3' dinucleotide overhang of the passenger strand (the strand with the 3' end at the thermodynamically less stable end of the siRNA duplex) leaving the 5' end of the guide strand free to be incorporated onto the argonaute protein. At the same time, R2D2 binds to the more stable 5' end of the passenger strand [138, 139, 180].

All Argonaute proteins identified to date contain a PAZ domain, which has a large binding pocket for the 3' dinucleotide overhang insertion [129]. Therefore it is most likely that in loading the siRNA duplex onto Argonaute, the 3' overhang of the guide strand will be loaded into the Argonaute PAZ domain which requires the 5' end of the passenger strand to be immobilized by R2D2. Similarly, the 5' end of the guide strand whose complementary 3' is held by Dicer-2 is incorporated into the deep pocket between the Argonaute MID and PIWI domain (Figure 5) [149, 150].

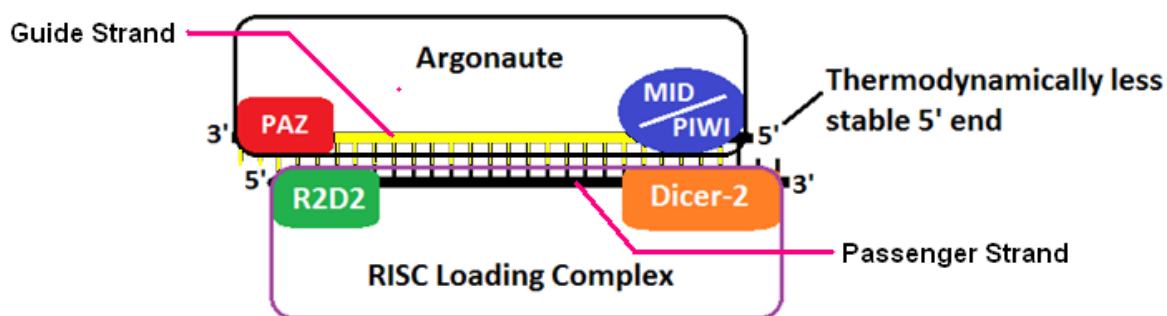


Figure 5: Loading of siRNA duplex onto Argonaute. Dicer-2 of RLC binds to the 3' end of the passenger strand (strand that does not have the 5' thermodynamically less stable end) and R2D2 binds to the passenger strand's 5' end. The Argonaute PAZ domain binds to the 3' end of the guide strand and the thermodynamically less stable 5' end of the siRNA duplex is held at the pocket between the MID and PIWI domain.

H. RISC Function

As in secondary siRNA synthesis, once the guide strand is loaded onto the argonaute protein of RISC, the passenger strand acts as a RNA target [158]. Once the passenger strand is cleaved and discarded, the RISC complex is ready to actively seek out target mRNAs for destruction.

The “seed region” of the guide strand, base pairs 2 to 6 from the 5’ end, is arranged in a near A-form conformation in the RISC complex protruding outward in an exposed orientation [166, 167]. Upon complementary binding to a target mRNA, the PIWI domain of the Argonaute protein initiates target RNA cleavage through nucleophilic attack between bases 10 and 11 of the guide strand from the 5’ end [95, 154].

I. Regulating the RNAi Mechanism

While much has been pieced together about the mechanism leading up to the RNAi mediated mRNA cleavage, very little information is known about how RNAi is regulated. All experimental data indicate that RNAi is transient with the exception of certain targeted genes in *C. elegans* whereby gene suppression is hereditary. At present, only bits and pieces of information exist to give a glimpse as to RNAi regulation.

One regulatory process is the expression of siRNA degradation proteins such as Enhanced RNAi (Eri)-1 in *C. elegans* [181]. In Eri-1 mutants, the presence of higher than normal concentrations of siRNA were noticed indicating either that more secondary siRNA are produced, or siRNA degradation is inhibited. Because Eri-1 is an evolutionary conserved protein containing a SAP/SAF-box domain and a DEDDh family exonucleases domain, it is highly probable that the function of Eri-1 is siRNA degradation [181]. Furthermore the nerve tissues cells of *C. elegans*, where Eri-1 is preferentially expressed, are refractory to RNAi, but in Eri-1 mutants

exogenously supplied dsRNA can elicit RNAi [181]. Thus neuronal tissue resistance to RNAi may be partially attributed to Eri-1 degradation of silencing triggers and in doing so preventing siRNA amplification and subsequent RNAi at a detectable level [181].

Another mechanism regulating RNAi is adenosine deaminase. The gene product of Adenosine Deaminase that Acts on RNA (ADAR) mutates the adenosines in the dsRNA into inosine. The progressively increasing amount of A-U pairs converted to I-U wobble pairs render the deaminated dsRNA more and more resistant to Dicer [182, 183]. Thus ADAR, like Eri-1, modulates RNAi by reducing the level of silencing trigger available for amplification and RISC assembly. Without an adequate supply of new RNAi triggers, the aforementioned RNAi regulation methods would result in the eventual removal of RNAi triggers. In addition, Tudor-SN substrate binds to and promotes the degradation of hyper-edited dsRNA.

IV. HISTORY OF RNAi IN PARASITIC NEMATODES

RNA interference was originally characterized in the free-living nematode *Caenorhabditis elegans* by Fire *et al.* in 1998 and emerged as a new technology to down-regulate target genes of interest using gene specific, double-stranded RNA (dsRNA) [94]. The RNAi technique has since been demonstrated in various other organisms including protozoa [184, 185], amphibians [186], insects [187, 188], and mammals [189]. Based on the successful implementation of RNAi in numerous organisms, it is logical to try and transfer the technology for use in the field of parasitic nematodes.

In *C. elegans*, the RNAi triggers (dsRNA or siRNA) can be delivered by several methods: microinjection, soaking, feeding of dsRNA-expressing bacteria, and electroporation. Although all four methods are effective in *C. elegans*, other *Caenorhabditis* species such as *C. briggsae* demonstrated varied susceptibility to both the soaking and bacteria feeding methods [190]. As such, to date the “gold standard” for RNAi in *Caenorhabditis* species remains microinjection.

In applying RNAi to parasitic nematodes of both human and veterinary interest, the use of a microinjection method would be impractical. In 2002, Hussein *et al.* utilized RNAi soaking method on the rodent gastrointestinal parasitic nematode *Nippostrongylus brasiliensis* [191]. The successful suppression of the secreted acetylcholinesterase B (AChE B) in adult *N. brasiliensis* was achieved. 1799 bp dsRNA derived from the full length AChE B cDNA suppressed AChE B mRNA level by 80%. The suppression was immediate, beginning on the first day of dsRNA exposure and rapidly returned to control levels over the next four days. The use of a shorter 240 base-paired dsRNA that corresponded to the 5' region of the AChE B coding sequence produced a more potent suppression of over 90% and the RNAi effect persisted throughout the 6 day culture period. Although no phenotypic abnormalities were observed in worms after either the

long or short dsRNA treatment, this experiment provided the first proof that RNAi is feasible in parasitic nematodes [191].

Following the success of Hussein *et al.*, Aboobaker and Blaxter successfully suppressed three genes of interest: β -tubulin (*Bm-tub-1*), RNA polymerase II large subunit (*Bm-ama-1*), and mf sheath protein 1/mf22 (*Bm-shp-1*) in the adult filarial nematode *Brugia malayi* *in vitro* using a soaking protocol. Suppression of all three target genes was confirmed using non quantitative RT-PCR with lethal phenotype observed for both *Bm-ama-1* and *Bm-tub-1*. dsRNA treatment of either *Bm-ama-1* or *Bm-tub-1* both resulted in unhealthy worms with reduced motility and death 24 hours post dsRNA treatment began. Suppression of *Bm-shp-1* resulted in a marked decrease in quantity of mf released in culture. Of the released mf, 50% exhibited fully elongated sheaths [112].

Following Aboobaker and Blaxter's successful demonstration of RNAi on the filarial nematode *Brugia malayi*, Lustigman *et al.* in 2004 carried out a similar *in vitro* RNAi experiment on the human filarial nematode, *O. volvulus*, to examine the role of cathepsin L and Z like cysteine proteases in L3 to L4 molting. Unlike the prior two RNAi experiments, Lustigman *et al.* demonstrated *O. volvulus* uptake of dsRNA in culture medium by using fluorescent-labeled Cy3 dsRNA for cathepsin L (cpl) and cathepsin z (cpz cysteine proteases). Cy3 labeled dsRNA was found in the esophagus and along the length of the intestine. In a few worms, Cy3 labeled dsRNA was also found in the cuticular and hypodermal regions. Although they did not perform RT-PCR to confirm RNAi mediated suppression, suppression was confirmed using immunoelectron microscopy. The immunostaining reduced significantly in cpl and cpz dsRNA exposed L3 worms in comparison to control worms. Furthermore, exposure to cpl or cpz dsRNA resulted in a 92% and 86% inhibition of L3 to L4 molting. In comparison to

normal L3 to L4 morphological changes, the dsRNA treated worms examined under electron microscopy revealed a lack of separation between the L3 cuticle and the newly synthesized L4 cuticles. The successful use of RNAi in *O. volvulus* was another positive step forward demonstrating the potential use of RNAi as an investigative tool in studying parasitic nematodes. Unfortunately, Lustigman *et al.* also noted that exposure to the *C. elegans* homologue of human Rh protein, *Ce-rhr-1*, or *O. volvulus* cpz intronic sequence *Ov-cpz-int2*, dsRNA also resulted in a 49.8% and 30% reduction in molting rate compared to control. This was an indication that RNAi in parasitic nematodes may not be as target specific as previously thought, or according to Lustigman *et al.* that it incurs the activation of a non-specific toxic effect by a yet unknown mechanism. [2]

With the successful demonstration of RNAi in three parasitic nematodes from 2002 to 2005, interest in RNAi as a potential tool for studying parasitic nematode biology increased such that there were seven publications between 2005 and 2006. All seven publications utilized RNAi soaking methods for dsRNA delivery with several groups also exploring the capability of several other delivery methods as well. The results collectively suggested that RNAi in parasitic nematodes may not be as straight forward as previous thought. Problems with reliability, consistency, and susceptibility arose in some of the parasitic nematode systems examined.

In 2005, Islam *et al.* published work investigating an inorganic pyrophosphatase in the pig gastrointestinal parasite *Ascaris suum* (*AsPPase*). L3 *Ascaris suum* were soaked in a concentration of 2 µg/ml *AsPPase* dsRNA containing culture medium for 24 hr and maintained in a dsRNA free culture medium for an additional 9 days. The dsRNA treatment completely abolished *AsPPase* mRNA level and immunofluorescence staining revealed that dsRNA exposure reduced the native enzyme activity by 56.3%. dsRNA exposed L3s also demonstrated a

reduced L3 to L4 molting by 31% compared to control in the *in vitro* molting assay. Exposure to AsPPase dsRNA resulted in no morphological defects or adverse effect on worm viability [192].

Following the successful suppression of cathepsin L and Z-like cysteine protease in the filarial worm *O. volvulus*, Ford *et al.* identified and investigated the function of a novel filarial serine protease inhibitor (*Ov-SPI*) on L3 stage *O. volvulus* using the RNAi soaking method previously described. A 200-fold suppression of the target gene, *Ov-SPI*, transcript was observed and immunoelectron microscopy confirmed the reduction in *Ov-SPI* expression. The RNAi mediated suppression of *Ov-SPI* transcript produced an 84.2% reduction in molting and a 39.4% L3 mortality compared to control which exhibited only 2.2% mortality. Closer examination of the dsRNA exposed L3s that failed to molt revealed incomplete separation between the L3 and L4 cuticle. Similar to the previous *O. volvulus* RNAi experiment, L3 to L4 molting was affected by non-target dsRNA though not as dramatically as with target dsRNA (dsRNA of *Plasmodium falciparum* gene *Pf-ebe-140* produced a 24.7% reduction in *O. volvulus* molting) [193].

During that same year, RNAi was carried out on the sheep gastrointestinal parasite *Trichostrongylus colubriformis*. Issa *et al.* attempted RNAi suppression of ubiquitin and tropomyosin by soaking, bacteria feeding and electroporation methods with varying success [194]. Feeding newly hatched L1 larvae in culture medium with bacteria expressing ubiquitin produced no defect on larval development, but ingesting tropomyosin dsRNA expressing bacteria resulted in a dramatic reduction in the number of worms developed into the infective L3 larvae from approximately 80% exhibited by control worms to 20%. Unlike previous RNAi on parasitic nematodes, Issa *et al.* utilized dsRNA as well as siRNA to evaluate the soaking and electroporation methods. For soaking and electroporation, only ubiquitin suppression was tested. Soaking L1 in ubiquitin dsRNA resulted in no developmental phenotype, but soaking in

ubiquitin siRNA resulted in a 69% reduction in number of worms that developed into L3s. Electroporation using ubiquitin dsRNA and siRNA both resulted in either death or developmental delays in more than 90% of the worms. Delivery of the RNAi trigger using electroporation produced abnormal phenotypes (shrunken and non-motile with granular appearance) as early as 2 hours after treatment began. This dramatic phenotypic effect was confirmed not to be the effect of electroporation as L1s exposed to either *C. elegans* ubiquitin dsRNA or siRNA produced no significant developmental defects. As the results imply, RNAi did take place in certain circumstances, but transcriptional suppression was not confirmed. [194]

In 2006, two groups independently investigated the feasibility of RNAi in the sheep and goat intestinal parasitic nematode, *Haemonchus contortus*. Kotze and Bagnall tested the potential for soaking mediated RNAi gene suppression on L3 *H. contortus* by targeting two β -tubulin genes of interest, *tub 8-9* and *tub 12-16*. A 1000 fold reduction in *tub 8-9* transcription level was observed that persisted for up to 6 days [195]. The RNAi mediated suppression of *tub 12-16* was much less dramatic with an approximate 10 fold decrease, but the suppression persisted beyond 6 days. The proportion of L3s that developed into L4s in both gene suppression groups was reduced, particularly for the *tub 12-16* dsRNA exposed group. A migration assay revealed that *tub 12-16* suppression resulted in a reduced migratory ability but *tub 8-9* RNAi exhibited no migration abnormality. While the successful use of RNAi on *H. contortus* adds another parasitic nematode to the growing list of RNAi tractable parasitic nematodes, the target specificity of the RNAi method is called to question as significant off-target suppression of none target genes were observed [195].

Simultaneously, Geldhof *et al.* explored the effectiveness of RNAi in *H. contortus* using soaking, bacteria feeding and electroporation methods. Contrary to the positive report by Kotze

and Bagnall, Geldhof *et al.* were only successful in suppression two of the five genes examined using the soaking method. B-tubulin isotype 1 (*Hc-ben-1*) transcription level in the L3 stage was completely suppressed following dsRNA exposure. COPII component (*Hc-sec-23*) targeted dsRNA exposure resulted in significant but not complete suppression of transcript. Unlike *Hc-ben-1* suppression, *Hc-sec-21* suppression proved unreliable because RNAi-mediated suppression was not achieved in two out of five experimental repeats. Microscopic examination of larvae *H. contortus* soaked in fluorescently labeled cathepsin L (*Hc-cpl-1*) dsRNA containing medium revealed very little dsRNA uptake. Thus, the soaking method appears to be unreliable with variation between experiments and recalcitrance for certain genes. Feeding *H. contortus* L1/L2 with bacteria expressing dsRNA was evaluated on four different gene targets. Suppression was not achieved for all four targets. 50 volt and 100 volt electroporation was used on the same four target genes with transcriptional level reduced but not abolished for *Hc-ben-1* and the Cu-Zn superoxide dismutase (*Hc-sod-1*), with no off-target effects noticed. Although the transcription level was suppressed for the two genes, worm survival was significantly compromised as a result of electroporation with high level of dead larvae found after 24 hours. [196] Thus it would appear that electroporation alone results in negative effects on the worms.

Between 2005 and 2006, the actin gene (*Ls-act*) of the rodent filarial parasitic nematode *Litomosoides sigmodontis* was investigated using RNAi soaking method. Adult *L. sigmodontis* were extracted from infected cotton rats and incubated in media containing *Ls-act* dsRNA for 24 hours before transfer into dsRNA free culture medium. *Ls-act* transcription level was reduced by 81% after the first 24 hours to 93% at 48 hours and 97% at 72 hours. Checking the mRNA level of two non-target genes resulted in no suppression thus confirming no off-target effects. Suppression of *Ls-act* transcription resulted in worm paralysis demonstrated by a stretched out

body and slow movements. The release of microfilariae (mf) was also dramatically reduced with *Ls-act* dsRNA treatment [197].

In 2006, Visser *et al.* evaluated RNAi capacity of the cattle gastrointestinal nematode *Ostertagia ostertagi* by soaking and electroporation. Eight genes were examined using exsheathed L3 *O. ostertagi* larvae. Five of the eight genes showed susceptibility to RNAi when animals were soaked in dsRNA. The reliability of the RNAi method was called into question when repetition of the RNAi experiments was proven unreliable as in the case of β -tubulin where suppression was confirmed only in three out of five repeats. Furthermore, suppression level varied between different batches of dsRNA used. Electroporation on the same eight genes produced suppression in only two targets, tropomyosin and β -tubulin, but neither was reproducible. [198]

Despite earlier success with RNAi, findings in 2005 and 2006 reveal that RNAi in parasitic nematodes is not as straightforward as previously thought. The recalcitrance of certain genes to RNAi and the unreliability of the RNAi mechanism diminished the hope of using RNAi as a tool to investigate genes of interest as well as screen for potential drug and vaccine targets. And interest in RNAi as a tool to study parasitic nematodes waned with no research papers related to RNAi in parasitic nematodes published in 2007.

In 2008, Lendner *et al.* attempted to suppress tropomyosin using RNAi on larvae of the murine intestinal parasitic nematode *Heligmosomoides polygyrus* by bacteria feeding, soaking and electroporation methods. Feeding L3 larvae with tropomyosin dsRNA expressing bacteria did not produce any effect, but feeding the same *H. polygyrus* tropomyosin dsRNA-expressing bacteria to *C. elegans* produced the characteristic phenotype of tropomyosin suppression. Thus it

would appear that RNAi mediated by bacteria feeding is ineffective in *H. polygyrus*. Electroporation on L1 larvae resulted in a 70% to 90% mortality. Closer examination using Cy3 labeled siRNA revealed that siRNA was not delivered into *H. polygyrus* by electroporation. Soaking larvae *H. polygyrus* in tropomyosin dsRNA reduced the lifespan of L1/L2 larvae and rendered L3s physiologically inactive. Worms soaked in tropomyosin dsRNA for 6 days exhibited morphological changes before dying with female worms exhibiting disintegrated ovaries and gut. In some cases, the presence of floating egg fragments in the pseudocoelomic cavity was observed. But quantitative PCR revealed no suppression of mRNA levels. As such Lendner *et al.* speculated that the observed RNAi phenotype might be a result of the stress and/or a toxic side effect induced by treatment rather than an RNAi effect [199].

In 2009, Ford *et al.* again performed RNAi on filarial parasitic nematodes but instead of *O. volvulus*, the function of cathepsin L and Z like cysteine proteases on adult female *B. malayi* was examined using similar soaking method as Aboobaker and Blaxter. Reduction in target mRNA levels ranging from 51% to 66.5% was confirmed for dsRNA exposed worms and 37.4% to 48.9% was confirmed for siRNA exposed worms using quantitative PCR. The uptake of both Cy3 labeled dsRNA and siRNA was confirmed using fluorescence microscopy. RNAi-mediated suppression of cathepsin L and Z resulted in a 61.3% to 92.8% reduction in microfilariae released. Closer examination of RNAi treated adult female *B. malayi* revealed malformed intrauterine embryos that were not fully developed with spacing between the embryo and the surrounding eggshell. Moreover, MTT viability staining revealed an 18% to 22% reduction in embryo viability [200].

In 2010, Xu *et al.* successfully suppressed a newly identified *Ascaris suum* gene (06G09) by soaking infective L3 larvae in dsRNA culture medium. Suppression was confirmed using non-

quantitative RT-PCR. The RNAi-suppressed L3s were then reintroduced into healthy BALB/c mice and larvae collected four days later. The RNAi mediated 06G09 suppression resulted in fewer larvae harvested from the livers and lungs of infected mice compared to controls, such that 71.43 ± 14.35 dsRNA treated worms were recovered compared to 164.29 ± 21.51 control worms. Examination of body length and width of dsRNA treated larvae reveal a reduced length of 200.57 ± 71.31 μm compared to 480 ± 105.77 μm of control larvae and a reduced width of 20.20 ± 2.43 μm compared to 23.93 ± 3.72 μm of control larvae [201].

The following year, Chen *et al.* published data on RNAi mediated suppression of *A. suum* enolase. Again, infective L3 were soaked in dsRNA and then reintroduced into healthy BALB/c mice. RNAi-mediated mRNA suppression was confirmed using non-quantitative RT-PCR. The number of dsRNA treated larvae extracted four days post infection from infected mice was not substantially different from that of worms extracted from control mice. As with the previous study by Xu *et al.*, the length of the extracted larvae was examined and dsRNA treated larvae exhibited a reduced length of 360 μm to 370 μm compared to 400 μm for control worms [202].

The same year, Samarasinghe *et al.* reported successful suppression of four of six L3 stage *H. contortus* genes by soaking in culture medium containing dsRNA. Exposure to dsRNA for the major excretory secretory protein *Hc-asp-1* or the highly protective gut amino peptides *Hc-H11* resulted in mRNA suppression of 80% and 76% respectively. Suppression was reproducible in at least 10 separate experiments but no phenotype abnormalities were detected within culture medium. Reintroduction of Hc-H11 RNAi suppressed worms into sheep resulted in a 57% reduction in fecal egg count, 40% reduction in worm burden and a 64% reduction in amino peptidase activity after 28 days post-infection when compared with control animals [203].

To date, the application of RNAi in parasitic nematodes has met with both success and significant problems. For some parasitic nematodes such as *Ascaris suum*, RNAi appears to be robust and reliable. But for other parasitic nematodes such as *H. contortus*, RNAi is both unreliable and sometimes unattainable. Thus as it stands, the unreliability of RNAi in parasitic nematodes preclude its potential as a powerful reverse genetic tool for studying parasitic nematodes.

V. OBJECTIVES

The general goal of the studies presented herein is to develop an *in vivo* RNAi method to study and better understand the biology of filarial worm *B. malayi*. Previous studies of parasitic nematodes have been hindered by the difficulty in gene manipulation and in keeping parasitic nematodes alive and healthy exo-host for the duration of the experiment. RNAi may provide an excellent means to study the function of target genes but unfortunately RNAi in parasitic nematodes has been riddled with problems. The problems may be due to the fact that parasitic nematodes once outside the host body undergo biological changes that render them resilient to exogenously induced RNAi. No matter how close the *in vitro* culture medium is to the host environment, there are still subtle differences that parasitic nematodes may require. As such, we hypothesized that RNAi may be more feasible and reliably carried out in *in vivo*.

The *in vivo* RNAi was accomplished by artificially infecting the *B. malayi*-susceptible *Aedes aegypti* (Liverpool strain) with *B. malayi* mf and exposing the developing parasite *in vivo* to RNAi triggers introduced into the mosquito host. Because mosquitoes have an open circulating system that effectively bathes developing *B. malayi* from the mf stage to the infectious L3 stage, it was my goal to take advantage of these and develop an *in vivo* RNAi method to study specific gene targets in juvenile *B. malayi*. The open circulatory system can be viewed as an *in vivo* culture solution. The infectious L3 stage *B. malayi* can be extracted from the mosquito host allowing for phenotype evaluation.

A second major objective of this study is to verify the reliability of the *in vivo* RNAi method. An *in vivo* RNAi method on *B. malayi* is critical in gaining a deeper understanding of *B. malayi* biology and the role specific genes play on the parasite's development and health.

A final major objective of the present study is to gain information concerning the importance of specific genes of interest to drug development. Many genes are predicted to be essential to parasite survival and are considered as potential drug targets. Through RNAi mediated knockdown and subsequent examination of the resulting phenotype, the relative potential of each gene for drug target development can be evaluated.

The present study is divided into two sections, each of which addresses one or more of the above stated objectives. Each section contains a Summary, Introduction, Materials and Methods, Results and Discussion. It is hoped that the technique developed and information gained from these studies will provide insights into juvenile *Brugia malayi* biology and provide a basis for future studies.

VI. THESIS ORGANIZATION

The above sections of this chapter provided a literature review that coincides with the research conducted herein. The subsequent chapter of this thesis contains papers that have either been accepted for publication or will be submitted to a peer-reviewed journal. Chapter 2 is a manuscript that has been published in PLoS Pathogens under the title “Development of an *In Vivo* RNAi Protocol to Investigate Gene Function in the Filarial Nematode, *Brugia malayi*”. The research was conducted and manuscript written by Chuanzhe Song under the supervision of Dr. Michael J. Kimber, Dr. Timothy A. Day and Dr. Lyric Bartholomay. The manuscript comprising Chapter 3 will be submitted under the working title “Confirming Robustness of the *In Vivo* RNAi Protocol Through Investigating Gene Function of Known and Potential Drug Targets in the Filarial Nematode *Brugia malayi*”. This paper confirms the robustness and consistency of the *in vivo* RNAi protocol and provides insights in the gene function of known or putative drug targets through application of the *in vivo* RNAi protocol. The research was conducted and the manuscript was written by Chuanzhe Song under the supervision of Dr. Michael J. Kimber, Dr. Timothy A. Day, and Dr. Lyric Bartholomay.

CHAPTER 2. Development of an *In Vivo* RNAi Protocol to Investigate Gene Function in the Filarial Nematode, *Brugia malayi*.

A paper published in *PLoS Pathogens*

Chuanzhe Song, Jack M. Gallup, Tim A. Day, Lyric C. Bartholomay, and Michael J. Kimber

I. ABSTRACT

Our ability to control diseases caused by parasitic nematodes is constrained by a limited portfolio of effective drugs and a paucity of robust tools to investigate parasitic nematode biology. RNA interference (RNAi) is a reverse-genetics tool with great potential to identify novel drug targets and interrogate parasite gene function but present RNAi protocols for parasitic nematodes, which remove the parasite from the host and execute RNAi *in vitro*, are unreliable and inconsistent. We have established an alternative *in vivo* RNAi protocol targeting the filarial nematode *Brugia malayi* as it develops in an intermediate host, the mosquito *Aedes aegypti*. Injection of worm-derived short interfering RNA (siRNA) and double stranded RNA (dsRNA) into parasitized mosquitoes elicits suppression of *Brugia malayi* target gene transcript abundance in a concentration-dependent fashion. The suppression of this gene, a cathepsin L-like cysteine protease (*Bm-cpl-1*) is specific and profound, both injection of siRNA and dsRNA reduce transcript abundance by 83%. *In vivo* *Bm-cpl-1* suppression results in multiple aberrant phenotypes; worm motility is inhibited by up to 69% and parasites exhibit slow-moving, kinked and partial-paralysis postures. *Bm-cpl-1* suppression also retards worm growth by 48%. *Bm-cpl-1* suppression ultimately prevents parasite development within the mosquito and effectively

abolishes transmission potential because parasites do not migrate to the head and proboscis. Finally, *Bm-cpl-1* suppression decreases parasite burden and increases mosquito survival. This is the first demonstration of *in vivo* RNAi in animal parasitic nematodes and results indicate this protocol is more effective than existing *in vitro* RNAi methods. The potential of this new protocol to investigate parasitic nematode biology and to identify and validate novel anthelmintic drug targets is discussed.

II. INTRODUCTION

Lymphatic filariasis is a disease caused by filarial nematodes including *Wuchereria bancrofti* and *Brugia malayi*, transmitted through the bite of infected mosquitoes. These parasites perpetuate socioeconomic instability in developing countries by inflicting crippling morbidity and debilitating stigmatization. The impact of this disease is vast - over 120 million people are infected in 81 endemic countries [24]. In an effort to alleviate morbidity and eliminate transmission of this disease, the Global Program for the Elimination of Lymphatic Filariasis (GPELF) has orchestrated a systematic mass drug administration (MDA) program centered on the repeated dosing of either diethylcarbamazine citrate (DEC) and albendazole or albendazole and ivermectin in areas where the other filarial worms, *Onchocerca volvulus* and *Loa loa* are co-endemic. This strategy has reduced prevalence in many areas [14] but lymphatic filariasis remains a significant global health concern. Many factors contribute to continued transmission, but central is the inadequate portfolio of effective drugs; none of the MDA drugs are effective against all life stages of the parasite with notable inefficacy against adult worms [15-17]. This means MDA must be provided annually for the duration of the lifespan of adult parasites. This situation is compounded by gaps in our understanding of mechanisms of drug action and pharmacology – the site of action of DEC is unknown despite being the drug of choice for lymphatic filariasis control for decades, and the filaricidal mechanism of ivermectin at therapeutic concentrations is also equivocal. There is a very real and significant need for additional and more effective antifilarial drugs, and a better understanding of the mode of action of existing drugs [206].

A major obstacle to the rational development of such drugs is the experimental intractability of parasitic nematodes. An example of this complication is RNA interference (RNAi), a reverse

genetic tool that allows researchers to rapidly and specifically ‘turn off’ genes of interest. RNAi has fast become a standard tool in rational drug discovery for the identification and validation of potential new drug targets [207, 208]. By suppressing specific genes and examining the resulting phenotype, it is possible to delineate gene function and appraise the potential value of encoded proteins as drug targets. Successful applications of present RNAi protocols to parasitic nematodes have been sporadically reported, limited in their effectiveness and seldom repeated [209]. Some success has been achieved with *Nippostrongylus brasiliensis* [210], *Ascaris suum* [211], *Trichostrongylus colubriformis* [212], *Ostertagia ostertagi* [198] and *Haemonchus contortus* [213, 214]. Germane to the study of filarial worms, RNAi has been described in *B. malayi* [112, 200], *Onchocerca volvulus* [2, 215] and *Litomosoides sigmodontis* [216]. The conclusion has been reached, however, that successful RNAi “only works on a limited number of genes, and in some cases the effect is small and difficult to reproduce” [213]. The inability to depend on present RNAi protocols with parasitic nematodes has proved a major stumbling block to the identification and validation of new drug targets, to a better understanding of anthelmintic mode of action, and to advancing our comprehension of parasite biology.

The recalcitrance of animal parasitic nematodes to RNAi is perplexing, given that *Caenorhabditis elegans*, a free-living nematode, and plant parasitic nematodes are readily susceptible to the technique [94, 217-223]. One hypothesis advanced to explain this recalcitrance is that because present RNAi protocols employ *in vitro* approaches including soaking nematodes in an RNAi trigger, feeding nematodes bacteria producing the trigger, or electroporating of the trigger into the parasite, the RNAi trigger is not provided in a manner conducive to systemic gene suppression [261]. Implicit in the use of these protocols is the removal of a parasite from the host and its maintenance in a liquid culture. Therefore these

protocols have distinct drawbacks such as difficulty maintaining healthy, viable worms that behave normally *in vitro*, limitation of use of parasites or life stages for which *in vitro* culture is defined, and poor efficacy in RNAi trigger delivery methods that can prove lethal to the parasite [225].

The aim of this study was to develop an innovative *in vivo* approach to RNAi in parasitic nematodes that overcomes the drawbacks associated with present *in vitro* experimental paradigms. Our approach is based on the filarial nematode *B. malayi*. We establish a *B. malayi* infection in an intermediate host, the mosquito *Aedes aegypti*, and then initiate suppression of parasite genes by injecting an RNAi trigger directly into the mosquito. The mosquito acts as an ideal culture and delivery system, ensuring the RNAi trigger is exposed to healthy, developing parasites. Using this approach we have effectively and quantifiably suppressed expression of *Bm-cpl-1*, a *B. malayi* gene encoding a cathepsin L-like cysteine protease. Dramatic aberrant phenotypes accompany this suppression, including a marked retardation of motility, an inhibition of normal parasite migration behavior within the mosquito and impaired parasite growth and development. Suppression is specific; non-target RNAi has no effect on nematode viability or behavior, and the level of gene suppression and extent of the resultant phenotypes suggest this new protocol is more effective than previous methods. The development of an *in vivo* RNAi protocol to reliably suppress gene expression in filarial worms has great potential for the identification and validation of novel drug targets, and more broadly, to explore parasitic nematode biology and host-parasite interactions.

III. MATERIALS AND METHODS

A. Mosquito Maintenance and Injection Protocol

Aedes aegypti (Liverpool strain), previously selected for susceptibility to filarial worms [253], were maintained in a contained environment at a constant temperature of 25°C, 80% relative humidity and a 14 h light to 10 h dark photoperiod. The mosquitoes were fed a diet of 0.3 M sucrose. Throughout the study mosquitoes to be injected were anaesthetized on ice and immobilized on a vacuum saddle before being microinjected intrathoracically at the base of the head using a pulled borosilicate glass pipette attached to a manual syringe for injection by volume displacement. A maximum volume of 0.5 µL can be injected using this approach with a high mosquito survival rate (>95%).

B. Establishing *Brugia* Infection

Brugia malayi microfilaria (mf) infected cat blood was obtained from the University of Georgia NIH/NIAID Filariasis Research Reagent Resource Center. To establish a consistent and repeatable parasitemia, mf were first purified using a filtration protocol [254]. Blood containing the parasites was diluted with phosphate buffered saline (1:5 ratio, blood:PBS) then syringe filtered through a 0.45 µm Millipore filter. Captured mf were washed three to five times with PBS then a further three to five times with *Aedes* physiologic saline [255] before centrifugation at 6,800 x g for five min. Supernatant was removed and the pelleted mf resuspended in fresh *Aedes* saline to a concentration of 40 worms per µL. To inoculate mosquitoes, 20 mf were injected as described. Microdissection of the mosquitoes throughout a 14 dpi period confirmed this method established a controlled infection that progressed in a predictable and consistent manner. We also tried a blood feeding approach to establish infection but this produced an

inconsistent worm burden that is too variable to reliably assess subsequent gene suppression experiments.

C. siRNA and dsRNA Generation and Injection

Short interfering RNAs (siRNA) targeting a *B. malayi* cathepsin L-like gene (*Bm-cpl-1* AF331035 [229]) were generated commercially (Qiagen, CA) and modified with a 3'-Cy3 fluorophore on the sense strand. The location of each siRNA was optimized using a proprietary algorithm and the sequence of each siRNA is as follows: BmCL1-1, AAGGCTTAGTTTCTTATACAA; BmCL1-2, CCGAATGGAAAGATTATGTAA; BmCL1-3, CAGAAGTGCATTGAAGGAATA; and BmCL1-4, CCGGTATTTACTCCAGTAATA. Equimolar amounts of each siRNA were combined and this mix was used for injection and gene suppression experiments. dsRNA duplexes were generated in-house using a T7 transcription-based approach. A 410 base pair transcription template was polymerase chain reaction (PCR) amplified from a *B. malayi* L3 stage cDNA library (kindly provided by Dr. S. Williams, Smith College, MA) using gene specific oligonucleotides designed to incorporate a T7 promoter sequence (TAATACGACTCACTATAGGGTACT) at both the 5' and 3' ends of the amplicon. For the *Bm-cpl-1* template, oligonucleotide sequence was: L1T7dsRNAF 5' TAATACGACTCACTATAGGGTACTACGGTTACCAAATTC 3' and L1T7dsRNAR 5' TAATACGACTCACTATAGGGTACTCGACAACAACAGGTC 3'. The location of this transcription template was carefully chosen so as to exclude the pro region of *Bm-cpl-1*, a domain with high sequence homology to other cathepsin L family genes, and consequently increase the specificity of this dsRNA duplex. Transcription templates were gel purified and dsRNA duplexes synthesized using the MEGAscript RNAi Kit (Ambion, TX) according to manufacturer's protocols. dsRNA species were quantified with a NanoVue spectrophotometer

(GE Healthcare, NJ) prior to use. The timing of siRNA or dsRNA injection into *B. malayi*-infected mosquitoes coincided with the presence of the parasite stage of interest: to target second larval stage (L2) parasites siRNA or dsRNA were injected five to eight dpi; to target third larval stage (L3) parasites siRNA or dsRNA were injected nine to 12 dpi (and for the lifespan of mosquito) [62]. The mosquitoes were processed to confirm suppression of the target gene, as described below, 48 h post-injection of siRNA or dsRNA.

D. Relative Quantitative RT-PCR

Brugia infected, RNA-treated and control mosquitoes were cold-anesthetized on ice. Total RNA was extracted from individual mosquitoes using RNAqueous Kit (Ambion, TX) before DNase treatment using the TURBO DNA-free Kit (Ambion, TX) in thin-walled PCR tubes. The RNA was stabilized with RNase Out Inhibitor (Invitrogen, CA) and stored in RNase-free microcentrifuge tubes at 4°C. This RNA was used as a template for a relative semi-quantitative multiplex RT-PCR using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, CA). The principle of this reaction is to amplify a target gene of interest and compare its intensity with a multiplexed and normalized internal standard during the linear phase of product amplification. A putative neuropeptide encoding gene, *Bm-flp-14* (Accession number AI508026) served this role. This gene was chosen as we had previously determined its stable transcript production during the *B. malayi* L3 stage by PCR (C. Song, unpublished). The oligonucleotide primers used to amplify *Bm-cpl-1* were: CPL-1 F 5' ACAGGGCAATATGACGAGAC 3' and CPL-1 R 5' ATCGAAGCAACGTGGCACAT 3'. These primer locations flank the region of *Bm-cpl-1* homologous to the dsRNA construct. The oligonucleotide primers used to amplify the *Bm-flp-14* internal standard were: FLP-14 F 5' CTCGTCCACTCTTATCACTG 3' and FLP-14 R 5' ACCGCAATGATATACAACATATA 3'.

The profile for this PCR was: cDNA synthesis at 50°C for 30 minutes; an initial denaturation phase of 94°C for 2 min; 38 cycles of 94°C for 30 s, 60°C for 30 s, 68°C for 1 min and a final extension phase of 68°C for 5 min. Reactions were visualized on a 1.2% agarose gel containing ethidium bromide.

E. Quantitative RT-qPCR

Total RNA was extracted from individual mosquitoes and DNase-treated as described above for three replicated RNAi experiments and before addition of RNase Out Inhibitor and storage, each RNA sample was quantified spectrophotometrically per a previous report [256]. This RNA served as a template in our RT-qPCR assays using the qScript One-Step Fast RT-PCR Kit with ROX (Quanta BioSciences, MD).

Establishing PREXCEL-Q Parameters

PREXCEL-Q, a qPCR assay development and project management software, was used to establish our RT-qPCR parameters and to determine valid working ranges for all of our samples per target and reference genes. A mixture of the RNA samples was used to determine the optimum template to use while avoiding RT-qPCR inhibition for each of the three targets at concentration ranges of between 0.01 and 0.08 ng/μL for the siRNA experiments and between 0.02 to 0.14 ng/μL for the dsRNA experiments. For subsequent quantitative assessment of transcript abundance, each RNA sample was diluted to 0.06 ng/μL for the siRNA RT-qPCR study and 0.11 ng/μL for the dsRNA RT-qPCR study, with 6 μL of sample used per 25 μL reaction.

Primers and Probes

The target sequence under evaluation in the RT-qPCR study was the *B. malayi* cathepsin L-like transcript previously described. Two reference genes were used, a neuropeptide-encoding gene *Bm-flp-14* also previously described, and *Bm-tph-1* (Accession number U80971), a tumor protein homolog-encoding gene that is a proven reference gene for qPCR of *Brugia* development in mosquitoes [228]. TaqMan minor groove binding (MGB) probes were used in this study to facilitate the use of shorter gene specific primer-probe sets. All probes and primers were designed using Primer Express v. 2.0 software (Applied Biosystems, CA) and synthesized by Applied Biosystems. The primer and probe sequences used are shown in Table 1.

Gene	Primer sequence (5' - 3')	Amplicon Size (bp)
<i>Bm-cpl-1</i>	Forward	GGTTACGGAACGCATCGAA
	Reverse	TGGGTTCCCCAGCTATTTTTAA
	Probe	6FAM-TCACGGTGATTACTGGAT-MGBNFQ
<i>Bm-flp-14</i>	Forward	TGGGAAGAGGAAGCATGAATACTT
	Reverse	TGCAGCGGGAACCTTTGATC
	Probe	6FAM-AGATTTGGTCGTAAGTAGTTG-MGBNFQ
<i>Bm-tph-1</i>	Forward	TTGCAACGATATGTTGATCTTCAA
	Reverse	ACGAGTCCGACGCAAGCT
	Probe	6FAM-ATGCATTCACAGATGAC-MGBNFQ

Table 1. Primer and TaqMan probe sequences for RT-qPCR experiment.

TaqMan RT-qPCR

25 μ L volume reactions were prepared in duplicate for each RNA sample, and 20 μ L of this reaction mixture is applied per well on a 96-well plate (using white-well reaction plates, Eppendorf, NY). Individual components of each RT-qPCR reaction were as follows: 6 μ L prediluted RNA (as determined by PREXCEL-Q), 6.25 μ L 4X One-step Fast Master Mix with ROX, 1.25 μ L qScript One-Step Fast RT, 775 nM each primer, 150 nM probe, nuclease-free water to 25 μ L. Cycling conditions included an initial cDNA synthesis step of 50°C for 5 min followed by an RT denaturation/Taq activation phase of 95°C for 30 s then 45 cycles of 95°C for 3 s and 58°C for 30 s. Four point standard curves were created for each target (within the ng/ μ L ranges already specified above) by diluting the RNA sample mixture in each case according to precise, PREXCEL-Q-determined parameters (eight-fold dilution from highest to lowest concentration). No-template control reactions substituted nuclease-free water for RNA, and thermocycling was performed on an ABI GeneAmp 5700 SDS (Applied Biosystems). Quantification cycle (C_q) values were obtained at an appropriate threshold per each target ($\sim 0.1 DR_n$ in all cases), and data were processed using custom Excel files by the efficiency-corrected ($E^{\Delta\Delta C_q}$) relative quantification method [227].

F. Phenotype Analysis

After confirmation of *Bm-cpl-1* suppression, multiple assays were performed to describe worm phenotypes. Each phenotypic assay was performed 14 dpi and at either four or seven d post-injection. Mosquitoes were cold-anesthetized then the wings and legs removed and discarded using a dissecting microscope. The head, thorax and abdomen were partitioned and further dissected to release the parasites. The following characteristics of dissected parasites were observed: (1) **Parasite location.** In order to be successfully transmitted, these parasites

have to actively migrate to the head of the mosquito and vigorously writhe free of the proboscis. Parasite migration through the mosquito was recorded and measured according to escape point from the mosquito body (abdomen, thorax or head). (2) *Worm motility*. A scoring schema of: one (immobile), two (compromised motility, immobile for stretches of time), three (sluggish, partial movement), four (in motion, some straight segments), or five (all parts of the worm in constant motion) was used to quantify parasite movement in a blind fashion by an independent evaluator. Additional observations of aberrant motility included knotting at one or both ends, paralysis of caudal region and presence of a distinct angular kink were also recorded. (3) **Parasite growth and development**. Digital images of RNAi and control worms were captured so that length and diameter could be calculated using NIS Elements D 2.30 software (Nikon, NY). (4) **Parasite viability**. The number of parasites that survived to the infectious stage was recorded so that infection prevalence and mean intensity could be calculated. (5) **Mosquito viability**. *We documented the* number of mosquitoes that survived through the development of parasites to the infectious stage because these parasites inflict significant pathology and decrease mosquito survival.

G. Microscopy

Nikon Eclipse 50i fluorescence microscope under UV light (EXFO, ON) equipped with a Hy-Q FITC filter set (Chroma, VT). Images were captured using a Digital Sight DS-2Mv camera and NIS Elements D 2.3 software (Nikon, NY).

H. Statistical Analysis

t-tests were used to analyze the effect of RNAi treatment on gene expression in the RT-qPCR experiments and parasite size, and ANOVA to analyze the effect of RNAi treatment on worm motility based on our one through five blind-scoring schema. Chi square tests were used to analyze the effect of RNAi treatment on all other worm and mosquito behaviors assayed. In all tests, *P* values ≤ 0.05 were considered statistically significant.

IV. RESULTS

A. A *Brugia* RNAi Trigger Rapidly Disseminates Throughout the Mosquito Host

Our hypothesis is that mosquitoes provide an optimal culture and delivery system for an RNAi trigger targeted to developing *Brugia malayi* parasites. Healthy, viable, developing parasites are subjected to the RNAi trigger because the parasites undergo growth and development in the mosquito intermediate host. In order to test the extent of dissemination of the RNAi trigger from the site of intrathoracic injection, 150 ng of an equimolar mix of four 3' Cy 3-labelled *Bm-cpl-1* siRNAs was injected into adult *Aedes aegypti* mosquitoes as described. The dissemination of this RNAi trigger through the mosquito was tracked over 15 d post-injection by periodic microdissection of the mosquito and evaluation of internal fluorescence compared to saline injected controls. The labeled siRNA mix spread rapidly from the site of injection and maximal fluorescence was observed 24 h post-injection (Figure 6). The intensity of fluorescence slowly decreased until reaching basal levels at five d post-injection after which fluorescence intensity was not appreciably different from control mosquitoes. Our observations closely parallel those of a previous report that describes the spread of 140 ng AlexaFluor 555-labeled siRNA in the mosquito *Anopheles gambiae* from an injection site to the midgut and pericardial cells 36 h post-injection [226]. Systemic dispersion and persistence of RNAi signal from the site of injection suggests *B. malayi* larvae are likely to be exposed to the RNAi trigger in our experimental model.

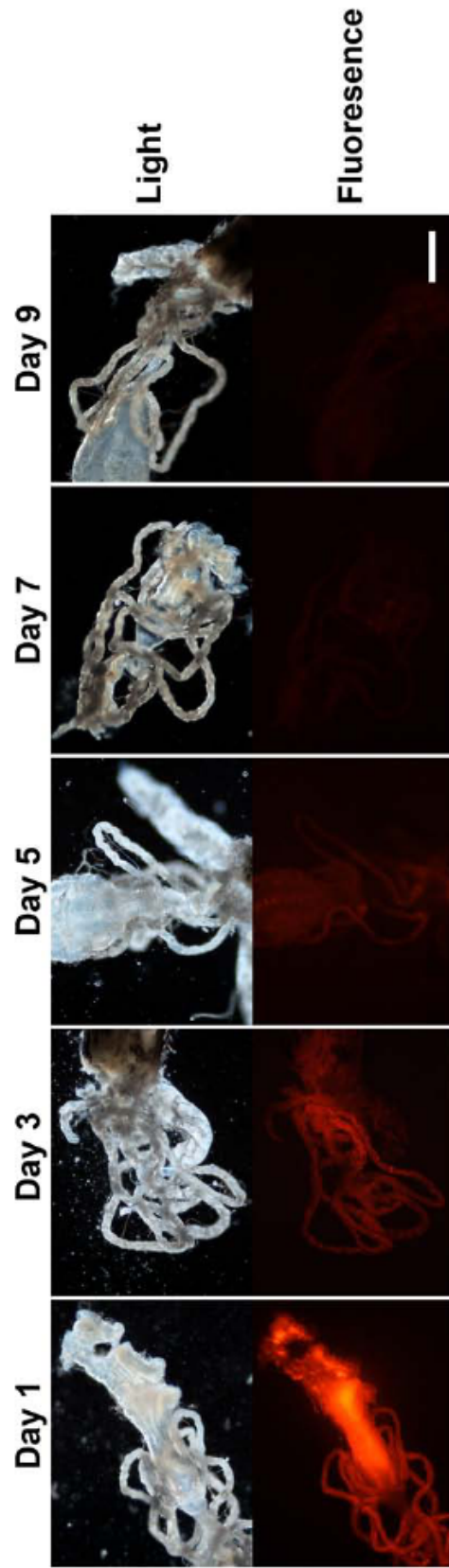


Figure 6. Dissemination and persistence of intrathoracically injected Cy 3-labelled *Brugia malayi* Cathepsin-L1 siRNAs in *Aedes aegypti*. Midgut and Malpighian tubule tissues are shown in light (upper panel) and fluorescence (lower panel) micrographs from 1 to 9 days post-injection (scale bar 100 μ m).

B. *Brugia* Gene Suppression *In Vivo* is Potent and Specific

Recently it has been shown that *B. malayi* genes encoding cathepsin L-like enzymes can be suppressed *in vitro* by soaking adult parasites in culture media containing siRNA [200]. We tested the capacity of our novel methodology to suppress larval stage *B. malayi* gene expression *in vivo* by injecting mixed siRNAs specific to the cathepsin L-like *Bm-cpl-1* gene directly into *Ae. aegypti* mosquitoes harboring L3 stage *B. malayi* parasites. Gene suppression was assayed 48 h post-injection using a semi-quantitative RT-PCR in which the intensity of *Bm-cpl-1* amplification in the linear phase of the reaction was compared to an internal *B. malayi* reference gene (*Bm-flp-14*) that is expressed stably and at comparable levels to *Bm-cpl-1*. Control mosquitoes were injected with equal volumes of *Aedes* physiologic saline. This methodology was optimized to amplify *Bm-cpl-1* from a heterogeneous mosquito/parasite total RNA preparation from a single mosquito. Suppression was concentration-dependent because injection of 0.15 ng siRNA did not appear to reduce *Bm-cpl-1* transcript levels. However, injection of 15 ng or 1.5 ng of siRNA decreased transcript levels, and injection of 150 ng mixed siRNA into mosquitoes profoundly suppressed *Bm-cpl-1* expression; the target parasite gene could not be amplified (Figure 7). This suppression was also specific; expression of the *Bm-flp-14* reference gene was unaffected by siRNA injection and target gene expression was normal in saline-injected controls.

Application of dsRNA is the commonly used method for triggering RNAi in parasitic nematodes and has advantages over siRNA; dsRNA can be generated in-house more quickly than commercially produced siRNAs at lower cost. *B. malayi*-infected mosquitoes were also subjected to treatment with dsRNA as an RNAi trigger. The effect of dsRNA was concentration-dependent such that injection of 15 ng dsRNA results in *Bm-cpl-1* suppression but

1.5 ng dsRNA had no appreciable effect. Injection of 150 ng of dsRNA potently suppressed *Bm-cpl-1* transcript abundance and suppression appeared specific, with *Bm-flp-14* expression unaffected by dsRNA (Figure 7).

RT-qPCR was used to quantify the level of *Bm-cpl-1* suppression relative to two reference genes (*Bm-flp-14* and *Bm-tph-1*) using the efficiency-corrected ($E^{\Delta\Delta C_q}$) relative quantification method [227]. PREXCEL-Q software was used to optimize the performance of the RT-qPCR assay; and important data pertinent to PCR efficiency, linear dynamic range and normalization of the assay are documented in Table 2. *Bm-tph-1* showed stable C_q values across the experiment and therefore was the most appropriate reference gene for these studies, as shown previously [228]. The suppressive effects of both RNAi treatments were almost identical; injection of 150 ng siRNA reduced *Bm-cpl-1* transcript by 83% compared to saline-injected controls ($P < 0.0001$) and 150 ng dsRNA also reduced *Bm-cpl-1* transcript by 83% ($P < 0.0001$) (Figure 8). *Bm-flp-14* reference gene transcript was slightly reduced by both RNAi treatments but these reductions were not significant (siRNA, 9%, $P = 0.38$; dsRNA, 12%, $P = 0.17$). These data support the gel-based semi-quantitative RT-PCR experimental findings and demonstrate the efficacy of this novel method of RNAi delivery.

	<i>Bm-cpl-1</i>			<i>Bm-flp-14</i>			<i>Bm-tph-1</i>		
	siRNA	dsRNA		siRNA	dsRNA		siRNA	dsRNA	
RT-qPCR efficiency (%)	84.7	65		108.2	116.5		104.2	96.4	
Calibration curve y intercept	30.6	32.9		33.9	35.9		29.5	33	
Calibration curve r^2	0.98	0.97		0.91	0.88		0.99	0.88	
Mean C_q	30.4±0.06	33.0±0.48		34.3±0.13	35.7±0.19		30.0±0.08	33.1±0.10	
NTC C_q	50	50		50	50		50	50	

Table 2. Reportable information on RT-qPCR experiment.

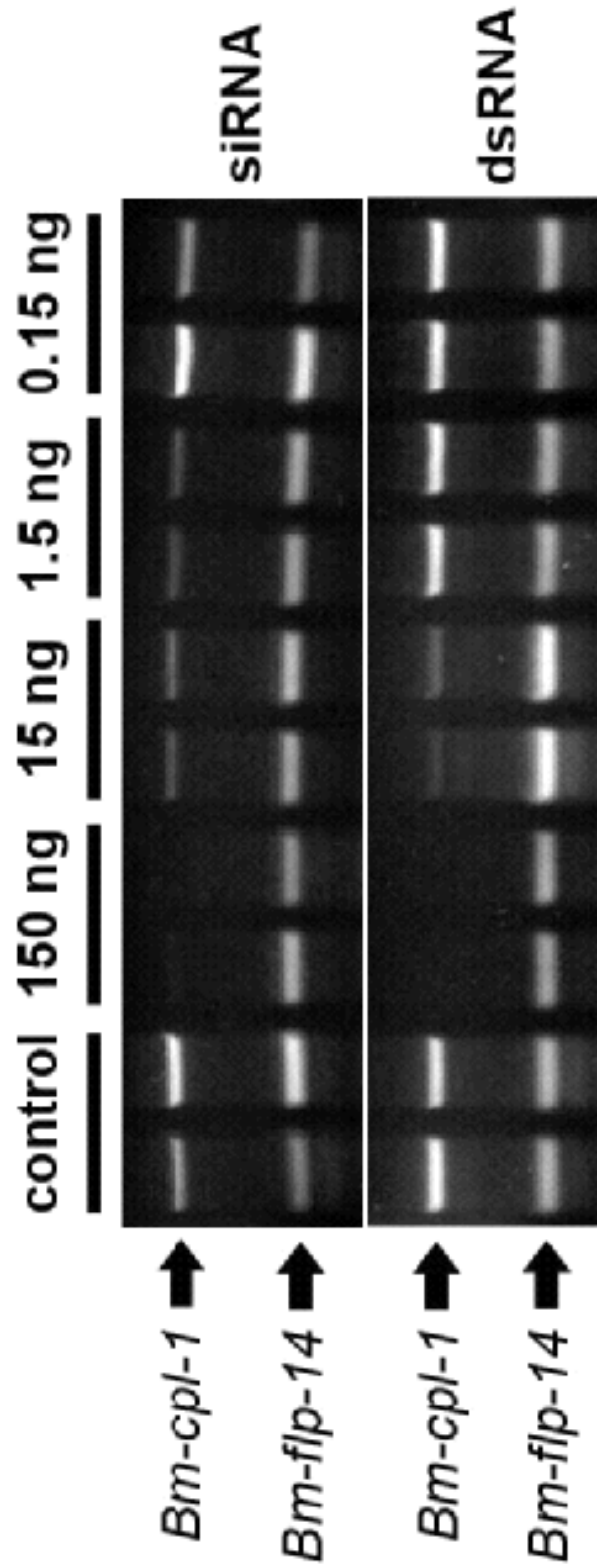


Figure 7. Concentration-dependent, *in vivo* suppression of *Brugia malayi* Cathepsin-L1 (*Bm-cpl-1*) using siRNA (Top) or dsRNA (Bottom) RNAi triggers. Micrograph shows ethidium bromide stained agarose gel electrophoresis of relative RT-PCR analysis of individual *B. malayi*-infected mosquitoes 48 h post-injection of RNAi trigger at 10 d post-infection. Amplified product for the target gene, *Bm-cpl-1*, is shown above a neuropeptide reference gene (*Bm-flp-14*).

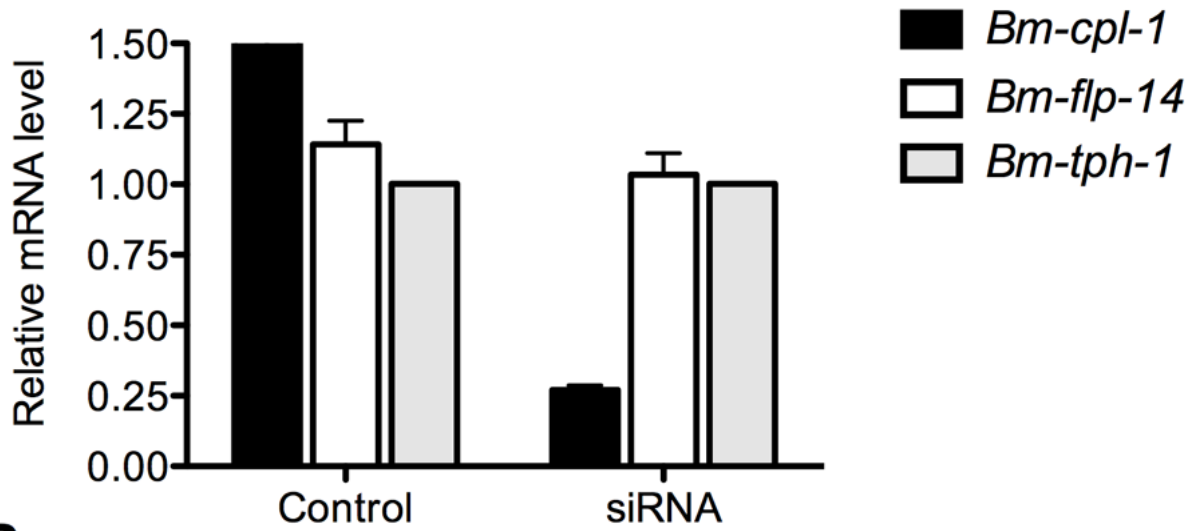
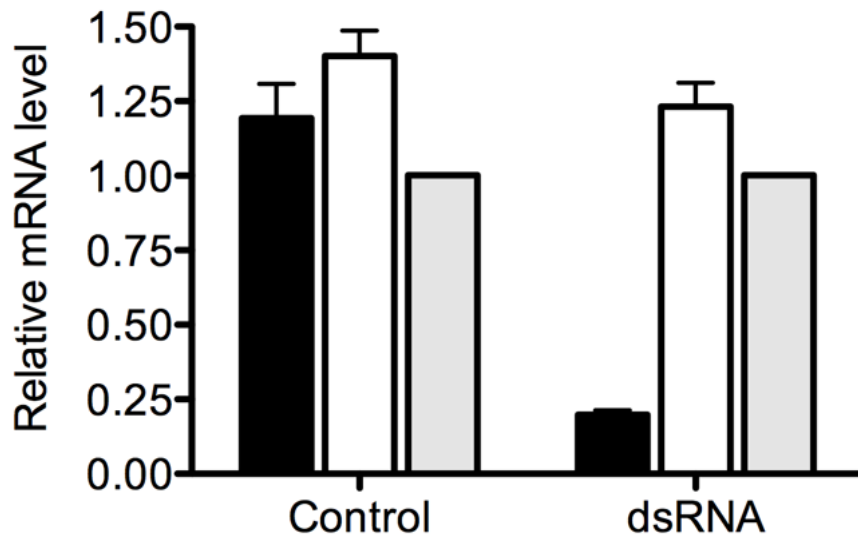
A**B**

Figure 8. Quantitative PCR demonstrates significant reduction in *Bm-cpl-1* transcript levels as a result of siRNA and dsRNA RNAi trigger injection into *B. malayi* infected *Aedes. aegypti*. Both siRNA (A) and dsRNA (B) injection reduces *Bm-cpl-1* transcript by 83% compared to controls (saline injected *Ae. aegypti* infected with *B. malayi*). *Bm-cpl-1* and control gene, *Bm-flp-14*, are normalized to a reference gene *Bm-tph-1*. qPCR was performed 48 h post-injection of RNAi trigger at 10 d post-injection. Each bar represents 13 mosquitoes from three biological replications.

C. *Bm-cpl-1* Suppression Elicits Marked Motility and Developmental Phenotypes

Previous studies have described aberrant filarial worm phenotypes associated with cathepsin L-like gene suppression *in vitro* including decreased microfilariae (mf) release from adult *B. malayi* [200] and an inhibition of the L3 to L4 molt in *Onchocerca volvulus* [2]. Based on these data, we predicted that *Bm-cpl-1* suppression would produce a phenotype *in vivo* in the mosquito host. Mosquitoes were injected with 150 ng *Bm-cpl-1* dsRNA 10 d post-infection (dpi) then microdissected four d post-injection to harvest L3-stage parasites. Worm motility was digitally recorded and scored according to a five-point schema of one (immobile), to five (all parts of the worm in constant motion). 100% of control worms from mosquitoes injected with *Aedes* physiologic saline were categorized as four or five on this scale. *Bm-cpl-1* suppression significantly inhibited this normal worm motility ($P < 0.001$), with only 67% of worms ranked as four or five on the scale (Figure 9). To confirm that this effect was *Bm-cpl-1* specific and not due to exogenous dsRNA impairing worm viability, this experiment was repeated with dsRNA for enhanced GFP (eGFP) as a random exogenous RNA. These worms were phenotypically identical to saline-injected controls (100% category four or five), confirming the specificity of the aberrant phenotype in *Bm-cpl-1* suppressed worms.

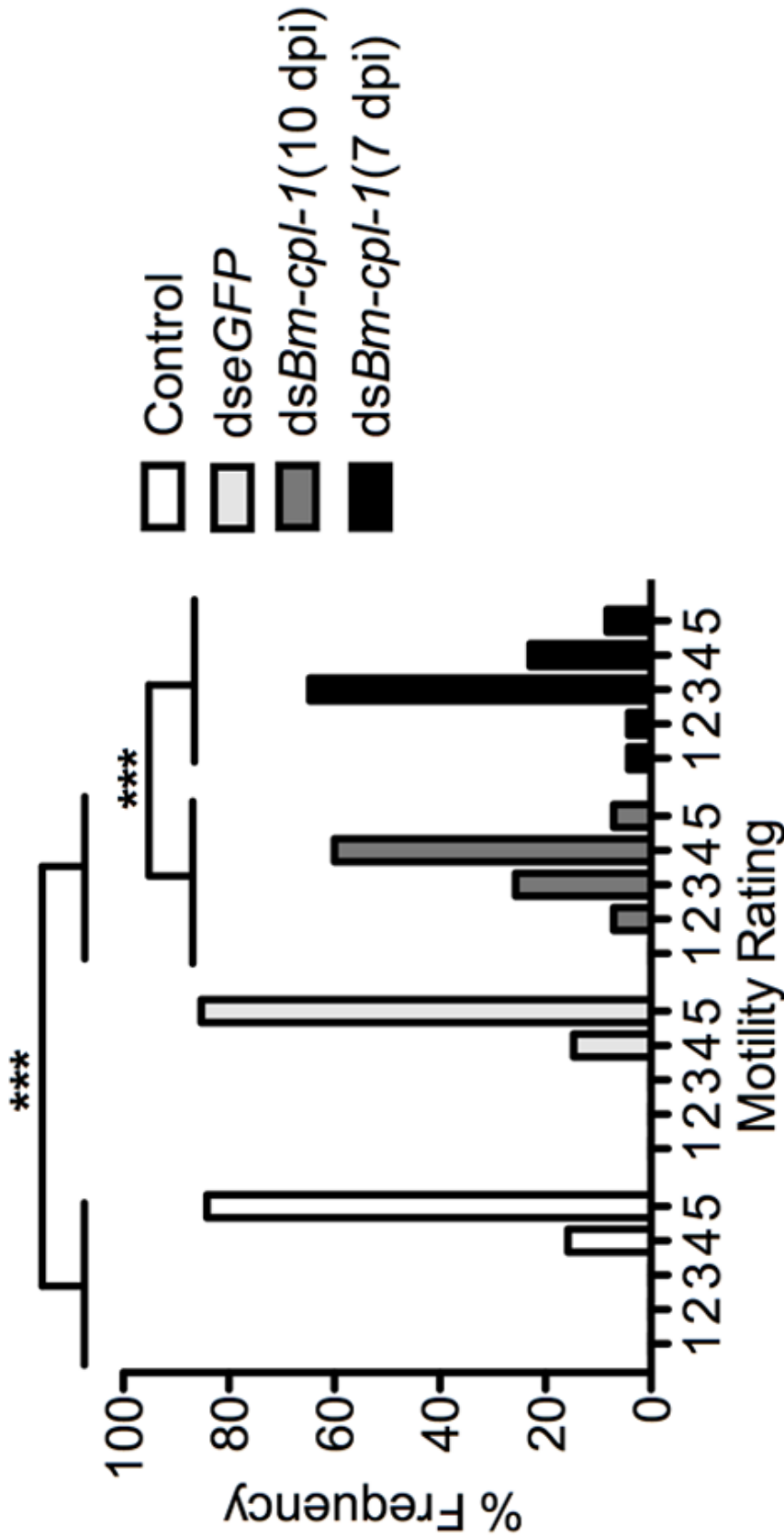


Figure 9 . Aberrant motility of dsRNA *Bm-cpl-1* exposed *B. malayi*. Frequency distribution for motility of L3 stage *B. malayi* recovered from *Ae. aegypti* showing significantly reduced motility of *Bm-cpl-1* suppressed worms. Parasitized mosquitoes were injected with saline (control), 150 ng *eGFP* dsRNA, or 150 ng *Bm-cpl-1* dsRNA at 7 or 10 d post-infection (dpi), then dissected to obtain parasites at 14 dpi. Parasite motility was scored on a 1-5 scale, with 1 = immobile and 5 = all parts of worm in constant motion (control n=101, ds*eGFP* n=68, ds*Bm-cpl-1* 10 dpi n=70, ds*Bm-cpl-1* 7 dpi n=48, P<0.001).

The effect of changing the timing of *Bm-cpl-1* suppression on worm motility was also examined. *Bm-cpl-1* transcript levels are elevated in L3 stage parasites, such that this gene has a purported role in the L3 to L4 molt [2]. The temporal expression of *Bm-cpl-1* was reported to be up-regulated during the L2 to L3 transition, at six to seven dpi [229]. Based on the timing of *Brugia* development in *Ae. aegypti* [62], infected mosquitoes were injected with *Bm-cpl-1* dsRNA at 10 dpi in order to target L3-stage worms (described above) and at seven dpi to target the L2 to L3 transition. Parasites exposed to *Bm-cpl-1* dsRNA at seven dpi showed significantly inhibited motility compared to saline controls ($P < 0.001$) with only 31% of worms displaying normal motility. The difference between parasites exposed to dsRNA at seven and 10 dpi was significant ($P < 0.001$), and may reflect an important biological role for *Bm-cpl-1* during the transition from L2 to L3 stages. More explicitly, earlier exposure to the RNAi trigger could impose more significant detrimental impact on the parasite by disrupting the L2 to L3 molt, or it may simply be a consequence of the longer period of time from gene suppression to phenotype assay, allowing Bm-CPL-1 rundown and maturation of the phenotype.

In addition to depressed activity, other morphological and motility phenotypes were apparent in *Bm-cpl-1* suppressed worms. A highly active, convoluted body form characterizes motility of healthy *B. malayi* L3s, both the heads and tails of the parasites in particular are conspicuously tortuous – curvature we described as ‘knotted’. Control worms from saline-injected mosquitoes frequently (86% of worms) displayed knotting at both ends. Suppression of *Bm-cpl-1* 10 dpi significantly inhibited this motility, because only 14% of worms presented with both ends knotted ($P < 0.001$) (Figure 10). This phenotype was enhanced by an early suppression of *Bm-cpl-1* at seven dpi such that no *Bm-cpl-1* suppressed

parasites exhibited this knotting morphology. The difference between L2 and L3 *Bm-cpl-1* suppression was significant ($P = 0.005$). Worms exposed to the exogenous eGFP dsRNA control confirmed that this phenotype was gene-specific because parasite motility was not significantly different from saline controls (85% knotted at both ends, $P = 0.2$). Another aberrant motility observed was the presence of a perturbed section of body wall slightly caudal to the midpoint of the worm. This abnormal kinked morphology was absent from control worms (4% of worms from saline-injected and 0% from eGFP-injected mosquitoes displayed this morphology), but evident with significantly greater frequency in 10 dpi *Bm-cpl-1* suppressed worms (47%, $P < 0.001$) (Figure 11). This kink rate increased with *Bm-cpl-1* suppression at seven dpi (63%), but compared to 10 dpi this was not significant ($P = 0.08$). Finally, partial paralysis of *Bm-cpl-1* suppressed worms was evident, presenting as immobility in the caudal third of the worm. This paralysis was observed in 61% of 10 dpi *Bm-cpl-1* suppressed worms, and 83% of seven dpi suppressed worms (this increase was significant, $P = 0.005$) but was generally absent from control worms (5% of worms from saline-injected mosquitoes and 3% of worms from eGFP-injected mosquitoes) (Figure 11).

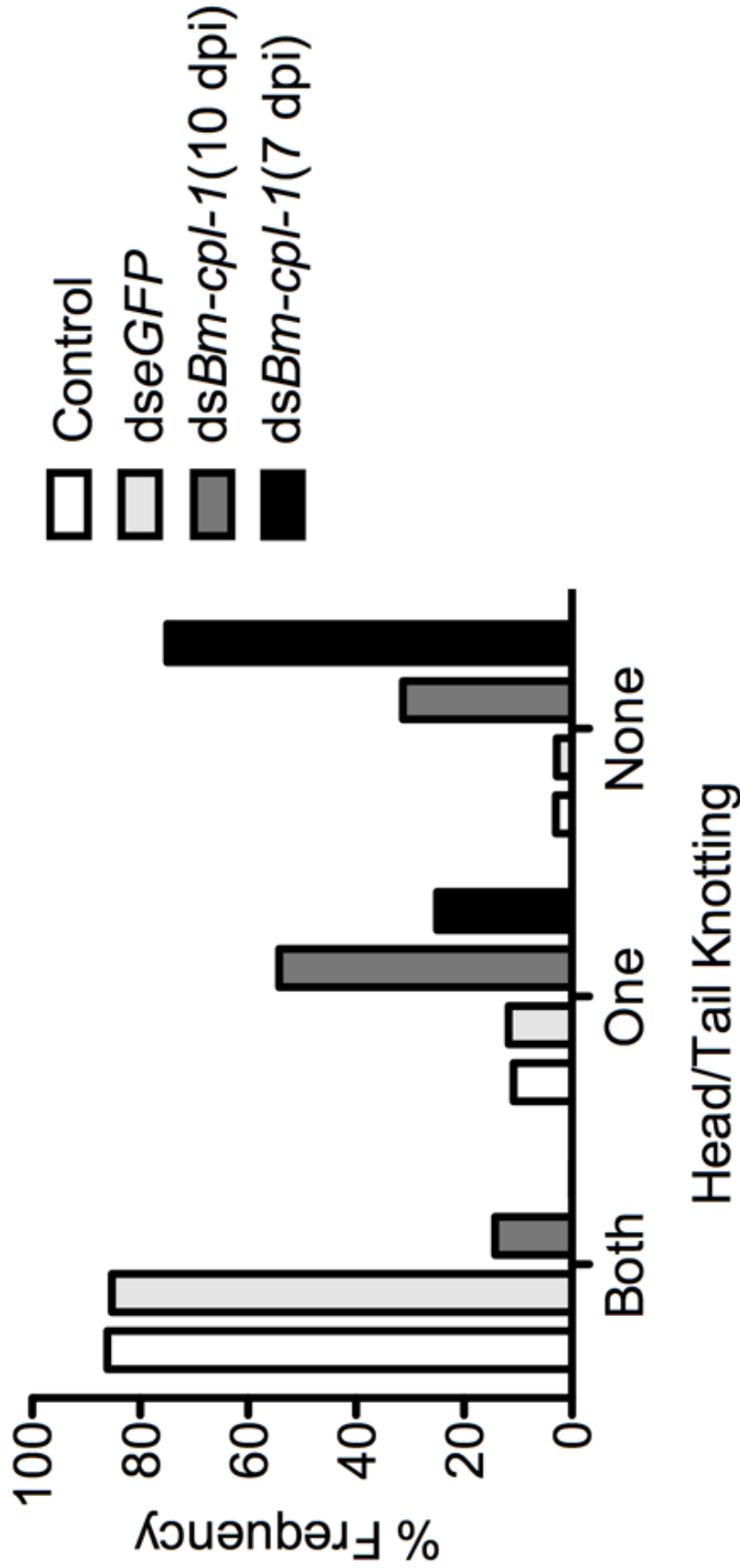


Figure 10. Disrupted motile phenotypes of dsRNA *Bm-cpl-1* exposed *B. malayi*. Frequency distribution for parasites exhibiting rigorous knotting behavior at both ends of worm, one end, or not at all showing normal terminal curvature is inhibited by *Bm-cpl-1* suppression. Parasitized mosquitoes were injected with saline (control), 150 ng eGFP dsRNA, or 150 ng *Bm-cpl-1* dsRNA at 7 or 10 d post-infection (dpi), then dissected to obtain parasites at 14 dpi (control n=101, dseGFP n=68, ds*Bm-cpl-1* 10 dpi n=70, ds*Bm-cpl-1* 7 dpi n=48).

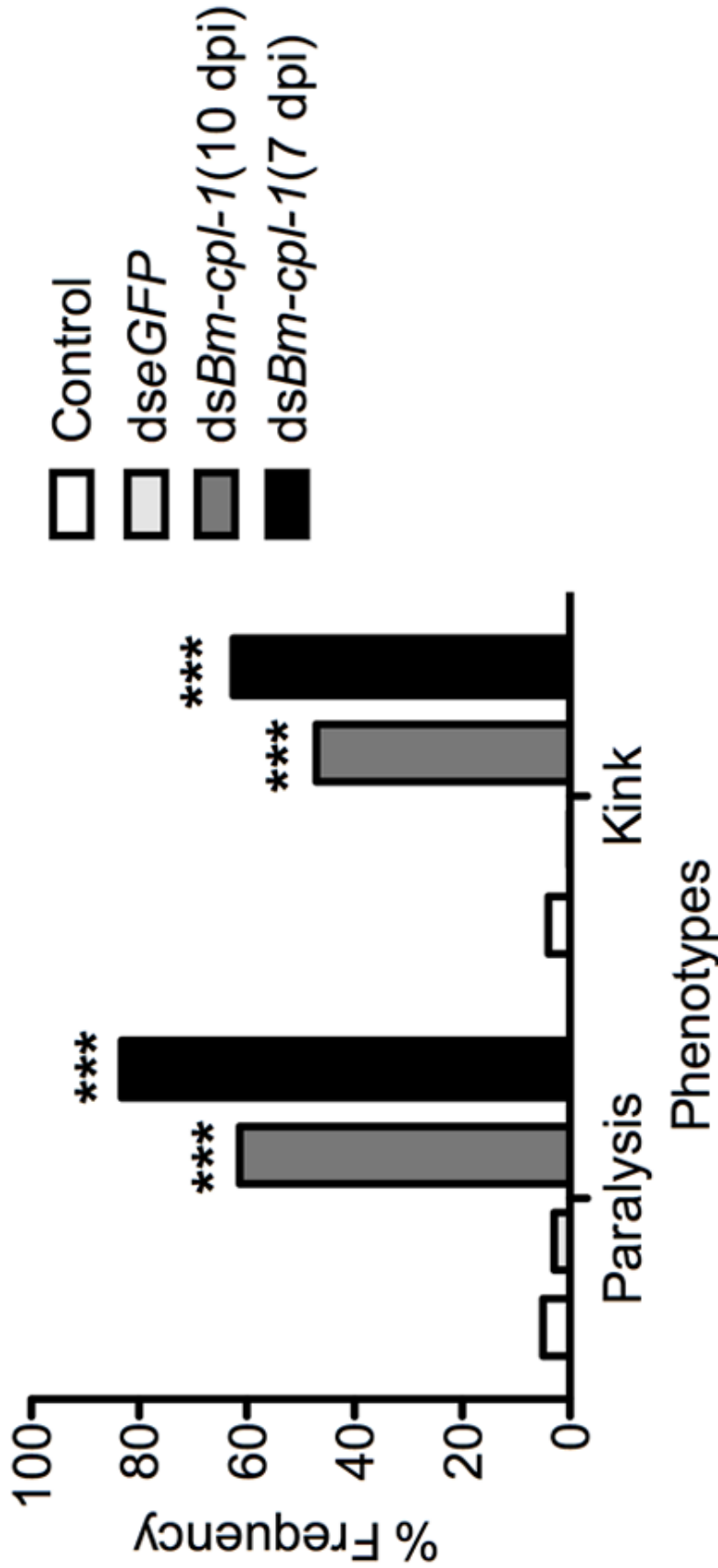


Figure 11. The frequency of caudal paralysis and kinked posture of dsRNA *Bm-cpl-1* exposed *B. malayi*. The frequency of both caudal paralysis and kinked posture is significantly increased with *Bm-cpl-1* suppression. Parasitized mosquitoes were injected with saline (control), 150 ng *eGFP* dsRNA, or 150 ng *Bm-cpl-1* dsRNA at 7 or 10 d post-infection (dpi), then dissected to obtain parasites at 14 dpi (control n=101, *dseGFP* n=68, *dsBm-cpl-1* 10 dpi n=70, *dsBm-cpl-1* 7 dpi n=48, $P < 0.001$).

To examine the consequence of this aberrant motility on *B. malayi* development, mosquitoes were injected with 150 ng *Bm-cpl-1* dsRNA 10 dpi then microdissected four d post-injection, partitioning the mosquitoes into head, thorax and abdomen preparations. Control worms from mosquitoes injected with either saline or eGFP dsRNA were found exclusively (100%) in head preparations as expected. *Bm-cpl-1* suppressed worms were most frequently observed escaping from the thorax and abdomen (Figure 12). Parasites in *Bm-cpl-1* dsRNA-injected mosquitoes, however, did not leave the thorax (94% of worms were found here) or abdomen (6%). *Bm-cpl-1* suppression, therefore, prevents worm migration to the head of the mosquito, effectively preventing normal progression of the parasite life cycle and thus abolishing the potential for parasite transmission.

A significant negative effect also was seen on growth and development of parasites subjected to *Bm-cpl-1* suppression. Parasitized mosquitoes were injected with 150 ng *Bm-cpl-1* dsRNA seven dpi then microdissected 14 dpi and the length, width and appearance of the worms recorded. Mosquitoes were injected seven dpi because the previous motility experiments dictated that this experimental timing generated the most pronounced motility phenotypes. *Bm-cpl-1* suppression significantly reduced the length of L3 worms by 48% ($P < 0.0001$) (Figure 13). The mean length of control L3, removed from mosquitoes seven d after saline injection and 14 dpi, was $1347 \pm 18 \mu\text{m}$. This was reduced to $700 \pm 49 \mu\text{m}$ after RNAi treatment. Unlike parasite length, width was not significantly affected by *Bm-cpl-1* suppression, although a slight decrease of 5% was observed ($P = 0.39$) from $31 \pm 1 \mu\text{m}$ in control worms to $30 \pm 2 \mu\text{m}$ in RNAi worms. In addition to worm length, the majority of *Bm-cpl-1* dsRNA parasites also presented with additional aberrant developmental phenotypes. Most evident was a disruption of the cuticle (Figure 13B), which extended significantly beyond the body of the worm. Some degree of this

cuticular sloughing was noticed in most worms but the severity of this phenotype was variable. Finally, the integrity of the gut appeared compromised in *Bm-cpl-1* worms. In such instances, the gastrointestinal tract of the parasites appeared incomplete and porous when examined at the light microscope level.

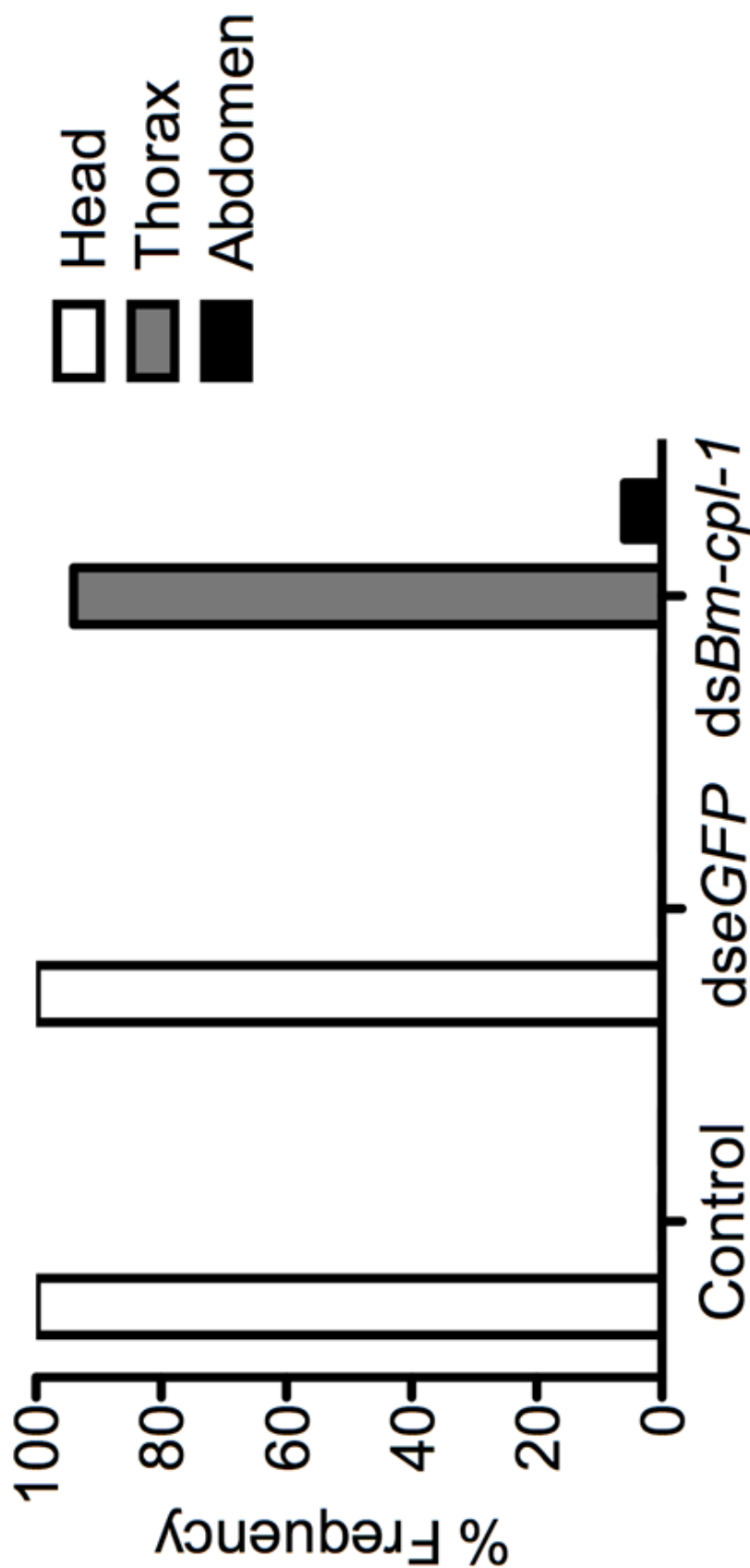


Figure 12. dsRNA *Bm-cpl-1* exposed *B. malayi* fail to migrate to the head of the mosquito. Frequency distribution of infectious (L3) stage *B. malayi* recovered from *Ae. aegypti* in the head, thorax or abdomen of the mosquito host. Parasites were dissected from *Ae. aegypti* mosquitoes 14 d post-infection and 4 d post-infection of saline (control), 150 ng *eGFP* dsRNA or 150 ng *Bm-cpl-1* dsRNA. One worm was recovered from each mosquito. Numbers of mosquitoes dissected from three biological replicates: n=18 (control), n=20 (*dseGFP*), and n=31 (*dsBm-cpl-1*).

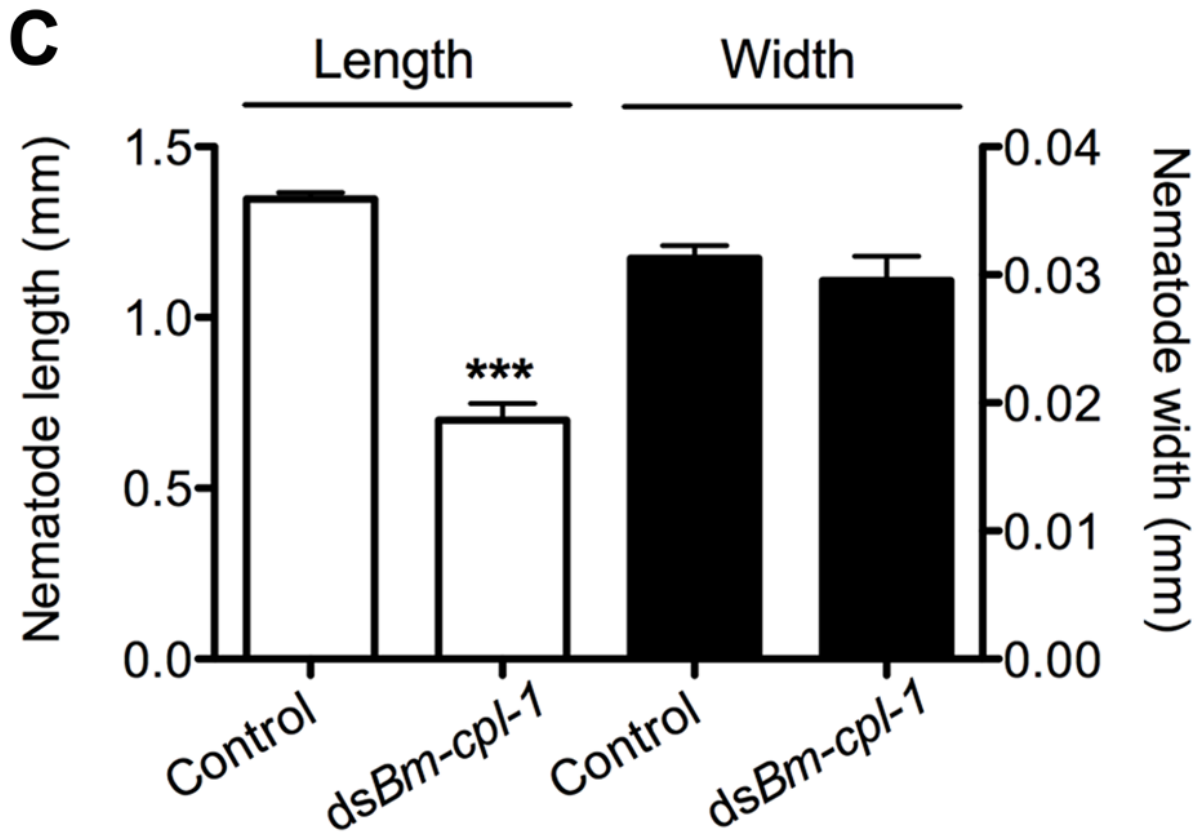
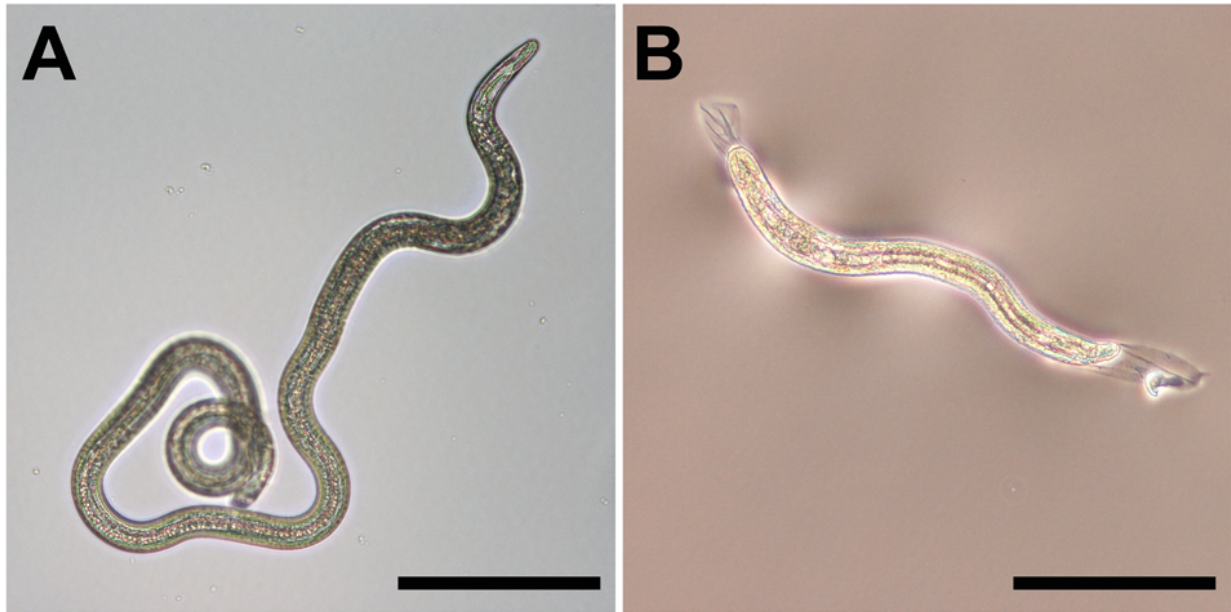


Figure 13. *Bm-cpl-1* suppressed *B. malayi* are significantly shorter than control worms. Parasites were dissected from *Ae. aegypti* mosquitoes 14 days post-infection and 4 d post-infection of saline (control, A) or 150 ng *Bm-cpl-1* dsRNA (B). Scale bar 250 μm . (C) RNAi exposed parasites are significantly shorter in length, but not width, than control worms. Numbers of parasites from three biological replicates: n=19 (control) and n=13 (ds*Bm-cpl-1*) ($P<0.001$).

D. *Bm-cpl-1* Suppression Enhances Mosquito Survival and Decreases Parasite Prevalence

Phenotype data resoundingly demonstrate that *Bm-cpl-1* suppression decreases *B. malayi* viability. It is logical to predict that this decreased viability would also have an impact on mosquito survival and prevalence of parasite infection. To examine this, mosquitoes were injected with 150 ng *Bm-cpl-1* dsRNA 10 dpi and the number of mosquitoes that survived through the development of parasites to the infectious stage, 14 dpi, was counted. Surviving mosquitoes then were microdissected to determine the proportion that harbored parasite infections. *Bm-cpl-1* suppression increases host mosquito survival. The survival rate of control mosquitoes injected with saline or eGFP dsRNA was 62% and 65% respectively ($P = 0.6$) as compared to 80% in *Bm-cpl-1* RNAi-exposed mosquitoes ($P < 0.001$) (Figure 14A). Early suppression of *Bm-cpl-1* at seven dpi enhanced the phenotype even more significantly such that 93% of mosquitoes were alive at the termination of the experiment ($P = 0.007$). This increased mosquito survival rate after *Bm-cpl-1* suppression may be as a result of the parasite's compromised ability to feed on, and migrate through, the host or may result from a more successful or effective host response against parasites with decreased viability. This hypothesis is supported by our observation that *Bm-cpl-1* suppression also decreased prevalence of infection – fewer surviving mosquitoes harbored parasites after *Bm-cpl-1* RNAi (Figure 14B). Every surviving mosquito injected with saline or eGFP was found to contain parasites 14 dpi, but 14 dpi *Bm-cpl-1* suppressed parasites (exposed to dsRNA at 10 dpi) were found in just 76% of mosquitoes, a significant reduction in prevalence ($P < 0.001$). Prevalence was further reduced to 62% in parasites exposed to *Bm-cpl-1* dsRNA at seven dpi, a statistically significant decrease compared to worms exposed to the dsRNA trigger at 10 dpi ($P = 0.03$).

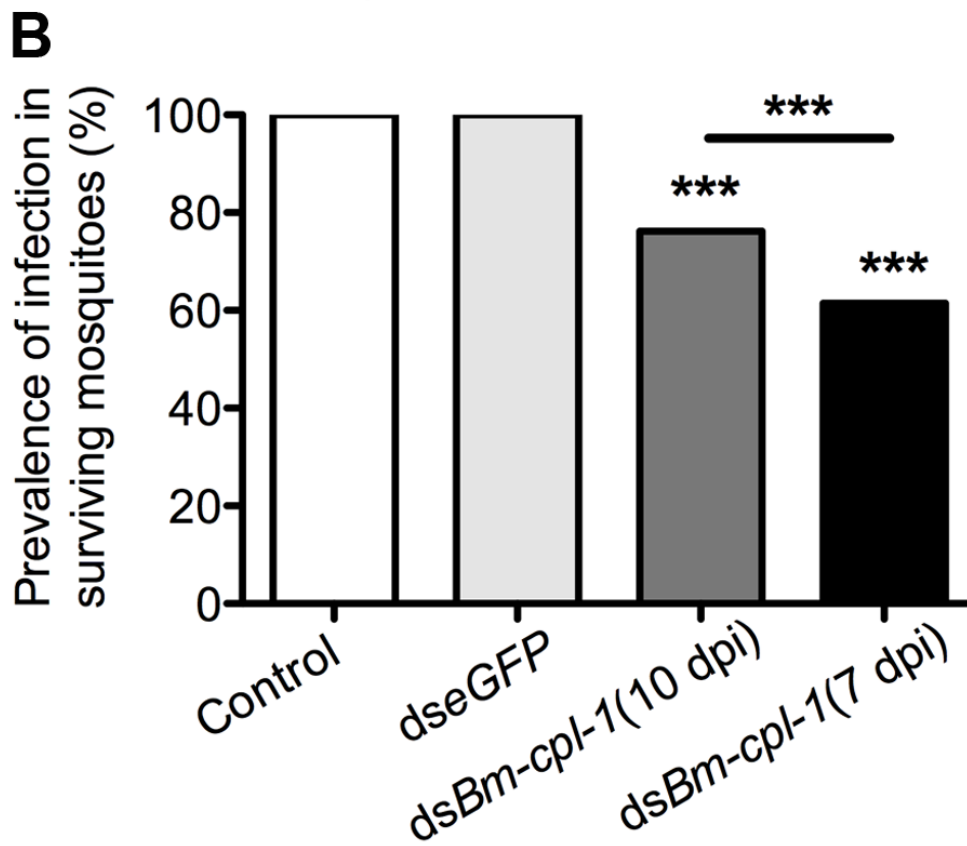
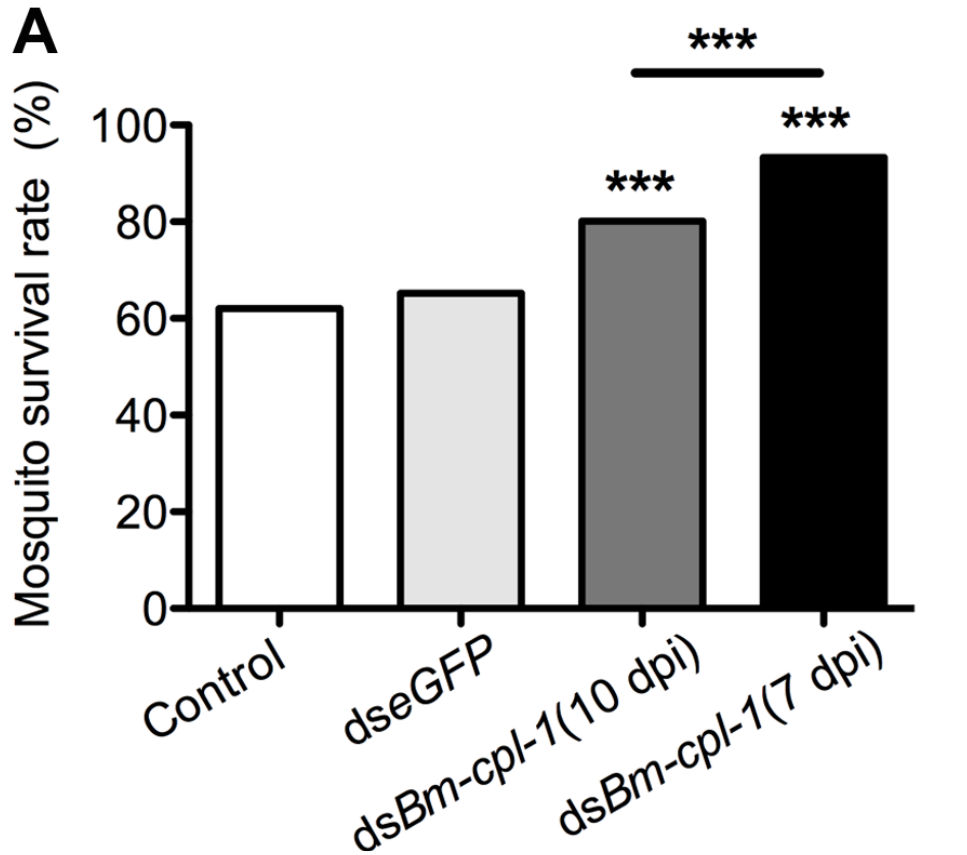


Figure 14. *Ae. aegypti* survival significantly increases as infection prevalence decreases in *Bm-cpl-1* dsRNA exposed mosquitoes. The effects on survival (A) and infection prevalence (B) are most profound in parasites exposed to *Bm-cpl-1* dsRNA at the L2/L3 transition (7 d post-infection, dpi). Parasites were exposed to saline (control), 150 ng *eGFP* dsRNA or 150 ng *Bm-cpl-1* dsRNA at 7 or 10 dpi, then were dissected from *Ae. aegypti* mosquitoes at 14 dpi.

V. DISCUSSION

Here we report the development of a novel *in vivo* approach to RNAi in the filarial nematode *Brugia malayi*, and describe its application first to suppress the expression of *Bm-cpl-1*, a *B. malayi* gene encoding a cathepsin L-like cysteine protease, then to validate this gene as a potentially potent anthelmintic drug target. To the best of our knowledge, this is the first description of *in vivo* RNAi in parasitic nematodes and represents an advance in the study of filarial nematode biology that may aid in the development of drugs to combat parasitic nematode infection. The rationale for developing an *in vivo* RNAi protocol stems from the hypothesis that RNAi is ineffective in animal parasitic nematodes because the supply of an RNAi trigger to the worms is inappropriate [224]. Our overarching hypothesis was that RNAi would work effectively and robustly if a trigger is supplied to healthy, viable worms in a host environment. Supporting this hypothesis, we were able to specifically reduce target gene transcript abundance in *B. malayi* larvae by 83% by supplying an RNAi trigger to parasites developing within the mosquito host. This level of transcript knockdown has not previously been reported using current *in vitro* RNAi soaking methods. The '*in squito*' approach to RNAi we describe is effective for the specific suppression of cathepsin genes in *Brugia* larval stages as they develop within their cognate mosquito host; it is therefore possible that this *in vivo* approach may represent a more effective means of eliciting gene suppression in filarial nematodes.

The mechanism by which the RNAi trigger is delivered to the parasites '*in squito*' is unclear, but could be a result of bathing the parasite in the trigger within a cell, or as a result of uptake by tissue ingestion. In support of the former, Cy3-labeled siRNA injected into the haemocoel rapidly disseminates throughout the mosquito supporting a hypothesis that the developing parasites are effectively incubating in a host milieu containing an RNAi trigger, essentially a

scenario analogous to *in vitro* RNAi by soaking. If this is the case, the '*in squito*' approach represents an efficient way to generate gene suppression by soaking. Most successful animal parasitic nematode *in vitro* soaking protocols use large amounts of ds- or siRNA with concentrations of 1 mg/ml typical, meaning anywhere between 25 µg and 2 mg of RNAi trigger are required per suppression experiment [2, 112, 198, 200, 210-215], with the exception of one report showing that lower trigger concentrations could still be effective at producing gene suppression by soaking [216]. Here we showed that gene suppression can be achieved using just 150 ng of ds- or siRNA per RNAi event, and indeed, a reduction in transcript abundance was observed after injecting as little as 15 ng dsRNA. In addition to the obvious cost saving advantages to performing RNAi experiments in this manner, such low RNA concentrations may also improve the specificity of gene suppression. Soaking plant parasitic nematodes in serial dilutions of ds- and siRNAs has been shown to reduce off-target effects in RNAi experiments [223, 230]. A second delivery hypothesis is that the developing parasites are ingesting the RNAi trigger. Microfilariae taken in during the blood meal rapidly penetrate the mosquito midgut [231], and migrate to the thoracic musculature where they grow and develop to the L3 stage [232, 233], a process completed in under two weeks [62]. From the L2 stage, the developing larvae are active feeders and consume host tissue [62, 234, 235], a behavior that would lead to the ingestion of an injected RNAi trigger in our experimental model. RNAi by feeding is a well-established method in free-living nematodes [217, 218, 236, 237]; by feeding these worms bacteria expressing dsRNA, systemic gene suppression can be effected in a relatively simple and efficient manner. This approach has not been successful with parasitic nematodes, however, as most parasitic species are not bacteriotrophic, and even for those species with bacteriotrophic life stages, this method is unreliable [225]. Resolution of the RNAi trigger delivery mechanism

afforded by our *in vivo* protocol may come through targeting *B. malayi* L1 worms in the mosquito. If target gene expression can be reduced in this non-feeding stage, this would support soaking as the prime mechanism.

The *in squito* suppression of *Bm-cpl-1* reveals new phenotypes associated with molting, growth and development, and motility that shed light on the important biological functions of this gene family in larval stages of *B. malayi*. Nematode molting is a three-stage process characterized by a shedding or separation of the old cuticle from the epidermis (apolysis), generation of a new cuticle, then the shedding of the old cuticle (ecdysis). The use of specific cysteine protease inhibitors markedly inhibits the L3 to L4 molt in filarial worms implicating cysteine proteases in general in this process [229, 238, 239]. More explicitly, both apolysis and ecdysis are disrupted giving rise to L4 parasites constrained within an L3 cuticle, termed an ‘accordion’ phenotype [229]. Multiple members of the cathepsin L-like family appear to be involved in molting as the specific suppression of *cpl-1* alone in *Onchocerca volvulus* reduced but did not abrogate the L3 to L4 molt [2]. We show that *Bm-cpl-1* is also involved in similar processes in *B. malayi* as its suppression manifested an aberrant cuticular phenotype in L3 worms. Examination of worms suppressed at seven dpi revealed an apparent sloughing of the cuticle without the accordion phenotype. As the L3 to L4 molt occurs in the vertebrate host this phenotype is not a disruption of the L3 to L4 molt, but rather a dysfunction in L3 cuticle formation, maintenance or development. Alternatively, we could be observing a disruption of the L2 to L3 molt. *Bm-cpl-1* expression is up-regulated in the L3 stage but the exact timing of this up-regulation as it relates to the transition from L2 to L3 stages is unclear. Guiliano *et al.* [229] report *Bm-cpl-1* up-regulation at six dpi, a window consistent with the L2 to L3 transition. If *Bm-cpl-1* performs the same function for the L2 to L3 molt as for the L3 to L4 molt, then the

sloughed cuticle we observe upon dsRNA injection at seven dpi could be that of the L2, with *Bm-cpl-1* suppression preventing ecdysis. Further examination of cuticle ultrastructure in these suppressed worms at the electron microscope level could provide evidence to this effect.

We observed a stunting of *Bm-cpl-1* suppressed L3 growth compared to control worms, a phenotype previously unreported either after chemical inhibition of cysteine proteases or gene suppression in other parasite stages. Normally at the end of the L2 stage parasites are 750 – 795 μm long and increase in length to approximately 1350 μm at the L3 stage within four d [232]. Our control L3, taken from mosquitoes injected with saline, had a mean length of 1347 μm corresponding closely with the published data. The mean length of *Bm-cpl-1* suppressed L3, however, was significantly shorter (700 μm). Suppression of this gene at the L2/L3 interface (seven dpi) arrests parasite growth and the L3 worms remain L2-sized within the mosquito. One explanation for this observation is that *Bm-cpl-1* suppression at the L2/L3 interface is inhibiting the L2 to L3 molt, L2 cuticle ecdysis is not successful and therefore the worms are constrained within it, unable to increase their length. Alternatively, the stunting may not be due to aberrant molting but rather an inhibition of normal CPL-regulated development or cellular remodeling post-molt as is seen in other nematodes [240]. RNAi suppression of *cpl-1* in *C. elegans* L3 by soaking produced significantly shorter and thinner adults [240] and the localization of *cpl-1* to the hypodermis in *C. elegans*, *O. volvulus* and *B. malayi* is consistent with a developmental role in nematodes [2, 229, 240]. Further, germline suppression of *cpl-1* in *C. elegans* by dsRNA injection generated an embryonic lethal phenotype but some embryos did progress to the L1 stage and those had incomplete gut development [240]. A repeatedly observed phenotype in our *Bm-cpl-1* suppressed L3 was a compromised gut that appeared fenestrated and poorly developed.

Finally, *Bm-cpl-1* suppression reduced normal L3 motility by up to 69%, increased atypical postural phenotypes including caudal paralysis, kinked appearance and reduced normal convolution at the head and tail of *B. malayi* L3 as compared to control worms. These behaviors made it impossible for the treated L3 to progress through to the culmination of development in the mosquito host, i.e., transfer to the definitive host. The dystaxic behaviors produced by the suppression of *Bm-cpl-1* suggest this gene has some role, directly or indirectly, in the neuromuscular activity of *B. malayi* L3 in the mosquito. It is certainly true that cathepsins are required for normal neuromuscular behavior in other helminths; suppression of a cathepsin L-like gene in the flatworm *Fasciola hepatica* generated several aberrant motile phenotypes including paralysis [241].

This study is the first to use the host as a delivery mechanism for animal parasitic nematode RNAi. The model of using the host as a delivery mechanism for RNAi has been established but has been restricted to plant pathology where the concept has an applied use with transgenic plants helping to control nematode infestation by RNAi mechanisms *in planta* [242-244]. An alluring corollary is that by generating transgenic mosquitoes capable of suppressing key nematode genes *in vivo* we may be able to abolish parasite transmission. We have already demonstrated here that *Bm-cpl-1* suppression *in vivo* prevents parasites migrating to the mosquito head and proboscis thus eliminating transmission potential. Transformation of a mosquito with an inverted-repeat (IR) transgene derived from *Bm-cpl-1* may result in endogenous transcription of a hairpin dsRNA, a trigger that conceptually would induce RNAi *in vivo* as described here and produce a mosquito incapable of transmitting lymphatic filariasis-causing worms. Methods to introduce transgenes into mosquito germlines are well established [245- 249] and proof of this principle has already been demonstrated for a mosquito-borne virus; transgenic lines of Dengue

virus-resistant mosquitoes were generated using a Dengue virus IR transgene driven by the carboxypeptidase A promoter, reducing virus transmission by an RNAi mechanism [250]. The viability of this approach is enhanced not only by the ability to transform important vector species but also by the identification of tissue-specific promoters to drive transgene expression in favorable tissues, for example, *act88F* [251] is a fly-specific promoter that drives gene expression in the flight musculature – the precise site of parasite development. Another positive impact this protocol may have on lymphatic filariasis control is as a means of better understanding the biology of current putative drug targets and generating new data that may validate proposed novel drug targets. This protocol introduces the ability to investigate mosquito-borne parasite life stages, allowing the critical examination of gene function in worms growing and developing in an optimum environment. This makes it possible to assay genes that encode known or proposed drug targets in a parasite within its native intermediate host, contextualizing the null phenotypes *in vivo* and accurately determining the consequences of target gene suppression producing a more valuable target validation. As an illustration, nematode cathepsins have been proposed as attractive novel drug targets [252] and we have further validated these drug targets *in vivo*, revealing new phenotypes, defining new biological roles and showing that *B. malayi* sans *Bm-cpl-1* are incapable of completing their life cycle. These data enhance the appeal of cathepsins as novel anthelmintic drug targets. Beyond cathepsins, this technique will have most utility in the investigation of known and potential antifilarial drug target genes expressed in both the mosquito-borne life stages and those life stages that are vulnerable to chemotherapeutic intervention.

CHAPTER 3. Confirming Robustness of the *In Vivo* RNAi Protocol Through Investigating Gene Function of Known and Potential Drug Targets in the Filarial Nematode, *Brugia malayi*

A paper to be submitted

Chuanzhe Song, Tim A. Day, Michael J. Kimber and Lyric C. Bartholomay

I. ABSTRACT

Lymphatic filariasis is a global public health concern, with over 120 million people infected in 81 endemic countries. Current efforts to control the spread of lymphatic filariasis are constrained by a limited portfolio of effective anti-filarial drugs and a paucity of tools to investigate parasitic nematode biology. The two currently available anti-filarial drugs, ivermectin and diethylcarbamazine citrate (DEC), are both inefficient at curing LF-causing worms like *Brugia malayi* from the host and function only to interrupt transmission through poorly understood microfilaricidal effects. As such, a deeper understanding of existing and new drug targets needs to be gained for the development of more efficient anti-filarial drugs. RNA interference (RNAi) is a reverse-genetics tool with great potential to identify novel drug targets and interrogate parasite gene function but RNAi on various parasitic nematodes has proven inconsistent and unreliable. Many current RNAi protocols are carried out *in vitro*, necessitating removal of the parasite from host prior to executing RNAi in an artificial environment. We have developed an alternative *in vivo* RNAi protocol (we term “*in squito*” RNAi) targeting the filarial nematode *B. malayi* as it develops in an intermediate host, the mosquito *Aedes aegypti*. To verify the consistency and potential of our previously developed *in squito* RNAi protocol to interrogate

potential novel drug targets, we suppressed the expression of two known and three putative anthelmintic drug targets in larval-stage *B. malayi*. The drug targets were genes encoding β -tubulin (*Bm-tub-1*), glutamate-gated chloride channel alpha subunit (*Bm-GluCl- α 3A*), G-protein coupled acetylcholine receptor 2 (*Bm-gar-2*), FMRFamide-like peptide 21 (*Bm-flp-21*), and prohormone convertase 2 (*Bm-pc-2*). Three repeated double-stranded RNA (dsRNA) injections of *dsBm-GluCl- α 3A*, *dsBm-tub-1*, *dsBm-gar-2*, or *dsBm-flp-21* resulted in phenotypic abnormalities including aberrant worm motility, survival and migration within the mosquito host.

II. INTRODUCTION

The filarial worm parasites, *Wuchereria bancrofti* (90% of cases) and *Brugia malayi* (10% of cases) [23] are the etiological agents of lymphatic filariasis (LF). The transmission of filarial worms is facilitated by mosquitoes that transmit infectious L3 stage filarial worms during blood feeding. LF causes damage to the infected individual's lymph system [29-35] that can lead to crippling morbidity and social stigmatization. The World Health Organization estimates that over 120 million or approximately 2% of the global population are infected and 1.2 billion in 81 endemic countries are considered at risk [24, 26]. Of the 120 million infected individuals, approximately 12.5% exhibit clinical symptoms associated with lymphedema such as elephantiasis, resulting in over 5 million Disability Adjusted Life Years (DALYs – healthy life years lost) annually [53].

Following the identification of LF as one of six potentially eradicable diseases by the World Health Assembly's International Task Force for Disease Eradication in 1997 [25], the Global Program for the Elimination of Lymphatic Filariasis (GPELF) has implemented a systemic mass drug administration (MDA) of diethylcarbamazine citrate (DEC) and albendazole, or ivermectin and albendazole in regions co-endemic with other filarial nematodes such as *Onchocerca volvulus*, in an effort to alleviate morbidity and eliminate the transmission of this disease. Despite reduced prevalence in many areas, LF remains a significant global health concern [45, 85] due, in part, to key weaknesses in MDA chemotherapy. Current control efforts can only interrupt disease transmission because DEC and ivermectin are only effective as microfilaricidal agents so do not function in a macrofilaricidal, curative manner [45, 86, 87]. Although drug resistance in lymphatic filarial nematodes has not been reported so far, a report in 2004 of onchocerciasis patients failing to respond to ivermectin treatments in Ghana raises concern about

possible development of drug resistance strains among *Onchocerca volvulus* [288, 289]. As such there is an urgent need for more effective anti-filarial agents. Unfortunately our current limited understanding of filarial worm biology and the mode of action of existing drugs hinders our ability both to rationally develop more effective anti-filarial drugs, and to make better use of existing compounds.

Since the 1998 characterization of RNA interference (RNAi) in the free-living nematode *Caenorhabditis elegans* by Fire *et al.*, RNAi has evolved into a powerful novel reverse genetic tool that enables researchers to rapidly and specifically “turn off” genes of interest. Since its characterization in *C. elegans*, RNAi have rapidly become a standard tool in the identification and validation of potential new drug targets for rational drug discovery [207, 208]. The ability for RNAi to suppression specific genes enables researchers to examine the resulting phenotypes to delineate specific gene functions and evaluate the encoded protein’s value as potential drug target. We have previously established a novel *in vivo* RNAi protocol to suppress gene expression in *Brugia malayi* where the intermediate host, the mosquito *Aedes aegypti*, is used as a culture and RNAi delivery mechanism [257]. Using this protocol, we demonstrated the important role of *Bm-cpl-1*, a gene encoding a cathepsin L-like cysteine protease, in the biology of larval stage *B. malayi*. Suppression of the gene produced potent behavioral and developmental worm phenotypes, supporting the contention that this protein has potential as a novel anti-filarial drug target.

Here we attempt to utilize our previously developed *in squito* RNAi protocol on two known drug targets, a glutamate-gated chloride channel alpha subunit (ivermectin) and β -tubulin (benzimidazole anthelmintics) as well as three putative drug targets, a G protein-coupled acetylcholine receptor (GAR), a prohormone convertase (PC), and a FMRFamide-like peptide

(FLP) to shed light on the biology of these genes by examining loss of function phenotypes. We used both semi-quantitative and quantitative RT-PCR to confirm and quantify suppression. To examine individual target gene functions, gene-suppressed L3 worms were compared phenotypically to normal control L3 worms. Phenotypic differences such as reduced worm motility, knotting behavior, frequency distribution, and mean intensity were observed between normal and dsRNA exposed worms.

III. MATERIAL AND METHODS

A. Mosquito Maintenance and Injection Protocol

The mosquito, *Aedes aegypti* (Liverpool strain), was selected based on its susceptibility to filarial worm infection [253]. *Ae. aegypti* were maintained in a contained environment under a constant temperature of 25°C, 80% relative humidity and a 14 h light to 10 h dark photoperiod. The mosquitoes were fed a diet of 0.3 M sucrose. Mosquitoes to be injected were anesthetized on ice and immobilized on a vacuum saddle before being microinjected intrathoracically. Intrathoracic microinjection took place at the base of the mosquito head via pulled borosilicate glass pipette attached to a manual syringe for injection by volume displacement. A maximum volume of 0.5 µl could be injected per injection using this approach.

B. Establishing *Brugia* Infection

Infected cat blood containing *B. malayi* microfilariae (mf) was obtained from the University of Georgia NIH/NIAID Filariasis Research Reagent Resource Center. As with our previous experiment [257], a filtration protocol [254] was used to isolate and purify the mf from the cat blood in order to establish a consistent and repeatable inoculum. *B. malayi* infected cat blood was first diluted with phosphate buffered saline (1:5 ratio, blood: phosphate-buffered saline (PBS)) before being syringe filtered through a 0.45µm filter to isolate microfilariae (mf). Filter-captured mf were subsequently washed three to five times with PBS followed by a further three to five times with *Aedes* physiologic saline [255]. Following wash, the mf containing supernatant was centrifuged at 6,800g for five min. Supernatant was removed and the pelleted mf was subsequently resuspended in fresh *Aedes* saline to a concentration of 40 worms per µl. To infect mosquitoes, 20 mf were microinjected as described.

C. Gene Selection & dsRNA Generation

The five target genes were: *Bm-tub-1* (XM001896580 [112]) for β -tubulin, *Bm-GluCl- α 3A* (XM001891558) for glutamate-gated chloride channel, *Bm-gar-2* (XP001893809) for a G protein-coupled acetylcholine receptor, *Bm-flp-21* (AA991111 [283]) for a FMRFamide-like peptide precursor, and *Bm-pc-2* (XM001901916) for a prohormone convertase (Table 3). Gene sequence for each of the five drug targets under investigation was obtained using the Basic Local Alignment Search Tool (BLAST) homology searches of the *B. malayi* expressed sequence tag (EST) dataset. Double stranded RNA (dsRNA) duplexes for each target gene were generated in-house using a T7 transcription-based approach as prior [257].

KNOWN DRUG TARGETS	
Beta-tubulin	<i>Bm-tub-1</i> (XM001896580)
Glutamate-gated Cl ⁻ Channel	<i>Bm-GluCl-α3A</i> (XM001891558)
PUTATIVE DRUG TARGETS	
G protein-coupled Acetylcholine Receptor	<i>Bm-gar-2</i> (XP001893809)
FMRFamide-like Peptide	<i>Bm-flp-21</i> (AA991111)
Prohormone Convertase-2	<i>Bm-pc-2</i> (XM001901916)

Table 3. Targets for *in squito* suppression

For each suppression target, a transcription template was PCR amplified from a *B. malayi* L3 stage cDNA library (kindly provided by Dr. S. Williams, Smith College, MA) using gene specific oligonucleotides designed to incorporate a T7 promoter sequence (TAATACGACTCACTATAGGGTACT) at both the 5' and 3' ends of the amplicon. These primer sequences can be found in Table 4.

Gene	Primer sequence (5' - 3')	Amplicon Size (bp)
<i>Bm-tub-1</i>	Forward TAATACGACTCACTATAGGGTACTTCCTTGTTGATCTGGA	319
	Reverse TAATACGACTCACTATAGGGTACTTTCGAGATCAGCAATG	
<i>Bm-GluCl- α3A</i>	Forward TAATACGACTCACTATAGGGTACTTTTGCCAACTTTTGA	542
	Reverse TAATACGACTCACTATAGGGTACTACCAAGAACGGGTAG	
<i>Bm-gar-2</i>	Forward TAATACGACTCACTATAGGGTACTATCGGAGGAAGTACG	432
	Reverse TAATACGACTCACTATAGGGTACTGTTTTCTTAAATTGCTGA	
<i>Bm-flp-21</i>	Forward TAATACGACTCACTATAGGGTACTCAAAGTCTGCGTTGTTTTTC	216
	Reverse TAATACGACTCACTATAGGGTACTAGATCGTTAGGAGGTGCT	
<i>Bm-pc-2</i>	Forward TAATACGACTCACTATAGGGTACTGGCATTGAAGAGGAC	514
	Reverse TAATACGACTCACTATAGGGTACTGATTTTCACCCCAAG	

Table 4. Primer sequence for dsRNA generation

The location of this transcription template was carefully chosen so as to exclude any region of high sequence homology within the *Ae. aegypti* genome to avoid off-target transcriptional suppression effects on the host. Transcription templates were gel purified and dsRNA duplexes synthesized using the MEGAscript RNAi Kit (Ambion, TX) according to the manufacturer's protocols. DsRNA species were quantified with a NanoVue spectrophotometer (GE Healthcare, NJ) prior to use.

For this experiment, we noticed that the progeny from newly infected *B. malayi* worms were exhibited higher survival rate in the mosquitoes with an increase in the number of worms that successfully develop to the L3 life stage as compared to parasites used in prior experiments that have expired due to natural aging. Using the new mf we found that our previously protocol was less effective in eliciting consistent suppression. Reoptimization of the protocol produced effective target suppression following an initial injection of target dsRNA at 6 day post-infection (dpi), at a time just prior to the molt from second larval stage (L2) parasites developing into the

third larval stage (L3) parasite [62], followed by two subsequent injections at 9 and 12 dpi. The mosquitoes were processed to confirm suppression of the target gene, as described below, 48 h post final injection of dsRNA.

D. Relative Quantitative RT-PCR

B. malayi infected mosquitoes injected with either saline (control) or dsRNA were cold anesthetized on ice. Total RNA from individual mosquitoes was extracted using the RNAqueous Kit (Ambion, TX) followed by DNase treatment using the TURBO DNA-free Kit (Ambion, TX) in thin-walled PCR tubes. The RNA was stabilized with RNase Out Inhibitor (Invitrogen, CA) and stored at 4°C in RNase-free microcentrifuge tubes. Relative semi-quantitative RT-PCR was performed using this RNA as template. Relative semi-quantitative RT-PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, CA). The *B. malayi* cathepsin L-like gene, *Bm-cpl-1* (Accession number AF331035 [229]), served as the internal standard on which the intensity of the target gene amplified from the same RNA sample is compared to. This gene was chosen as we had previously determined its stable transcript production during the *B. malayi* L3 stage by PCR [257].

The oligonucleotide primers used to amplify targets were designed to flank the gene regions covered by the dsRNA. These primer sequences can be found in Table 5. The profile for *Bm-cpl-1*, *Bm-gar-2*, *Bm-pc-2*, *Bm-flp-21*, and *Bm-tub-1* PCR were: cDNA synthesis at 50°C for 30 minutes; an initial denaturation phase of 94°C for 2 min; 42 cycles of 94°C for 30 s, 60°C for 30 s, 68°C for 1 min and a final extension phase of 68°C for 5 min. The profile for *Bm-GluCl-α3A* PCR was: cDNA synthesis at 50°C for 30 minutes; an initial denaturation phase of 94°C for 2 min; 32 cycles of 94°C for 30 s, 60°C for 30 s, 68°C for 1 min and a final extension phase of 68°C for 5 min. Reactions were visualized on a 1.2% agarose gel containing ethidium bromide.

Gene	Primer sequence (5' - 3')	Amplicon Size (bp)
<i>Bm-tub-1</i>	Forward ATATGTGCCACGAGCAGTC Reverse TGGATACTCCTCACGAATTT	307
<i>Bm-GluCl- α3A</i>	Forward TATGTTGGTCGTCGTTTCCT Reverse TGAGGTCAACACGTTTGCTA	431
<i>Bm-gar-2</i>	Forward TGGAACGTGAAAGTACAGCA Reverse TAGCCATGGCATAGCAAAAT	564
<i>Bm-flp-21</i>	Forward AAGATCACCAGATGTCAACATTTTAT Reverse GTAACGCCAGGTTTTCCCAGTC	342
<i>Bm-pc-2</i>	Forward ACGGTAGATGGACCACGTAA Reverse ATCTGGCAAGTCTCTGCATC	465
<i>Bm-cpl-1</i>	Forward AACAGGGCAATATGACGAGA Reverse CAGCATGCTTAAGTCCCAGT	810

Table 5. Primer sequence for relative quantitative RT-PCR

E. Quantitative RT-qPCR

Total RNA from each individual *B. malayi* infected mosquito injected with saline (control) or dsRNA was extracted and DNase-treated as described above for three replicated RNAi experiments. Following DNase treatment, RNase Out Inhibitor was added to each sample. Each RNA sample was quantified spectrophotometrically. This RNA served as a template in our RT-qPCR assays using the qScript One-Step Fast RT-PCR Kit with ROX (Quanta BioSciences, MD).

Establishing PREXCEL-Q Parameters

To establish our RT-qPCR parameters and determine the valid working ranges for all of our samples (target and reference genes), PREXCEL-Q, a qPCR assay development and project management software, was employed. To determine the optimum template concentration to use while avoiding RT-qPCR inhibition for each of the seven targets, a mixture of the RNA samples at concentration range between 0.02 to 0.14 ng/ μ l was used. For subsequent quantitative assessment of transcript abundance, each RNA sample was diluted to 0.11 ng/ μ l with 6 μ l of sample used per 25 μ l reaction.

Primers and Probes

The target sequences under evaluation in the RT-qPCR study were the *B. malayi* β -tubulin 1, glutamate-gated chloride channel alpha-3A subunit, G-protein coupled acetylcholine receptor-2, FMRamide-like peptide-21, and prohormone convertase-2 transcript previously described. Two reference genes were used, a cathepsin L-like gene *Bm-cpl-1* also previously described, and *Bm-tph-1* (Accession number U80971), a tumor protein homolog-encoding gene that is a proven reference gene for qPCR of *Brugia* development in mosquitoes [228]. To facilitate the use of

shorter gene specific primer-probe sets for this study, TaqMan minor groove binding (MGB) probes were used. All primers and probes were designed using Primer Express v. 2.0 software (Applied Biosystems, CA) and synthesized by Applied Biosystems. The primer and probe sequences used are shown in Table 6.

Gene	Primer sequence (5' - 3')		Amplicon size (bp)
<i>Bm-tub-1</i>	Forward	GAACGCCGATCTCCGTAAAC	62
	Reverse	AGAAATGCAACCGTGGAAATG	
	Probe	6FAM-TGCCGTCAATATGG-MGBNFQ	
<i>Bm-GluCl-α3A</i>	Forward	GGATGCTGTACCTGCTCGTGTA	71
	Reverse	ATACCTGATGCTTGTGTAGTCATTGTT	
	Probe	6FAM-CATTAGGTGTAACAACATTG-MGBNFQ	
<i>Bm-gar-2</i>	Forward	CCGCCTCGGATTGAGGTTA	62
	Reverse	CGTACTTCCTCCGATGTTACCAA	
	Probe	6FAM-CGAGGAACCAAAGCA-MGBNFQ	
<i>Bm-flp-21</i>	Forward	CAGTGCTACAAACTGCTAAGGATGA	71
	Reverse	TGCTGCTGGAATAGCGTGAT	
	Probe	6FAM-TGATAGTACTCAGTATTCTAC-MGBNFQ	
<i>Bm-pc-2</i>	Forward	CCCTCGACCGGAAATCTTGT	61
	Reverse	CTGTCCGAGTCCCAAGCA	
	Probe	6FAM-CTGGAATTGATTACTGATGC-MGBNFQ	
<i>Bm-cpl-1</i>	Forward	GGTTACGGAACGCATCGAA	62
	Reverse	TGGGTTCCTCCAGCTATTTTTAA	
	Probe	6FAM-TCACGGTGATTACTGGAT-MGBNFQ	
<i>Bm-tph-1</i>	Forward	TTGCAACGATATGTTGATCTTCAA	62
	Reverse	ACGAGTCCGACGCAAGCT	
	Probe	6FAM-ATGCATTCACAGATGAC-MGBNFQ	

Table 6. Primer and TaqMan probe sequences for RT-qPCR experiment.

TaqMan RT-qPCR

For each RNA sample, 25 μ l volume reactions were prepared in duplicate and 20 μ l of this reaction mixture was applied per well on a 96-well plate (using white-well reaction plates, Eppendorf, NY). Individual components of each RT-qPCR reaction were as follows: 6 μ l prediluted RNA (as determined by PREXCEL-Q), 6.25 μ l 4X One-step Fast Master Mix with ROX, 1.25 μ l qScript One-Step Fast RT, 775 nM each primer, 150 nM probe, nuclease-free water to 25 μ l. Cycling conditions included an initial cDNA synthesis step of 50°C for 5 min followed by an RT denaturation/Taq activation phase of 95°C for 30 s then 45 cycles of 95°C for 3 s and 58°C for 30 s. Four point standard curves were created for each target (within the ng/ μ l ranges already specified above) by diluting the RNA sample mixture in each case according to precise, PREXCEL-Q-determined parameters (eight-fold dilution from highest to lowest concentration). Nuclease-free water was substituted for RNA in No-template control reactions. Thermocycling was performed on an ABI GeneAmp 5700 SDS (Applied Biosystems). Quantification cycle (C_q) values were obtained at an appropriate threshold per each target ($\sim 0.1 DR_n$ in all cases), and data were processed using custom Excel files by the efficiency-corrected ($E^{\Delta\Delta C_q}$) relative quantification method [227].

F. Phenotype Analysis

After confirmation of target gene suppression, multiple assays were performed to describe worm phenotypes. Each phenotypic assay was performed 14 dpi. Infected mosquitoes were inoculated with target specific dsRNA at 6, 9, and 12 dpi. Mosquitoes were cold-anesthetized on ice before the wings and legs were removed and discarded. The head, thorax and abdomen were partitioned and further dissected to release the parasites. The following characteristics of dissected parasites were observed: (1) **Parasite location:** In order to be successfully transmitted,

these parasites have to actively migrate to the head of the mosquito and vigorously writhe free of the proboscis. Parasite migration through the mosquito was recorded and measured according to escape point from the mosquito body (abdomen, thorax or head). (2) **Worm motility:** A scoring schema ranging from one (immotile) to five (constantly, highly motile) was used to quantify parasite movement in a blind fashion by an independent evaluator. (3) **Kinked morphology:** The number of parasites with a perturbed section of body wall slightly caudal to the midpoint of the worm was noted. (3) **Parasite survival / viability:** The number of parasites that survived to the infectious stage was recorded so that infection prevalence and mean intensity could be calculated. (4) **Mosquito survival / viability:** The number of mosquitoes that survived through the development of parasites to the infectious stage was recorded because *B. malayi* inflict significant pathology and decrease mosquito survival.

G. Statistical Analysis

To analyze the effect of RNAi on gene expression in the RT-qPCR experiments and parasite size, *t*-tests were used. To analyze the effect of RNAi on worm motility based on our one through five blind-scoring schema and worm viability based on the number of parasites harvested from each infected mosquito, ANOVA was employed. To analyze the effect of RNAi on all other worm and mosquito behaviors assayed, chi square tests were used. For all tests, *P* values ≤ 0.05 were considered statistically significant.

IV. RESULTS

A. Target Genes were Expressed During Mosquito Borne Life Cycle Stages

The target genes were checked for expression during the intermediate host life cycle using *Brugia malayi* microfilaria, L2 and L3 cDNA libraries (kindly provided by Dr. S. Williams, Smith College, MA) as PCR templates. *Bm-tub-1* was expressed at all three life stages (Figure 15A) but *Bm-GluCl- α 3A* was expressed only at the L3 life stage (Figure 15B). Similarly, *Bm-gar-2* was strongly expressed at the L3 larvae life stage with faint expression at the mf life stage and no expression at the L2 life stage (Figure 15C). *Bm-flp-21* was found to be expressed at all three stages (Figure 15D) and *Bm-pc-2* was expressed weakly at the mf life stage and strongly at the L3 life stage with no expression at the L2 life stage (Figure 15E). The expression of all gene targets during the mosquito-borne life stages provides a basis to evaluate their potential and effectiveness as drug targets using our mosquito-based *in squito* RNAi protocol.

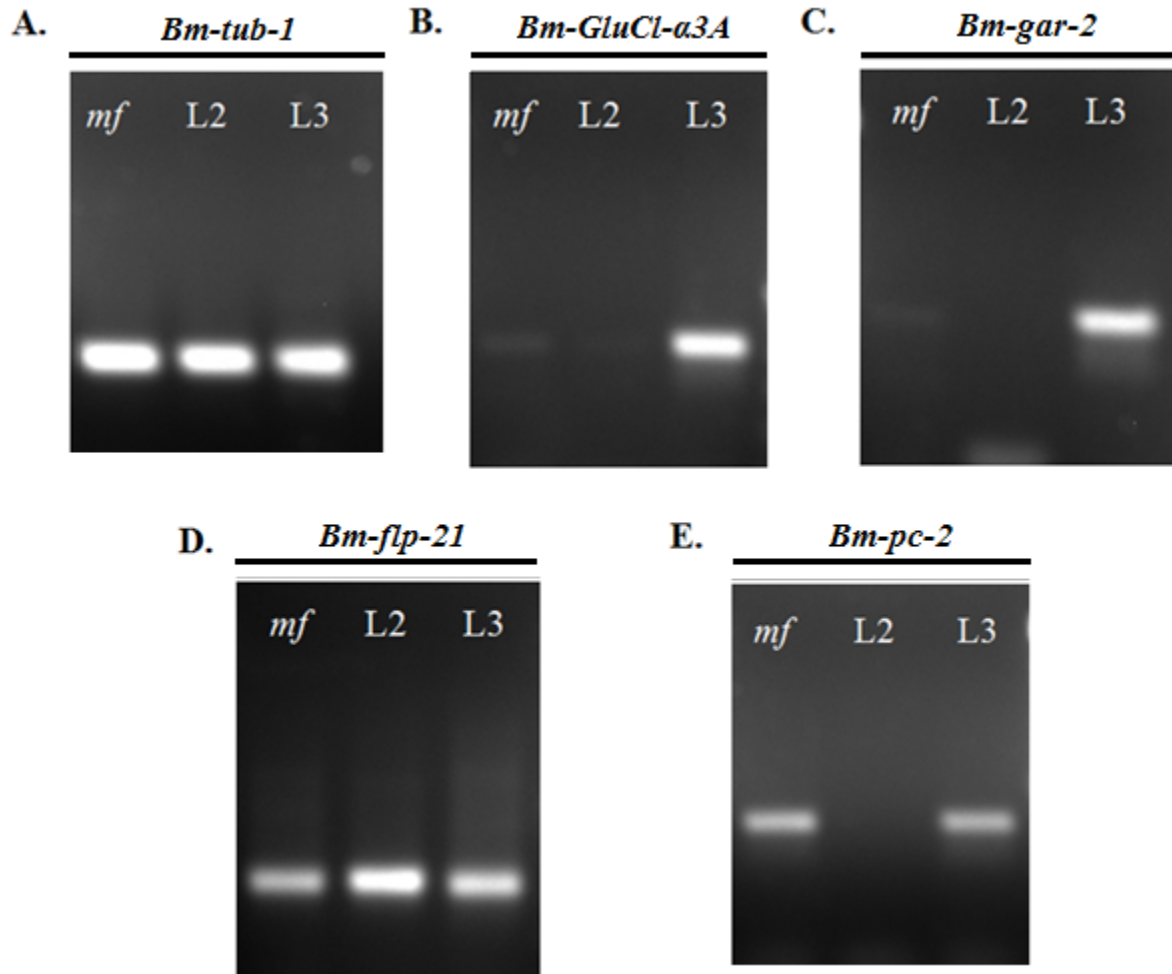


Figure 15. Target genes are expressed during mosquito life cycle stages. (A) *Bm-tub-1* was expressed at all three life stages (microfilaria (mf), L2 and L3 stages). (B) *Bm-GluCl- α 3A* was expressed only at the L3 stage. (C) Similarly, *Bm-gar-2* was expressed strongly at the L3 larvae life stage with faint expression at the mf life stage and no expression at the L2 stage. (D) *Bm-flp-21* was expressed at all three life stages. (E) *Bm-pc-2* was expressed weakly at the mf life stage and strongly at the L3 stage with no expression at the L2 stage.

B. *In squito* Suppression of Target Genes was Potent and Specific

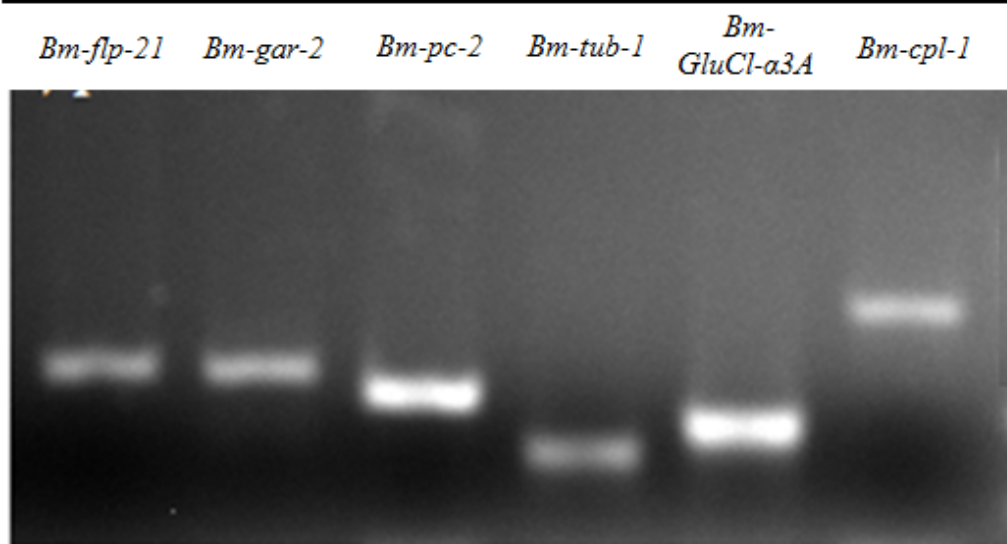
Infected mosquitoes were separated into six different groups: control, *Bm-tub-1*, *Bm-GluCl- α 3A*, *Bm-gar-2*, *Bm-flp-21*, and *Bm-pc-2*. Each group was injected with 150 ng of the appropriate dsRNA at 6, 9, and 12 dpi except for the control group, which was injected with an equal volume of *Aedes* physiological saline. Gene suppression was assayed 48 h post last dsRNA injection using a semi quantitative RT-PCR in which the intensity of the target gene amplification in the linear phase of the reaction was compared to an internal *B. malayi* reference gene (*Bm-cpl-1*) that is expressed stably. Suppression was consistent for four of the five target genes; *Bm-pc-2* could not be consistently suppressed but rather was potently up-regulated (Figure 16).

Real time quantitative PCR (RT-qPCR) was used to quantify the level of *Bm-tub-1*, *Bm-GluCl- α 3A*, *Bm-gar-2*, and *Bm-flp-21* suppression relative to two reference genes (*Bm-cpl-1* and *Bm-tph-1*) using the $E^{\Delta\Delta C_q}$ relative quantification method [227]. PREXCEL-Q software was used to optimize the performance of the RT-qPCR assay; and important data pertinent to PCR efficiency, linear dynamic range and normalization of the assay are documented in Table 7. *Bm-tph-1* showed stable C_q values across the experiment and therefore was the most appropriate reference gene for these studies, as shown previously [228]. *Bm-pc-2* mRNA abundance after RNAi was quantified relative to *Bm-cpl-1* only, as *Bm-tph-1* transcript level was reduced by 38.2% ($P < 0.0001$) after ds*Bm-pc-2* treatment. The suppression of *Bm-tub-1*, *Bm-GluCl- α 3A*, *Bm-gar-2*, and *Bm-flp-21* was potent and specific. Thrice RNAi reduced *Bm-tub-1* transcript by 37% compared to saline injected controls ($P < 0.0001$) (Figure 17A), thrice *Bm-GluCl- α 3A* by 28% ($P < 0.001$) (Figure 17A), thrice *Bm-gar-2* by 53% ($P < 0.0001$) (Figure 17A) and thrice *Bm-flp-21* by 45% ($P < 0.0001$) (Figure 17A). Thrice injection of 150 ng *Bm-pc-2* dsRNA surprisingly increased *Bm-pc-2* transcript by 341% compared to saline injected controls ($P < 0.0001$) (Figure

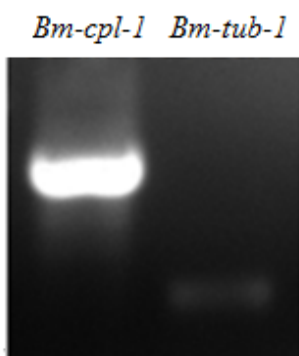
17B). *Bm-cpl-1* reference gene transcript was slightly reduced by *Bm-tub-1*, *Bm-GluCl- α 3A*, *Bm-gar-2*, and *Bm-flp-21* RNAi treatments but these reductions were not significant (*Bm-gar-2* dsRNA, 10.0%, P=0.06; *Bm-GluCl- α 3A*, 9.4%, P=0.071; *Bm-tub-1*, 13.4%, P=0.47; *Bm-flp-21*, 12.1%, P=0.55). These data support the gel based semi-quantitative RT-PCR experimental findings and provided evidence that *Bm-pc-2* dsRNA exposure resulted in an up-regulation of *Bm-pc-2*.

A.

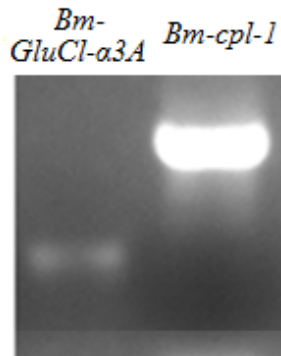
Control Worm



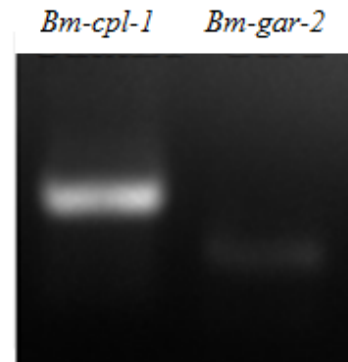
B.

Bm-tub-1 RNAi

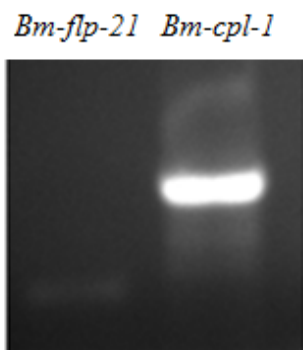
C.

Bm-GluCl- α 3A RNAi

D.

Bm-gar-2 RNAi

E.

Bm-flp-21 RNAi

F.

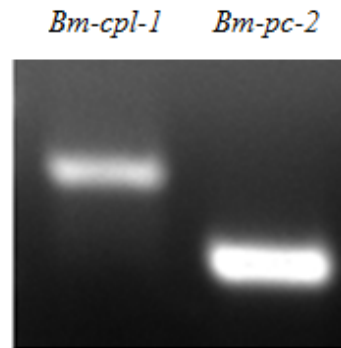
Bm-pc-2 RNAi

Figure 16. Target genes are suppressed using *in squito* RNAi protocol. Micrograph shows gel electrophoresis of relative RT-PCR analysis of individual, *B. malayi*-infected mosquitoes 48 h post-injection of RNAi trigger. (A) amplification product of the reference gene *Bm-cpl-1* and all six target genes: *Bm-flp-21*, *Bm-gar-2*, *Bm-pc-2*, *Bm-tub-1*, and *Bm-GluCl- α 3A* of control *B. malayi*-infected mosquito injected with saline (B) Amplified product for the target gene, *Bm-tub-1*, is shown right of the reference gene *Bm-cpl-1* (C) Amplified product for the target gene, *Bm-GluCl- α 3A*, is shown left of the reference gene *Bm-cpl-1* (D) Amplified product for the target gene, *Bm-gar-2*, is shown right of the reference gene *Bm-cpl-1* (E) Amplified product for the target gene, *Bm-flp-21*, is shown left of the reference gene *Bm-cpl-1* (F) Amplified product for the target gene, *Bm-pc-2*, is shown right of the reference gene *Bm-cpl-1*.

	<i>Bm-tub-1</i>	<i>Bm-GluCl-α3A</i>	<i>Bm-gar-2</i>	<i>Bm-ftp-21</i>	<i>Bm-pc-2</i>	<i>Bm-cpl-1</i>	<i>Bm-iph-1</i>
RT-qPCR efficiency (%)	84.36	30.12	97.34	77.33	75.89	83.53	105.01
Calibration curve y intercept	32.35	34.18	33.8	27.99	31.24	27.6	27.54
Calibration curve r^2	0.95	0.93	0.85	0.99	0.89	0.99	0.99
Mean C_q	31.56±0.13	37.04±1.65	35.89±0.51	26.13±0.15	25.17±0.30	28.55±0.15	28.34±0.07
NTC C_q	50	50	50	50	50	50	50

Table 7. Reportable information on RT-qPCR experiment.

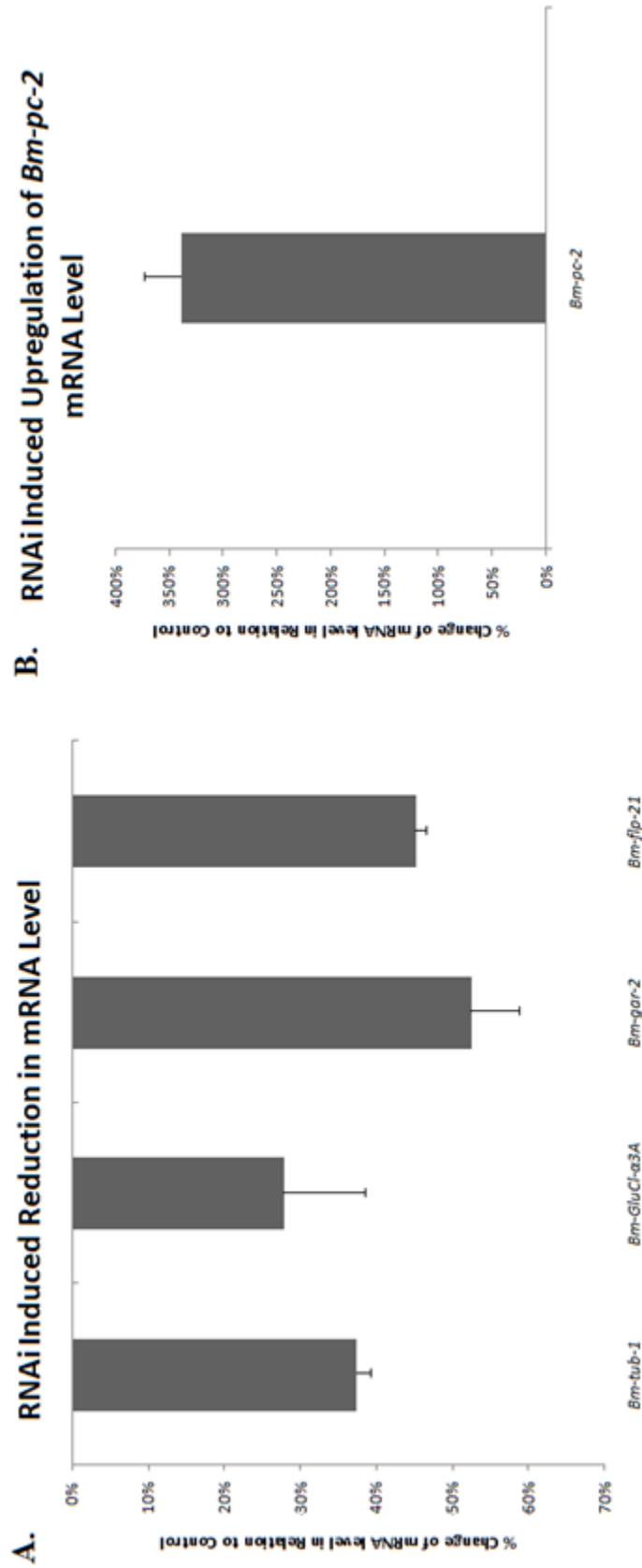


Figure 17. Quantitative RT-PCR demonstrates significant reduction in transcript abundance of *Bm-tub-1*, *Bm-GluCl- α 3A*, *Bm-gar-2*, and *Bm-flp-21* and a dramatic up-regulation of *Bm-pc-2* following dsRNA. (A) Injection with *Bm-tub-1* dsRNA decreased *Bm-tub-1* transcript by 37% compared to control. Injection with *Bm-GluCl- α 3A* dsRNA reduced *Bm-GluCl- α 3A* transcript by 33% compared to control. Injection with *Bm-gar-2* dsRNA reduced *Bm-gar-2* transcript by 55% compared to control. Injection with *Bm-flp-21* dsRNA decreased *Bm-flp-21* transcript by 45% compared to control. *Bm-GluCl- α 3A*, *Bm-tub-1*, *Bm-gar-2*, and *Bm-flp-21* are normalized to two reference genes *Bm-tph-1* and *Bm-cpl-1*. (B) Injection with *Bm-pc-2* dsRNA increased *Bm-pc-2* transcript by 341% compared to control. *Bm-pc-2* is normalized to *Bm-cpl-1* reference gene since *Bm-pc-2* dsRNA injection resulted in a significant *Bm-tph-1* reduction of 38% ($P < 0.001$). Quantitative RT-PCR was performed 48 h post last injection of RNAi trigger at 14 dpi. Each bar represents 10 mosquitoes from three biological experimental replicates.

C. *Brugia* Gene Suppression Result in Aberrant Motility

Mosquitoes were injected with 150 ng of target dsRNA or saline (control) at 6, 9, and 12 dpi then microdissected at 14 dpi to harvest L3-stage parasites. Worm motility was digitally recorded and scored according to a five-point schema of one (immobile) to five (all parts of the worm in constant motion). *Bm-tub-1* suppression decreased worm motility by 11.0% compared to control ($P < 0.001$). Similarly, *Bm-GluCl- α 3A*, *Bm-gar-2*, and *Bm-pc-2* suppressions also elicited 9.5% ($P < 0.001$), 6.7% ($P = 0.001$), and 5.5% ($P = 0.017$) reduction in worm motility. With *Bm-flp-21* suppression, an increased motility of 1.8% was observed, but this increase was not statistically significant ($P = 0.420$) (Figure 18).

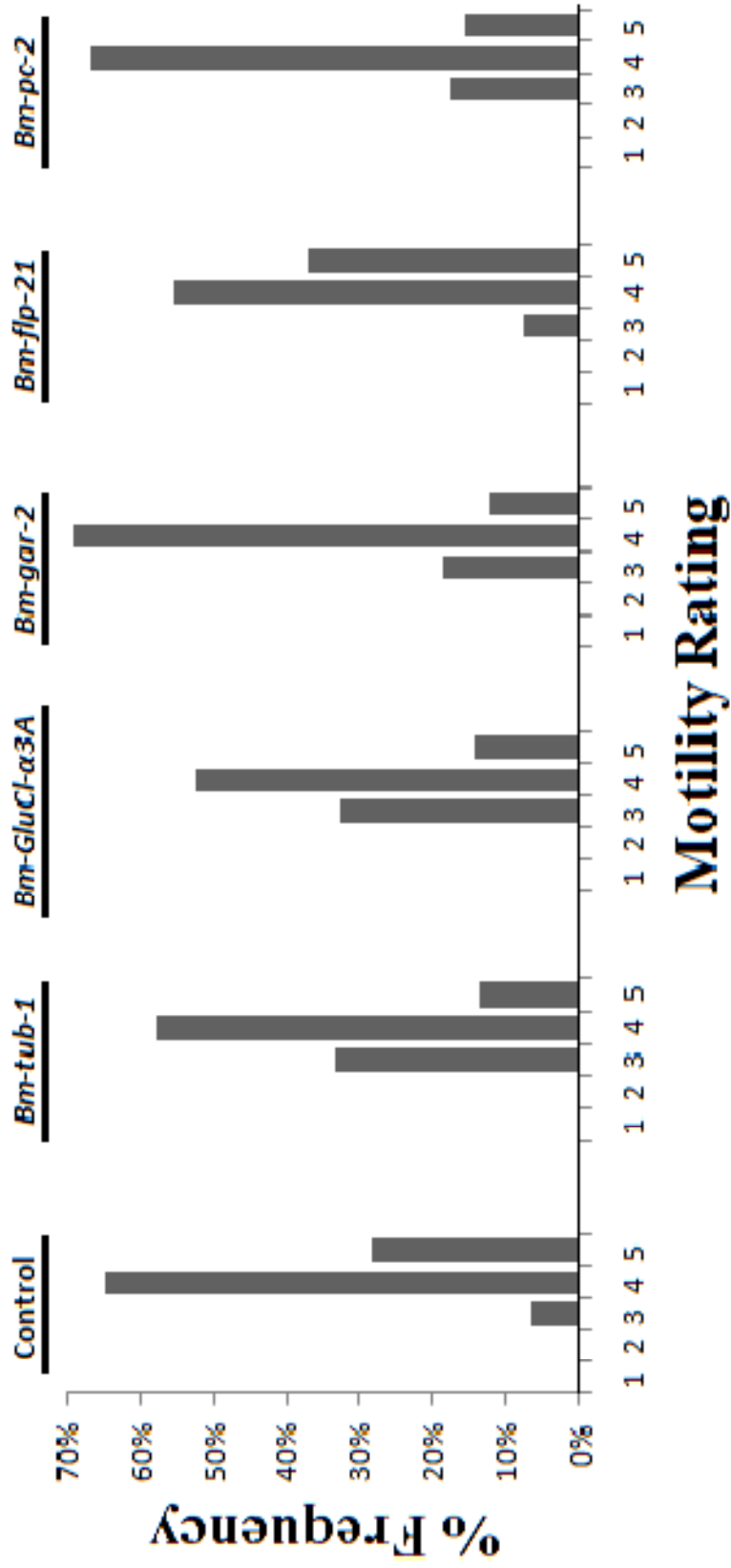


Figure 18. *Bm-tub-1*, *Bm-GluCl- α 3A*, *Bm-gar-2*, and *Bm-pc-2* dsRNA exposure elicited reduced motility. Frequency distribution for motility of L3 stage *B. malayi* recovered from *Ae. Aegypti*. *Bm-tub-1*, *Bm-GluCl α 3A*, *Bm-gar-2*, and *Bm-pc-2* dsRNA exposure resulted in 11.0%, 9.5%, 6.7%, and 5.5% motility reduction compared to control. *Bm-flp-21* suppression did not alter worm motility. Parasitized mosquitoes were injected with saline (control) or 150 ng *B. malayi* target dsRNA at 6, 9, and 12 dpi, then microdissected to obtain parasites at 14 dpi. Parasite motility was scored on a 1-5 scale, with 1 = immobile and 5 = highly mobile. Control n=88, ds*Bm-tub-1* n=57(P < 0.001), ds*Bm-GluCl- α 3A* n=75 (P<0.001), ds*Bm-gar-2* n=106 (P=0.001), ds*Bm-flp-21* n = 65 (P=0.420), and ds*Bm-pc-2* n=56 (P=0.017)

D. Effect of dsRNA Exposure on Normal Knotting Behavior of L3 *B. malayi*

Healthy *B. malayi* L3 are characterized by a highly, continuously active and convoluted body form with either one, or both ends of the parasite being noticeably tortuous; we describe this curvature phenotype as “knotted”. Using the control and RNAi suppressed worms extracted for motility evaluation we took note of whether the worm exhibited zero, one, or both ends knotted. Control worms exhibited 1% zero ends knotting, 51% one end knotted, and 48% both ends knotted. Compared to controls, gene suppression elicited a marked decrease in knotting behavior. *Bm-tub-1* suppression elicited an 8-fold increase in zero ends knotted and a 30% decrease in both ends knotted ($P<0.001$). *Bm-GluCl- α 3A* suppression resulted in a 19-fold increase in zero end knotting frequency and decreased both ends knotted frequency by 58% ($P<0.001$). The incidence of zero ends knotted was increased 12-fold following *Bm-gar-2* suppression. One and both end knotting among *Bm-gar-2* suppressed worms also decreased by 13% and 13% ($P<0.001$). *Bm-flp-21* suppression resulted in a 26 fold increase in incidence of zero ends knotted and a decrease in one and both ends knotted frequency of 16% and 45% ($P<0.001$). *Bm-pc-2* dsRNA exposure produced a 4-fold increase in zero end knotting frequency and a 9% reduction in one end knotting frequency ($P=0.011$) (Figure 19).

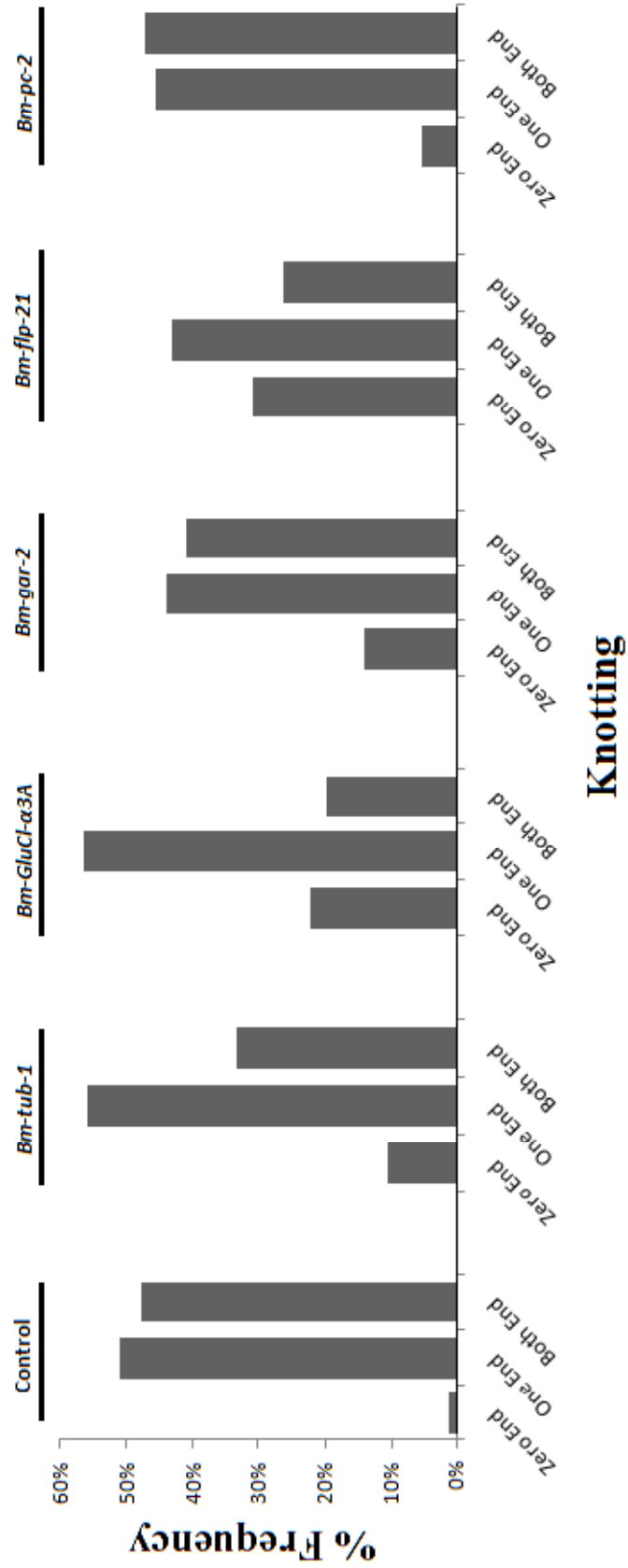
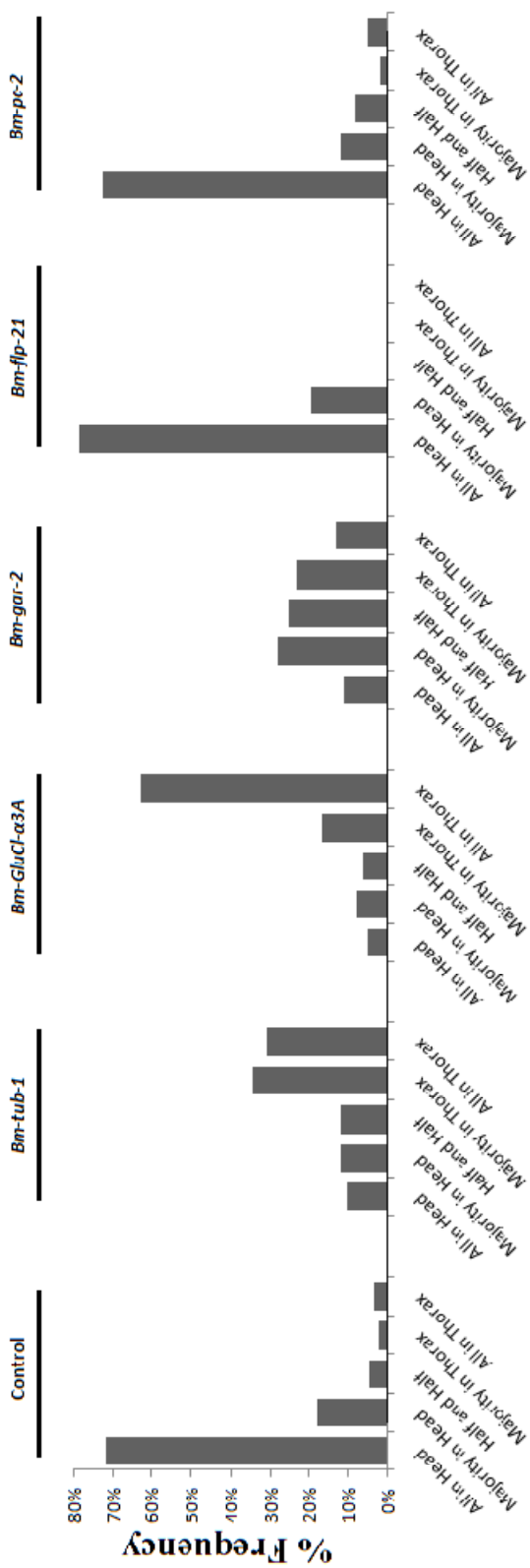


Figure 19. Suppression of target *Brugia malayi* genes altered normal knotting behavior. Frequency of control worms exhibited zero, one, and both end knotting are 1%, 51%, and 48%. *Bm-tub-1* suppression resulted in zero, one, and both end knotting frequency of 11%, 56%, and 33%. *Bm-GluCl- α 3A* suppression resulted in zero, one, and both end knotting frequency of 23%, 57%, and 20%. *Bm-gar-2* suppression resulted in zero, one, and both end knotting frequency of 14%, 44%, and 42%. *Bm-flp-21* suppression resulted in zero, one, and both end knotting frequency of 31%, 43%, and 26%. *Bm-pc-2* dsRNA exposure resulted in zero, one, and both end knotting frequency of 5%, 46%, and 48%. Infected mosquitoes were injected with *Aedes* physiological saline (control) or 150 ng *B. malayi* target dsRNA at 6, 9, and 12 dpi, then microdissected to obtain parasites at 14 dpi. Control n = 88, ds*Bm-tub-1* n=57 (P<0.001), ds*Bm-GluCl- α 3A* n=75 (P<0.001), ds*Bm-gar-2* n=106 (P<0.001), ds*Bm-flp-21* n=65 (P<0.001), and ds*Bm-pc-2* n=56 (P=0.011).

E. Worm Distribution is Significantly Altered with RNAi Suppression.

To further examine the effect of *B. malayi* gene suppression, worm distribution was examined. Two days after RNAi treatment, each mosquito was partitioned into head, thorax, and abdomen and the number of worms harvested from each segment was counted. Since no control or RNAi suppressed worms were found in the abdomen, the dissected mosquitoes were further divided into five categories based on where the worms were harvested: all worms found in the head, majority of worms found in the head, half of the worms found in head and half in thorax, majority of worms found in the thorax, and all worms found in the thorax. Worms in control mosquitoes were predominantly found in the head with 90% of the mosquitoes having either all or the majority of worms extracted from the head region. *Bm-tub-1*, *Bm-GluCl- α 3A*, and *Bm-gar-2* suppression significantly disrupted worm migration with only 22% (*Bm-tub-1* $P < 0.001$), 13% (*Bm-GluCl- α 3A* $P < 0.001$), and 39% (*Bm-gar-2* $P < 0.001$) of mosquitoes having all or the majority of worms present in the head region. No statistically significant change in worm migration was noted with *Bm-flp-21* and *Bm-pc-2* dsRNA exposure (Figure 20).



Frequency Distribution of Worms

Figure 20. Frequency distribution of worms extracted from control and dsRNA injected mosquitoes. Control mosquitoes: 72% with all worms present in head region, 18% with majority of worms present in head region, 5% with half of worms present in head region and half of worms present in thorax region, 2% with majority of worms present in thorax, and 3% with all worms present in the thorax. *Bm-tub-1* dsRNA injected mosquitoes: 10% with all worms present in head region, 12% with majority of worms present in head region, 12% with half of worms present in head region and half of worms present in thorax region, 35% with majority of worms present in thorax, and 31% with all worms present in the thorax. *Bm-GluCl- α 3A* dsRNA injected mosquitoes: 5% with all worms present in head region, 8% with majority of worms present in head region, 7% with half of worms present in head region and half of worms present in thorax region, 17% with majority of worms present in thorax, and 63% with all worms present in the thorax. *Bm-gar-2* dsRNA injected mosquitoes: 11% with all worms present in head region, 28% with majority of worms present in head region, 25% with half of worms present in head region and half of worms present in thorax region, 23% with majority of worms present in thorax, and 13% with all worms present in the thorax. *Bm-flp-21* dsRNA injected mosquitoes: 78% with all worms present in head region, 20% with majority of worms present in head region, and 2% with majority of worms present in thorax. *Bm-pc-2* dsRNA injected mosquitoes: 72% with all worms present in head region, 12% with majority of worms present in head region, 9% with half of worms present in head region and half of worms present in thorax region, 2% with majority of worms present in thorax, and 5%

with all worms present in the thorax. Infected mosquitoes were injected with saline (control) or 150 ng *B. malayi* target dsRNA at 6, 9, and 12 dpi, then microdissected to obtain parasites at 14 dpi. Based on where the majority or all of the worms are harvested, mosquitoes were each sub-divided into five categories: all worms found in the head, majority of worms found in the head, half of the worms found in head / thorax, majority of worms found in the thorax, and all worms found in the thorax. Control n = 88, ds*Bm-tub-1* n=58 (P<0.001), ds*Bm-GluCl- α 3A* n=77 (P<0.001), ds*Bm-gar-2* n=107 (P<0.001), ds*Bm-flp-21* n=65 (P=0.064), and ds*Bm-pc-2* n=58 (P=0.335).

F. Effect of *B. malayi* Target Gene Suppression on Worm Survival

Parasitized mosquitoes were injected with saline (control) or 150 ng *B. malayi* target dsRNA at 6, 9, and 12 dpi, and then microdissected at 14 dpi. The number of extracted worms per mosquito was counted. The mean intensity of worms found among n=88 control mosquitoes was 3.44 worms per mosquito. *Bm-tub-1*, *Bm-GluCl- α 3A*, and *Bm-pc-2* dsRNA exposure significantly reduced the average number of worms found per mosquito by 19% (*Bm-tub-1*, $P<0.001$), 17% (*Bm-GluCl- α 3A*, $P<0.001$), and 13% (*Bm-pc-2*, $P=0.004$). *Bm-gar-2* suppression had no effect on worm survival, but *Bm-flp-21* suppression increased worm survival by 30% ($P<0.001$) (Figure 21).

G. Effect of *B. malayi* Target Gene Suppression on Mosquito Survival

To examine mosquito survival, the number of mosquitoes that survived to 14 dpi for each of the dsRNA and saline injected groups was counted. Mosquito survival rate was determined based on the ratio of surviving mosquitoes to total infected mosquitoes per dsRNA or saline injection group. We found that *Bm-tub-1*, *Bm-GluCl- α 3A*, *Bm-gar-2*, and *Bm-pc-2* dsRNA injection did not significantly alter mosquito survival rate, but *Bm-flp-21* suppression resulted in a 22% reduction in mosquito survival rate ($P=0.004$).

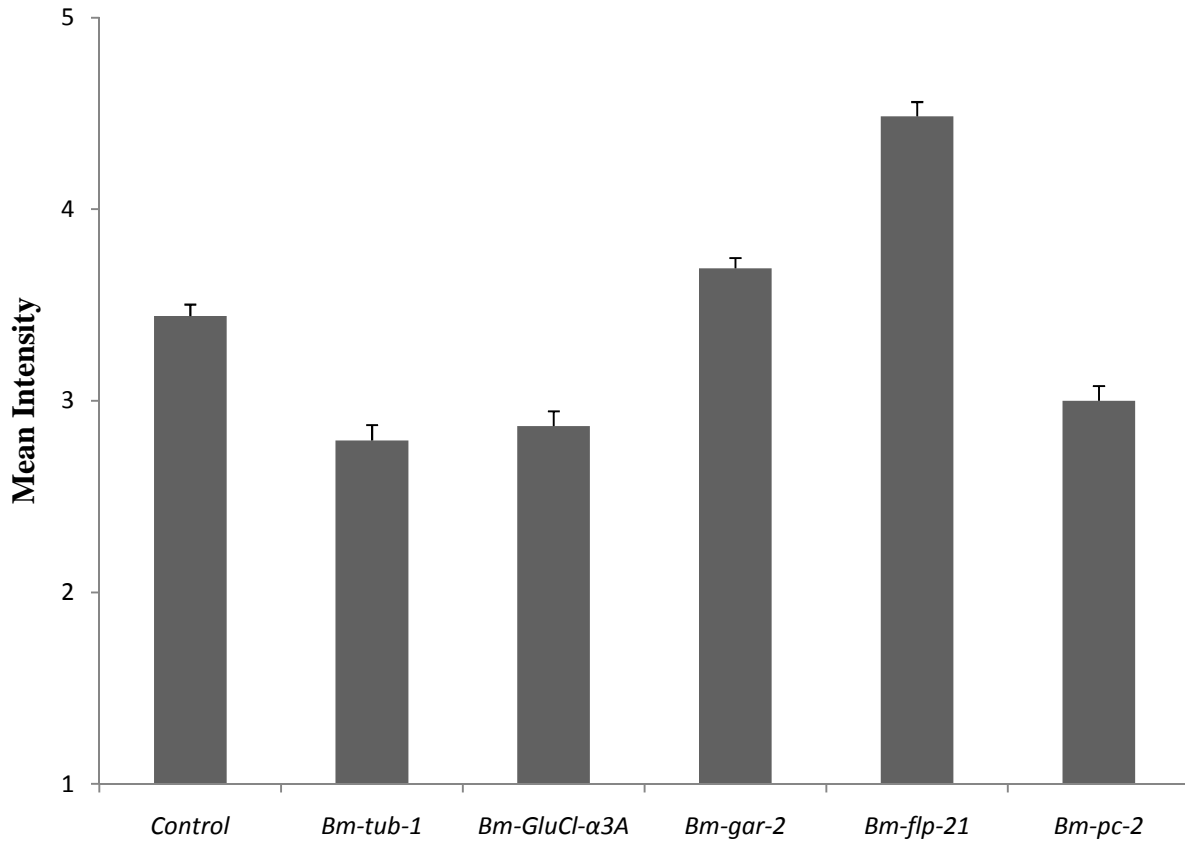


Figure 21: Parasite mean intensity of control and dsRNA injected *Aedes aegypti*. Average number of worms extracted from control group mosquitoes was 3.44. Average number of worms extracted from *Bm-tub-1*, *Bm-GluCl-α3A*, *Bm-gar-2*, *Bm-flp-21*, and *Bm-pc-2* dsRNA injected mosquitoes were 2.79, 2.87, 3.69, 4.48, and 3.00. Infected mosquitoes were injected with saline (control) or 150 ng *B. malayi* target gene dsRNA at 6, 9, and 12 dpi, and then microdissected to obtain an infection count. Control n = 88, ds*Bm-tub-1* n=58 (P<0.001), ds*Bm-GluCl-α3A* n=77 (P<0.001), ds*Bm-gar-2* n=107 (P=0.055), ds*Bm-flp-21* n= 65 (P<0.001), and ds*Bm-pc-2* n=58 (P=0.004).

V. DISCUSSION

A. The Robustness of the *in squito* RNAi Protocol

In this study we examined five *B. malayi* genes encoding known and putative drug targets using *in squito* RNAi. Of the five target genes, consistent RNAi mediated suppression was achieved with *Bm-tub-1* (37%), *Bm-GluCl- α 3A* (28%), *Bm-gar-2* (53%), and *Bm-flp-21* (45%) and up-regulation of 341% was observed with *Bm-pc-2* dsRNA RNAi trigger exposure. The relatively reliable knock down of target gene expression compares favorably with other RNAi methodologies applied to a broad cross section of parasitic nematodes that have met with limited success. The consistency of our RNAi protocol may be a result of *in vivo* RNAi suppression within the host because all previous RNAi protocols introduce RNAi triggers within artificial *in vitro* RNAi environments.

B. *Bm-tub-1*

Benzimidazole-type anthelmintics, such as albendazole, exert their effect by binding to β -tubulin and inhibiting microtubule formation. Microtubules have a wide variety of functions such as intracellular transport, nutrient (glucose) uptake, cell shape, and synaptic formation among others [266, 270-273]. Thus the disruption of microtubule formation interrupts parasite survival in a multitude of ways including preventing the uptake of glucose, inhibiting egg production and impairing muscle movement leading to nematode death [266, 270-273]. While albendazole alone has no anti-filarial effect, when giving in conjunction with either ivermectin or DEC it has proven to enhance both anti-filarial drugs' potency [80]. Based on this proven activity, *B. malayi* β -tubulin (*Bm-tub-1*) was selected as a gene target for our evaluation. In our *in squito* RNAi suppression of *Bm-tub-1*, we observed a reduction in worm motility. In addition, the parasite mean intensity for *Bm-tub-1* suppressed mosquitoes was significantly reduced compared to

control. Worms from *Bm-tub-1* infected mosquitoes were more predominantly found in the thorax. The decrease in worm motility and survival as well as disruption to worm migration indicates that *Bm-tub-1* plays an important role in normal worm survival and transmission. It should be noted that suppression of *Bm-tub-1* in the mosquito stages of the parasite lifecycle did not result in a lethal phenotype as observed by Blaxter and Aboobaker using *in vitro* RNAi method on adult female *B. malayi* [9].

C. *Bm-GluCl- α 3A*

At present, of the two anti-filarial drugs available, only ivermectin's potential site of action is known. Based on studies using various non-filarial nematodes, ivermectin has been found to selectively bind to the glutamate-gated chloride channel alpha subunit exerting complementary, and potentially additive, effects with glutamate on the glutamate-gated chloride channel resulting in confirmation changes and the observed slow and irreversible channel opening [93, 97, 99, 101, 111, 258]. This irreversible activation of glutamate-gated chloride channel leads to the two observed effects of ivermectin on non-filarial nematodes: paralysis of body wall muscle [294] and pharyngeal musculature [259, 260, 262]. As such gastrointestinal nematodes experience compromised motility and feeding, and are subsequently expelled from the host body [263]. The mode of action for ivermectin is less certain for filarial nematodes where it is only effective as a microfilaricide [264, 265]. Thus, at present, little is known about anti-filarial drug actions.

The exposure of ds*Bm-GluCl- α 3A* resulted in a 28% reduction in *Bm-GluCl- α 3A* transcript level. The mean Cq value for *Bm-GluCl- α 3A* in our quantitative RT-qPCR experiments was 37.04 ± 1.65 , a Cq value typically associated with approximately one copy of the target transcript, given such low initial transcript abundance, quantification by RT-qPCR is likely to be less reliable than for the other targets and given that the reaction efficiency for this gene was low

(30%) it is reasonable to expect this 28% reduction is an underestimate of the actually knock down achieved, which may actually be closer to 50-60%.

Bm-GluCl- α 3A suppression significantly reduced the worm survival within infected mosquito. *Bm-GluCl- α 3A* suppression also compromised the L3 worms' capability to navigate within the mosquito host body toward the head region. Studies using the filarial worm *B. pahangi* demonstrated that ivermectin effectively blocked amphid response to chemical stimuli [267, 268]. Amphids are the sensory organs within the nematode head that contains sensory neurons. Additionally in *H. contortus*, the ivermectin resistant strains were found to have defective amphids [269]. The phenotypes observed in *Bm-GluCl- α 3A* suppression are as expected of worms with sensory disruption thus hinting at a potential role for ivermectin in disrupting the worms' sensory system.

It would be expected that compromised navigation would result in worms migrating to the abdomen as well, but this is not the case. Lack of migration to the abdomen could be attributed to suppression of *Bm-GluCl- α 3A* merely diminishing the worm's capability in distinguishing sensory stimuli directly related to migration out of the head.

Moreno *et al.* reported that ivermectin acts to prevent *B. malayi* microfilarial excretory-secretory capability of microfilaria [282]. In *B. malayi*, the glutamate-gated chloride channel, which is the target site for ivermectin, was found to be expressed only in the muscle structure surrounding and controlling the microfilarial excretory-secretory vesicles [282]. Moreno *et al.* postulated that the suppression of glutamate-gated chloride channel, which regulates the release of excretory-secretory vesicle contents, prevents the release / secretion of proteins that facilitate in parasite host immune evasion [282]. The reduction in the number of parasites found within

Bm-GluCl- α 3A dsRNA injected mosquito may be a result of *Bm-GluCl- α 3A* suppressed worms' diminished capability to release host immune evasion proteins.

In non-filarial nematodes, the paralysis of body wall muscle is due to ivermectin's action on inhibitory motor neurons. The irreversible opening of glutamate-gated chloride channel result in the irreversible hyperpolarization of inhibitory motor neurons. This hyperpolarization renders the inhibitory motor neuron incapable of producing the inhibitory action potentials that is necessary for muscle relaxation [263]. This capacity for muscle relaxation is required for normal worm movement [263]. Here we observed that *Bm-GluCl- α 3A* suppression resulted in reduced worm motility which is consistent with a reduced capacity to regulate inhibitory neuromuscular signaling. The lack of worm paralysis with *Bm-GluCl- α 3A* suppression could be due to the presence of multiple glutamate-gated chloride channel subtypes and that suppression of *Bm-GluCl- α 3A* renders only those channel subtypes consisting of the *Bm-GluCl- α 3A* subunit defective. The defective channel subtypes could be compensated for to a certain extent by replacement with other channel subtypes, thus *Bm-GluCl- α 3A* suppression would not completely abolish inhibitory motor neuron regulation.

D. *Bm-gar-2*

Neurotransmitter gated G protein-coupled receptors such as GARs regulate cellular activities via cytoplasmic second messengers. Because of the fast action of direct ligand gated channels, the slower indirect GPCRs like GARs have been largely ignored in the past. Recent studies on GARs demonstrate their importance in regulating neuromuscular movement in organisms such as *C. elegans* have spurred interest in GARs as potential anthelmintic drug targets [280]. Dittman and Kaplan (2008) demonstrated that *Ce-gar-2* plays a role in inhibiting cholinergic motor neurons in *C. elegans* and the loss of *Ce-gar-2* function result in a decrease of both reversal and

large-angle (omega) turning behavior [280]. Stegger and Avery (2004) found evidence suggesting that *Ce-gar-3* may play a role in regulating *C. elegans* pharyngeal pumping [281]. The removal of *Ce-gar-3* resulted in an increased pharyngeal pump rate [281]. Conversely, over activation of *Ce-gar-3* resulted in the pharyngeal muscle unable to relax to the closed position thus inhibiting the effective transport of bacteria into the worm intestine [281]. As such, interruption in the regulatory component of the parasitic nematodes may impair its survival capability. Thus a putative *B. malayi* GAR (*Bm-gar-2*) was selected to evaluate whether GARs in *B. malayi* are sites for potential anti-filarial drug development.

In our study, suppression of *Bm-gar-2* elicited reduced motility and disrupted worm migration. These observed phenotypes correlate with the neuronal regulatory function of GARs and *gar-2*'s role in inhibiting cholinergic motor neurons as demonstrated in *C. elegans* [280]. A reduced ability to inhibit cholinergic motor neurons would disrupt normal smooth body motion in line with the observed reduction in worm motility accompanying *Bm-gar-2* suppression. It is also observed that *Bm-gar-2* suppression diminished worm migratory capability. Unlike *Bm-GluCl- α 3A*, and *Bm-tub-1* suppression, *Bm-gar-2* suppression did not result in a predominance of incidence where all or majority of worms found in thorax, but instead, *Bm-gar-2* suppressed worms had relatively equal occurrence of majority or all of the harvested worms found in the head as occurrence of majority or all of the harvested worms found in the thorax. Why this is remains a puzzle and is something that warrants further investigation. At present it can only be speculated that the suppression of *Bm-gar-2* possibly disrupted the worm's migratory and navigation capability. While much more needs to be investigated about the role GARs play in filarial nematodes, the information gleaned from our *Bm-gar-2* suppression study shines light on

Bm-gar-2 as a potential drug target that merits further consideration for GARs as potential new anthelmintic drug targets.

E. *Bm-flp-21*

FMRFamide-like peptides (FLPs) are the largest and most diverse family of neuropeptides within invertebrates [221, 274, 284, 285]. FLPs are highly conserved between parasitic nematodes [283] and have a broad role within its nervous system [283] and are associated with parasite motor and sensory functions [274]. Studies on FLPs using various nematode systems including such as *A. suum* and *C. elegans* have demonstrated that they play a role in both inhibitory and excitatory modulation of motor neuron activities [286, 290, 291]. In *A. suum*, the FLP gene *af1* was found to function in abolishing spontaneous motor neuron activities [292-294]. Similarly, the peptide encoded by *A. suum* FLP gene *af8*, predicted from the *C. elegans flp-6* gene, was found to elicit ventral somatic muscle contraction and dorsal somatic muscle relaxation [295]. The *C. elegans flp-13* peptide has been shown to elicit both ventral and dorsal somatic muscle relaxation in *A. suum* [296]. As such, based on the findings from previous studies, we postulate that FLPs may prove to be an important gene for proper filarial worm function and a potentially overlooked anti-filarial drug target. Of the *B. malayi* FLP genes considered, *Bm-flp-21* is the only one whereby dsRNA at sufficient concentration can be consistently produced. As such for this study we chose to investigate the importance of *Bm-flp-21* in juvenile *B. malayi* and evaluate its potential as anti-filarial drug target.

In *C. elegans*, the peptides encoded by the *flp-18* and *flp-21* genes were also found to activate both neuropeptide receptor (NPR)-1 215F and NPR-1 215V receptors [278, 279]. Moffett *et al.* found that peptides encoded in *C. elegans Ce-flp-21* gene elicited excitatory effect on ovijector of *A. suum* causing tissue lengthening and the termination of muscle activity leading

to flaccid paralysis [297]. In this study, *Bm-flp-21* suppressed worms were observed to exhibit an increase in worm survival. As expected with the increase in worm survival, mosquito survival diminished. The exact reason for the increase in worm survival needs to be further investigated.

F. *Bm-pc-2*

Many biologically important proteins are synthesized as larger inactive polypeptides that require prohormone convertase to activate them through proteolytic cleavage at the carboxyl side of the mono-, di- or oligomeric basic amino acid sites [287] thus making prohormone convertase a potential drug target of interest. Thus for this study we targeted the *B. malayi* PC2 (*Bm-pc-2*). Suppression of prohormone convertase 2 (PC2) in *C. elegans* through RNAi elicited aberrant phenotypes such as deficient egg laying and uncoordinated movement [287]. The *in squito* *Bm-pc-2* suppressions were observed to elicit up-regulation of target gene transcription. Quantitative RT-qPCR demonstrated significant up-regulation (341%) of *Bm-pc-2* with *dsBm-pc-2* exposure. Why *Bm-pc-2* dsRNA exposure resulted in a three to four fold transcription increase is puzzling. Geldhof *et al.* in 2005 reported that attempt to RNAi suppress the *Haemonchus contortus* vacuolar ATPase *Hc-vha-10* using electroporation and soaking resulted in the up-regulation of *Hc-vha-10* expression [213]. Despite the 3.4 fold up-regulation, *Bm-pc-2* dsRNA exposure did result in a reduction in worm survival. In addition *dsBm-pc-2* exposure also elicited a 38% reduction in *Bm-tph-1* expression not seen with other gene targets investigated in this study. Thus it is very possible that the difference in worm survival and motility may be a result of the *Bm-tph-1* suppression.

In summary, in this study we examined five existing and putative drug targets *Bm-tub-1*, *Bm-GluCl- α 3A*, *Bm-gar-2*, *Bm-flp-21* and *Bm-pc-2* using our *in squito* RNAi protocol. Of the five drug targets, consistent RNAi mediated suppression was achieved with *Bm-tub-1*, *Bm-GluCl-*

α3A, *Bm-gar-2*, and *Bm-flp-21* RNAi and up-regulation was observed with *Bm-pc-2* dsRNA exposure. Various phenotypes have been observed with each of the target gene dsRNA exposure allowing us to glean a deeper understanding of these gene targets. In the case of *Bm-tub-1*, *Bm-GluCl-α3A*, and *Bm-gar-2*, RNAi mediated suppression disrupted normal worm motility and migration thus severely compromised parasite transmission potential. The significant (30%) increase of worm survival rate associated with *Bm-flp-21* suppression and the up-regulation of *Bm-pc-2* both warrants further investigation. Of puzzling interest is the fact that up-regulation of *Bm-pc-2* resulting from *Bm-pc-2* dsRNA exposure resulted in a decrease in worm motility. Why this is needs further investigation.

CHAPTER 4. GENERAL CONCLUSIONS

The development of an *in vivo* RNAi method to study and better understand the biology of filarial worm *Brugia malayi* was achieved and verified. *In vivo* RNAi was achieved using the mosquito host as a culture and delivery system. RNAi trigger was introduced into the infected mosquito host via intrathoracic injection and subsequently the developing *B. malayi* larva is exposed to the RNAi trigger. Suppression was potent and specific for five target genes that represent either known or putative drug targets: *B. malayi* cathepsin L-like cysteine protease (*Bm-cpl-1*), *B. malayi* β -Tubulin (*Bm-tub-1*), *B. malayi* glutamate-gated chloride channel alpha-3A subunit (*Bm-GluCl- α 3A*), *B. malayi* G-protein coupled acetylcholine receptor-2 (*Bm-Gar2*) and *B. malayi* FMRamide-like peptide-21 (*Bm-flp-21*). Up-regulation of transcript was observed with *in vivo* dsRNA exposure for *B. malayi* prohormone convertase-2 (*Bm-PC2*). The consistency and reliability of the *in vivo* RNAi protocol may be due to exposing the filarial worm within its host environment unlike previous *in vitro* RNAi protocols which requires the worm to be extracted from its host prior to carrying out RNAi.

Bm-cpl-1 suppression revealed new phenotypes associated with molting, growth and development, and motility that shed light on the important biological functions of this gene family in larval stages of *B. malayi*. *Bm-cpl-1* suppression significantly inhibited worm motility and inhibited normal worm terminal curvatures with a significant reduction of exhibited rigorous knotting behavior. In addition, *Bm-cpl-1* suppression compromised normal worm migration within the mosquito intermediate host. With *Bm-cpl-1* suppression, mosquito host survival was significantly increased as parasite prevalence decreased. *Bm-cpl-1* suppression also resulted in a significantly shorter worm length. Thus cathepsin L-like cysteine proteases may have potential as targets for the development of more efficient anthelmintic drugs.

Bm-tub-1 and *Bm-GluCl- α 3A* suppression showed we could reduce the expression of *B. malayi* genes that encode proven drug targets and also showed we could reveal phenotypes associated with drug target knock down through the use of an array of simple assays. *Bm-tub-1* suppression revealed the importance of β -tubulin for normal parasite motility, survival & development, and migration *in vivo*. *Bm-tub-1* suppressed worms exhibited increased kinked posture. Furthermore, *Bm-tub-1* suppression was found to compromise normal worm motility, migration and survival. *Bm-GluCl- α 3A* suppression revealed effects on normal parasite motility, survival and development. *Bm-GluCl- α 3A* suppressed worms were found to compromise normal worm motility, migration and survival.

Bm-gar-2 suppression confirmed the importance of G-protein coupled acetylcholine receptor as potential site for novel anthelmintic drug development. *Bm-gar-2* suppressed worms exhibited compromised normal worm motility and migration.

Bm-flp-21 suppression confirmed the importance of FMRFamide-like peptide for normal parasite movement, survival & development, and migration implicating it as peptides of interest for further investigation and consideration as basis for anthelmintic drug development. *Bm-flp-21* suppressed worms were found to enhance worm survival and detrimentally impact host survivability.

Bm-pc-2 dsRNA exposure did not result in suppression but instead incidences of what would appear to be up-regulation was observed using relative quantitative RT-PCR. Quantitative RT-qPCR confirmed ds*Bm-pc-2* exposure resulted in up-regulation of *Bm-pc-2* expression level by 3 to 4 fold. This up-regulation is not unprecedented as Geldhelf *et al.* in 2005 also noticed similar phenomenon with *H. contortus* vacuolar ATPase gene *He-vha-10* [213]. Although motility,

worm behavior and development were not altered with *Bm-pc-2* dsRNA exposure, worm survival was reduced by 13%.

In conclusion, previous RNAi protocols for parasitic nematodes were unreliable and inconsistent. We have developed a new *in squito* RNAi protocol whereby instead of removing parasite from its host to perform *in vitro* RNAi as previous protocols we here target the filarial nematode *B. malayi* as it develops within the intermediate host, the mosquito *Aedes aegypti*. The consistency and reliability of our *in squito* RNAi protocol was verified on five target genes of interest and its validity as a reverse-genetic tool for identifying novel drug targets and elucidating parasite gene functions was also confirmed. In addition *in squito* RNAi allowed us to gain further insight into the biology of targeted genes in larval *B. malayi*.

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