# HOST-PARASITE INTERACTIONS OF THE FILARIAL NEMATODE *BRUGIA MALAYI* (NEMATODA: FILARIOIDEA)

by

ERICA JANELLE BURKMAN

(Under the Direction of Andrew Moorhead)

## ABSTRACT

The works presented in this thesis aimed to investigate clinical, in vitro, and molecular processes on host-pathogen interactions within the filarial nematode *Brugia malayi*. The hypothesis of the first study was whether we would observe early clinical indicators of *B. malayi* infection in the cat distinguishing between those that would become microfilaremic (mf+) versus amicrofilaremic (mf-). Though there was no significant difference between mf+ and mf- cats, there were 2 animals that presented with a delayed phenotype that differed from these two groups. Dogs were also infected with *B. malayi* $L_3$ to determine whether they could be experimental hosts for this parasite, along with measuring inter- and intraspecies differences with the cats. Dogs became infected, though mf was transient. Dogs that were mf- had elevated lymphocytes, implicating lymphocyte clearance of these parasites. Between cats and dogs, eosinophilia was elevated in the cats, but not dogs, suggesting these granulocytes may play a protective role for the parasite in a primary infection. The aim of the second study was to determine whether the addition of

heavy metal ions would facilitate development, molting, and survival in an in vitro culture assay. The hypothesis was zinc and iron ions will aid *B. malayi* $L_3$ molting and survival, thereby facilitating development into the $L_4$ stage while copper ions will hinder development. Copper ions were found to kill *B. malayi* $L_3$ in culture, zinc encouraged development, but not survival, and iron facilitated survival but did not develop. The final study aimed at identifying parasite-derived miRNAs found in infected feline plasma. We hypothesized that we could identify host immunity gene transcripts targeted by *B. malayi* secreted miRNAs. Nine gene targets were identified that targeted potential immune processes, suggesting that miRNAs secreted by *B. malayi* may affect the immune response of the host.

INDEX WORDS: *Brugia malayi*, Lymphatic filariasis, Host-pathogen interaction, MicroRNA, Eosinophilia, Excretory/secretory

# HOST-PARASITE INTERACTIONS OF THE FILARIAL NEMATODE *BRUGIA MALAYI (*NEMATODA: FILARIOIDEA)

by

ERICA JANELLE BURKMAN

B.S., Florida International University, 2002

M.S., Georgia Southern University, 2009

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2019

# HOST-PARASITE INTERACTIONS OF THE FILARIAL NEMATODE *BRUGIA MALAYI* (NEMATODA: FILARIOIDEA)

by

ERICA JANELLE BURKMAN

| | |
|---|---|
| Major Professor: | Andrew R. Moorhead |
| Committee: | Adrian Wolstenholme |
| | Bridget Garner |
| | David Peterson |
| | Timothy Geary |

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 2019

DEDICATION

To my husband and son, Brad and Brandon, I could not have done this without you both.

All my love.

ACKNOWLEDGMENTS

I would like to thank my major professor Dr. Andrew Moorhead for his guidance and endless patience. I would like to thank my committee members Dr. Adrian Wolstenholme, Dr. Bridget Garner, Dr. David Peterson, and Dr. Timothy Geary for their invaluable advice and suggestions. And endless thanks to my lab colleagues Dr. Dzimianski, Mr. Chris Evans, and Mr. Bob Storey for their vital technical help in collecting materials and data analysis during my research.

Family and friends are paramount to making through a Ph.D. with your sanity intact. I have been fortunate to have had both love and support from all my family, but notably my mother, grandmother, and my aunt Cindi who have supported me throughout the entirety of my educational career. Also, my close group of friends that have been there for me for game nights, painting parties, and when I needed time to de-stress and have some fun. Lastly, to my husband Brad and my son Brandon, to the endless joy these two have brought to my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# ABBREVIATIONS

| | |
|---|---|
| ADL | Adenolymphangitis |
| AFL | Acute filarial lymphangitis |
| ALB | Albendazole |
| Bands | Immature neutrophil |
| CBC | Complete blood count |
| DEC | Diethylcarbamazine |
| Dpi | Days post-infection |
| E/S | Excretory/secretory |
| Eos | Eosinophils |
| HBSS | Hanks balanced salt solution |
| Hct | Hematocrit |
| Hgb | Hemoglobin |
| IP | Intraperitoneal infection route |
| IRS | Indoor residual spraying |
| IVM | Ivermectin |
| jA | Juvenile-adult stage |
| $L_1$ | First larval stage |
| $L_3$ | Third larval stage / infective stage |
| $L_4$ | Fourth larval stage |
| LF | Lymphatic filariasis |
| LLIN | Long-lasting insecticidal bed nets |
| Lymphs | Lymphocytes |
| M2 | Alternatively activated macrophages |
| MCH | Mean corpuscular hemoglobin |
| MCHC | Mean corpuscular hemaglobin concentration |
| MDA | Mass drug administration |
| Mf | Microfilaria/microfilariae |
| miRNA | MicroRNA |
| Monos | Monocytes |
| mRNA | Messenger RNA |
| NTDs | Neglected tropical diseases |
| Segs | Segmented neutrophil |
| SQ | Subcutaneous |
| UTR | Untranslated region |
| WBC | White blood cells |

# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## Neglected tropical diseases and their impact

Neglected tropical diseases (NTDs) are a group of viral, bacterial, and parasitic diseases that affect more than one billion people globally. These groups of diseases are neglected due to largely affecting only the non-developed world and persist in the poorest and most remote communities [1]. There are 20 NTDs prioritized by the World Health Organization (WHO) affecting more than 1.4 billion people in 149 endemic countries. Of these 20, 8 are caused by helminths: cysticercosis-taeniasis (tapeworm), echinococcosis, dracunculiasis (guinea-worm), food-borne trematodiases (flukes), onchocerciasis (river blindness), schistosomiasis, soil-transmitted helminthiases (STH), and lymphatic filariasis (nematodes) [2]. These NTDs can cause substantial illness, impairing both physical and cognitive development. Because of this, farming and/or earning a living can be difficult, thus trapping the most vulnerable in a cycle of poverty with few options of escape.

## Lymphatic filariasis

One of the most disfiguring and disabling neglected diseases is lymphatic filariasis (LF). LF is a mosquito-borne disease that can lead to disfiguring conditions such as lymphedema, hydrocele, and elephantiasis [3]. Human LF is caused by the parasitic nematodes *Wuchereria bancrofti* (~90% of cases), *Brugia malayi* (~10% of cases) and *B.*

*timori* (~<1% of cases) [2]. These parasites are a major cause of morbidity throughout the tropics, with an estimated 120 million people infected in 72 countries and 40 million disfigured and/or incapacitated by this disease [1-3]. These filarial nematodes are obligate parasites that can persist in the infected host for up to 5-12 years [4] and are transmitted via a mosquito vector, which varies geographically [5]. LF can result in an altered lymphatic system leading to lymphangiectasia and chronic lymphedema causing pain and severe disability. Medications may not reverse the symptoms of LF, but education and patient management of disease, such as washing and elevation of affected limbs, can help improve a patient's overall condition [6].

**History of Lymphatic filariasis**

The history of lymphatic filariasis, referred to colloquially as elephantiasis, is speculated to be first recognized in ancient Egyptian times. Pharaoh Mentuhotep II ruled Egypt from approximately 2046 BC to 1995 BC. He is depicted in a statue (Figure 1.1) with enlarged limbs, a characteristic symptom of elephantiasis. Later, at approximately 500 AD, West African artifacts from the Nok civilization show scrotal swelling or hydrocele, another common symptom of LF infection in males [7]. The first written accounts of this disease emerged from ancient Roman and Greek civilizations. The term elephantiasis originated from the Greek word for elephant - eléfantas, notably due to the thickened, elephant's hide-like appearance of the skin. It was not until the 16th century while a Dutch merchant and historian named Jan Huygen van Linschoten was exploring Goa in Western India that he noted the clinical symptoms of LF. Here he wrote that "all borne with one of

their legges and one foote from the knee downewardes as thick as an Elephantes leg, the other legge and all their members without any deformitie, being well proportioned" [7-8].

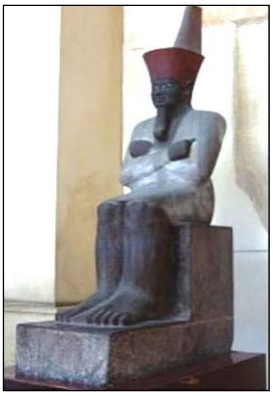


Figure 1.1: Statue of Pharaoh Mentuhotep II. This statue depicts swollen limbs suggesting possible elephantiasis [9].

**Discovery of microfilaria**

Several hundred years later, in 1843, an English physician William Prout published the book "On the nature and treatment of stomach and renal diseases" describing a condition referred to as chylo-serous urine. He noted the appearance of this urine was opaque and milky-white. One of the unique features of this observation was those individuals that presented with chylous urine had few symptoms and were notably less marked and severe than was expected in renal disease patients. Most of the cases exhibiting these symptoms were patients from hot climates such as the West India Islands [10]. Twenty years later, in 1863, a Parisian surgeon named Jean-Nicolas Demarquay had an 18-year-old patient originally from Havana, Cuba presenting with a left-side scrotal tumor. The fluid aspirated from the tumor was a milky yellow-whitish fluid. Once the fluid was removed, the patient was declared healthy and returned home. One year later, the same patient returned presenting with a hydrocele now on the right side. Again, the fluid was

aspirated and was found to be a milky-blue-white fluid. Intrigued, Demarquay had this fluid examined by a fellow colleague and surgeon, Dr. Lemoine. The composition of the chylous urine contained pus, fat globules, fibrin, and many small transparent worms. Samples of the fluid were sent to a well-respected microbiologist/parasitologist Casimir Joseph Davaine. Unfortunately, Dr. Davaine was unable to detect the worms in the sample fluid that Dr. Lemonie had witnessed. Fortunately, Dr. Lemoine had also sent drawings and descriptions of the worms with the patient's urine samples and Dr. Davaine remarked that from these images and descriptions the worms seen were likely some species of nematode. Specifically, he noted they were the larval form of a nematode due to the transparent phenotype and lack of visible organs. The only known nematodes of humans at that time were *Trichina* (=*Trichinella*) *spiralis* and *Filaria* (=*Dracunculus*) *medinensis* [11]. Unable to confirm their findings, Demarquay's group's discovery went largely unnoticed for many years. In 1866, Otto Wucherer, while searching for *Schistosoma haematobium* in patient's urine in Bahia, Brazil, also found these same thread-like, transparent worms.

**Nocturnal periodicity**

In 1872, Timothy Lewis, a British physician working in Calcutta, was reviewing blood slides from a male patient with diarrhea when he “observed nine-minute nematoid worms in a state of great activity, on a single slide” as seen in figure 1.2 (Lewis, 1872). When he showed a colleague, Dr. Cunningham, this slide, they both agreed they were the same nematodes that were seen previously in chylous urine. This was a confirmation of what Dr. Otto Wucherer had observed in peripheral blood samples. Unfortunately, this

patient checked himself out of the hospital and Dr. Lewis was unable to find him to continue his observations. Fortuitously, encountered a female patient with haematochyluria where he noted the presence of these same worms. Afraid that this patient would disappear like his previous case, he visited her at her home during the evening and obtained another blood sample. Lewis kept this patient under observation for two months. He observed that during the evening, there was a transient surge of microfilariae into the bloodstream after being absent during the day. He had come to discover that these helminths had a nocturnal periodicity. Interestingly he noted as well that the clinical condition of the female patient did not worsen during this time [10-11].

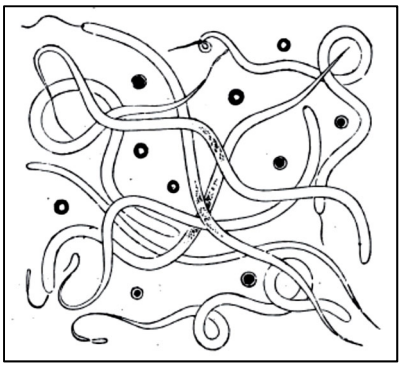


Figure 1.2: Living filariae observed by Lewis in a single blood preparation of a woman suffering from chyluria. RBCs were added to the drawing to compare sizing to that of the adult worms (Lewis, 1872).

**Adult worm & vector discovery**

Joseph Bancroft was a British surgeon and parasitologist living in Queensland, Australia. In 1876, he was the first to discover the adult filaria in a lymphatic abscess in a patients arm. On March 21$^{st}$ the following year, he also found the adult worms while examining the fluid from a patient with a hydrocele, thus confirming this initial discovery [11]. Thomas Spencer Cobbold, a colleague of Bancroft's, wrote to the Lancet in 1877

about this discovery where he named the worm *Filaria Bancrofti* in honor of Joseph Bancroft [13]. Bancroft had not only found the adult worms but was one of the first scientists to suggest that disease was transmitted by mosquitoes. However, it was Patrick Manson (1844-1922) who reported the development of filarial embryos in the mosquito [11]. Once it was known in the medical field that these microfilariae were periodically detectable in the blood, the Scottish doctor Patrick Manson hypothesized that night-biting mosquitoes might be responsible for transmission. Manson worked as a doctor in Kaohsiung, in the south of Formosa, present-day Taiwan. In subsequent years, he carried out various experiments in Amoy (Xiamen), China. One of these experiments involved allowing mosquitoes to bite his filarial-infected gardener. After these mosquitoes fed, he dissected them to look for microfilariae. In doing this, he was able to follow the metamorphosis of the parasites in the mosquito host. Manson, however, thought that mosquitoes fed on blood only once in their lifetime. So, there was a problem in explaining transmission. He assumed that drinking water contaminated by dead mosquitoes was the source of transmission [11]. This mystery was solved by Manson's assistant George Carmichael Low in 1900, who found the third-stage larvae ($L_3$) in the proboscis of the mosquito [14]. This indicated that the parasites are transmitted via the bite of infected mosquitoes, primarily by the night-biting *Culex* and *Anopheles* mosquitoes [11]. This biting behavior is important as the numbers of microfilariae in the peripheral blood systematically fluctuate over a 24-hour period reaching their highest levels at night. The density of parasites is greatest at the time when the chance of transmission is greatest (at night). In the Pacific Islands, transmission occurs via the daytime-biting *Aedes* mosquitoes so there is no or less diurnal variation (= subperiodic form). Manson is regarded by many

as the father of tropical medicine due to the elucidation of the filarial life cycle and other discoveries in parasitology.

**Lymphatic filariasis: Elimination efforts**

In 2000, the World Health Organization's (WHO's) Global Programme to Eliminate Lymphatic Filariasis (GPELF) was formed in response to a World Health Assembly resolution (WHA50.29) to eliminate LF as a public health threat by 2020 [15]. The goals of GPELF are two-fold, one is to interrupt transmission of disease with mass drug administration (MDA), and the second is to manage morbidity and prevent disability in those populations affected [15]. Along with these efforts, ectoparasite control such as integrated vector management can also be used to reduce the vector, the mosquito in the case of LF, from transmitting of the disease [16]. Currently, there are 73 countries in which LF is endemic, with a further 17 considered non-endemic and under surveillance [17]. Although these efforts are strategic and backed by a wealth of research, there are still huge roadblocks in executing LF elimination in areas that have little to no organized health systems, the concern of the use of only a few medications for the MDA program, varying degrees of financial support, and political and/or civil unrest [18–21]. Due to these hurdles and the sheer scope of the GPELF, there is considerable skepticism in the LF community on whether the lofty goal of global elimination of LF by 2020 will indeed be reached.

Fifteen years have passed since GPELF was formed by the WHO. Within the first ten years, 2.8 billion treatments for LF were distributed to more than 800 million people in 53 countries [6, 20]. During the next decade, the same strategic approach will remain unchanged, maintaining the same goals and targets. However, since 2000, the global health

environment has changed drastically, with GPELF as a part of a comprehensive program of NTD elimination and control where MDA, morbidity management, and vector control are integrated as a whole package at the global, national and local levels [22]. The benefits of such synergy among health and elimination programs would work by combining preventative measures for both malaria and other mosquito-borne NTDs through vector control via bed nets. As of 2017, 51 countries still require MDA, and 5 of these countries (Equatorial Guinea, Gabon, Sao Tome and Principe, South Sudan, and New Caledonia) had not started MDA programs [23]. Initially, the 2020 GPELF goal was 100% of all endemic countries will have entered the post-intervention surveillance [22]. However, even with expanded global coverage of MDA programs, these programs will need to continue administering drug treatments past the 2020 goal [23]. As such, there still remains the need for adequate education and awareness of the importance of NTDs. Both the population and those in power in these endemic countries need to support these programs so that adequate financial resources are provided to accomplish the crucial break in the transmission cycle.

There are three drugs used in the LF MDA program: albendazole (ALB), ivermectin (IVM) and diethylcarbamazine (DEC) [21-22]. Those living in endemic areas can take a yearly dose of the medications ALB plus either IVM or DEC that kills the microfilariae circulating in the blood [6]. The reasoning for using these two drug combinations is that clinical trials have shown that ALB in combination with DEC or IVM is more effective than a single treatment drug, yielding up to 99% clearance of the microfilariae up to one-year post-treatment [25]. In 2018, a study by King *et al* published the efficacy of one-dose triple-drug therapy with DEC, ALB, and IVM [26]. This triple-drug therapy resulted in a 96% mf clearance for three years after only one treatment, compared to 83% clearance

after three years with the two-drug regimen. However, the two-drug regimen administered annually resulted in mf clearance of 98-99% of individuals suggesting this as the superior method for LF control. However, in locations where annual administration of medications is difficult, the triple-therapy would be an effective solution. These drugs target the microfilariae but can also affect the adults by reducing reproduction and affecting the production of additional microfilariae long after the drug has dispersed within the patient [24-25]. The strategy of using these drugs is based on reducing the number of microfilariae circulating in people in the endemic regions to levels below which transmission cannot occur [25]. By annual administration of these medications (or every 3 years with triple-drug therapy), the goal is to maintain low microfilariae levels for the duration of the fecundity of the adult female worms (4-6 years) [25]. These drugs, which are readily available through donations made by Merck, GlaxoSmithKline (GSK), and Eisai are distributed to the entire at-risk population through the MDA program [6]. For albendazole, GSK has committed to an unlimited supply for as long as the drug is needed to combat LF [22]. The pharmaceutical company Eisai Co., Ltd has donated to the WHO up to 2.2 billion tablets of DEC for the period of 2014-2020 [22]. Merck & Co., Inc., has also promised an unlimited supply of Ivermectin for as long as is needed and is donated direction to countries for not only LF but for onchocerciasis as well [22]. As of 2017, Merck & Co. expanded their donation of Ivermectin by up to 100 million additional treatments through 2025 towards LF and onchocerciasis elimination [23].

The second major goal of the GPELF was in reducing morbidity and to prevent disability caused by LF in those populations affected [15]. Though the MDA program is

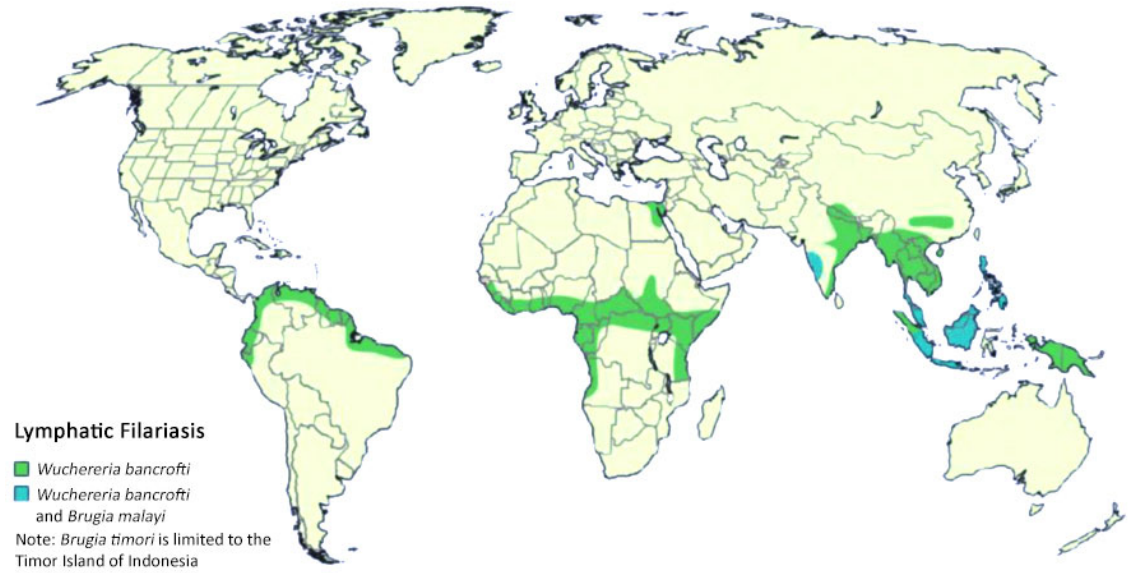



Figure 1.3: Global distribution of Lymphatic Filariasis (Ghedin et al., 2007).

now well advanced, morbidity management remains limited with only 26 of the 73 endemic countries having programs focusing on living with the disease [21]. *Wuchereria bancrofti* is found geographically throughout the equatorial belt and *B. malayi* is confined to regions of South and Southeast Asia. *Brugia timori*, however, is localized on the Lesser Sunda Islands of Indonesia (Figure 1.3) [29]. Infection with either *W. bancrofti* or either *Brugia* species can result in both acute and chronic clinical manifestations such as lymphoedema, adenolymphangitis (ADL), and bacterial infections causing significant pain and fever during the acute phase [20, 28]. This is then followed by chronic lymphoedema, hydrocele and potentially elephantiasis [30]. Other less reported conditions that are often hidden due to social stigma are lymphoedema of the breast and vulva, rheumatic, and respiratory problems [21]. Though MDA drugs kill circulating microfilariae, they do little in regards to treating the disease once the worms have reached sexual maturity. Morbidity programs

educate and advocate for self-management of symptoms to reduce suffering caused by LF. These measures are simple, such as regular washing and elevation of affected limbs, in addition to exercise [1]. For hydrocele, surgery is an option but is often too prohibitively expensive [1]. Among these clinical manifestations, most debilitating are those associated with lymphatic dysfunction.

**Vector Management for Lymphatic filariasis**

In addition to the MDA and morbidity programs, 68 vector-borne disease-endemic countries have established national policies for integrated vector management for LF as an additional control method in reducing mosquito populations [21, 28]. For example, in areas where LF is co-endemic with *Loa loa,* preventative treatments are limited due to adverse reactions to the available drugs. Therefore, vector control is crucial for disease management [22]. These methods are also important in areas of inadequate MDA coverage or where the cost of treatments is prohibitive [31]. Vector control relies on the use of low-risk pesticides that are recommended by the WHO Pesticide Evaluation Scheme (WHOPES) [21, 29]. These pesticides are either sprayed into the environment (aerosol sprays), inside homes (residual insecticides) or imbued in bed nets as preventative measures [15, 29]. Although vector control has a proven record of reducing the burden of vector-borne diseases, its benefits are far from being realized. Complications such as the scarcity of skills in management and implement vector control, the development of insecticide resistance in disease vectors, and minimal or lack of collaboration between infrastructure development programs and the health sector are only a few difficulties in establishing these vector programs [31]. Additionally, the majority of these programs have

been with countries with well-established health systems. Information and data in remote or post-conflict settings are scarce or unknown when healthcare delivery structures are lacking or non-existent. For instance, in South Sudan, due to years of violence and instability, their public health system and other services remain devastated, making intervention methods extremely difficult [31]. In addition, the country is rural, water-logged with minimal roads, combined with the nomadic behavior of the people, adds to the difficulty of deployment of these preventative measures [31]. Utilization of long-lasting insecticidal bed nets is compromised by their abuse or misuse and most homes are not amenable to indoor residual spraying (IRS) [31]. Though integrated vector management has been shown to be an effective tool towards the goal of LF elimination, there remain fundamental roadblocks for many countries in establishing these programs.

**Anthelminthic resistance**

The scope of work organized and facilitated by the GPELF has not been a small feat by any measure. Those working from the local all the way to the global scale should all be lauded for the incredible efforts accomplished over the years. Billions of people have been impacted by this program in improving the overall health and quality of life throughout the world. However, there are a number of roadblocks that actively work against LF elimination. Within the past several decades, there has been increasing evidence of other helminths, such as *Haemonchus contortus* and *Onchocerca volvulus* gaining resistance to one or more classes of drugs [27]. A concern with the current LF MDA program is the use of only a few drugs given to an increasingly larger number of people. Ivermectin, one of the drug treatments for LF, may select for a nematode β-tubulin allele,

which is also responsible for resistance to benzimidazole anthelmintics such as ALB [27]. Because LF MDA programs rely on these two-drug combinations, the repeated dosing year after year may enhance selection pressure for the benzimidazole class resistance. By continuing to use these measures, it not only potentially jeopardizes the LF program, but also STH treatment programs that rely upon these drug classes as well [27]. Having only three drugs (IVM, ALB & DEC) used in the LF MDA program it is not surprising that research efforts into new drug classes for these and other nematodes are a global priority. A lack of efficacy in any of these three medications would result in a resurgence of transmission ensuring that elimination of LF as a public health concern would not be accomplished by the 2020 GPELF goal.

**Filarial life cycle & morphology**

Lymphatic filarial parasites provide a unique challenge in treatment and elimination due to the complexities of their life cycle and their obligate requirement for a mosquito intermediate host. The life cycle of these filarioids are a series of morphologically distinct forms in both the mosquito vector and vertebrate host comprised of five major stages separated by four molts. In each endemic area, there are local vector species belonging to the genera *Culex, Aedes*, *Anopheles,* and *Mansonia*. Figure 1.4 depicts the life cycle of *Wuchereria bancrofti* in humans and mosquito vectors. Life cycles of other lymphatic nematodes (i.e., *B. malayi and B. timori*) are identical, while the life cycles for other filarial worms differ in the body location of adult worms, the microfilariae present, and the arthropod intermediate hosts and vectors.

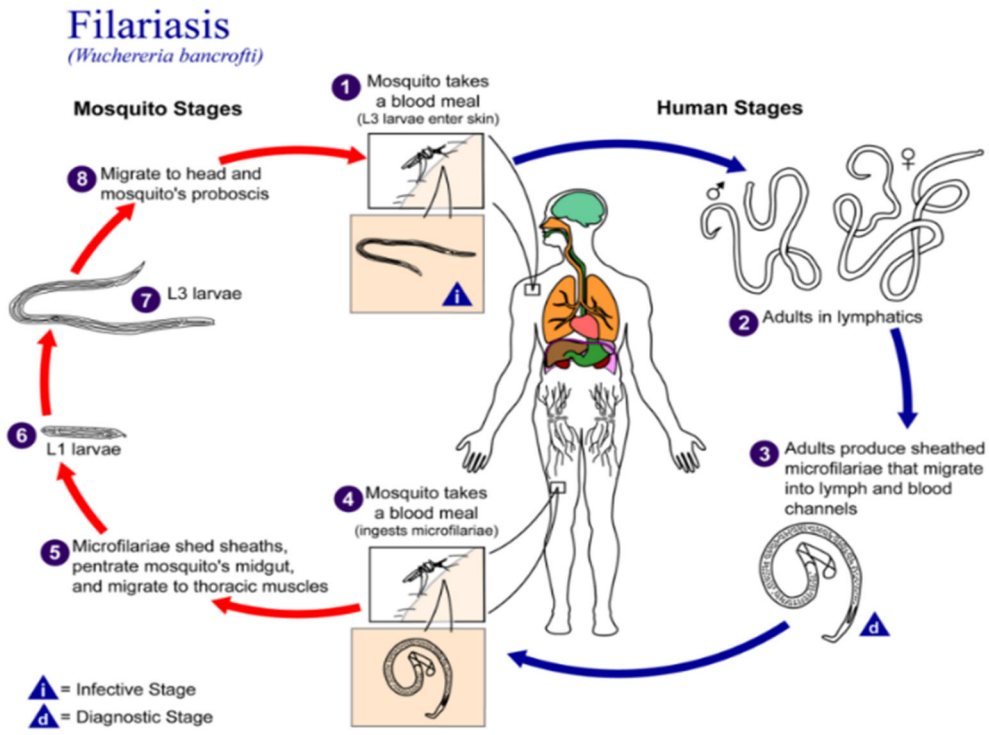



Figure 1.4: Filariasis life cycle using *Wuchereria bancrofti* as the main example [1].

The life cycle for both *W. bancrofti* and *B. malayi* begins with the mosquito vector biting an infected host and ingesting microfilariae found in the blood. These microfilariae retain the egg membrane as a protective sheath [33]. Once inside the mosquito vector, the microfilariae enter the mid-gut with the blood meal where they exsheath. The microfilariae then penetrate the wall of the mid-gut and migrate to the thoracic muscles where they develop through two molts from an $L_1$ to an infective third-stage larva ($L_3$) (1,500 x 20 µm) [34]. After approximately 10-14 days, the $L_3$ migrate to the proboscis where it awaits for the mosquito to feed on its next host. A few morphological distinct characteristics of $L_3$ are a sealed buccal cavity and the absence of an open gut and gonads. Once the infected

mosquito bites another human host, the $L_3$ enter the bite wound and migrate through the host to the lymphatic vessels molting twice more into the fourth-larval stage ($L_4$) and juvenile adults, finally maturing into adults. Characteristics of the $L_4$ stage are an average length of 2 mm, fully formed gut and gonads, along with an open buccal cavity [35]. The adult females are ovoviviparous, thereby producing thousands of microfilariae after copulation with the adult males [33].

According to Simonsen (2008), an adult *W. bancrofti* female on average measures 80-100 mm long and 0.20 mm wide [34]. The males are smaller at 40 x 0.1 mm. *Brugia* adults in comparison are typically half the size of *W. bancrofti* adults. Circulating microfilariae in the peripheral blood of the host are observed on an average of 8 months after infection with *W. bancrofti* and 3 months for *B. malayi*. *Wuchereria* microfilariae measure on average 260 x 8 μm; whereas, *Brugia* microfilariae are slightly smaller [34]. These microfilariae circulate within the host's peripheral bloodstream for up to a year circulating within the host where they are taken up in the blood meal by another mosquito, thus completing the life cycle. Most lymphatic filarial species have a nocturnal periodicity (microfilariae counts peak between 10 p.m. and 2 a.m.). The adult worms can survive and produce microfilariae for up to 20 years, though the typical lifespan is 5 years. Due to the longevity and fecundity of these worms, halting transmission remains a formidable challenge.

**Pathology of LF**

Lymphatic filariasis pathology consists of both chronic and acute manifestations of disease. The primary instigator of LF morbidity is the adult worms and their location within

the lymphatics of the infected host. Movement of the worms within the lymphatic system and the resulting immunological response has the potential to elicit the chronic form of LF resulting in lymphedema, elephantiasis, and hydrocele [31, 33]. Fortunately, the chronic form of LF occurs in only approximately 10% of the infected population. The remaining 90% of those living in endemic regions are generally asymptomatic [36]. In these individuals, high microfilaremia is often observed, which is paradoxical due to their high worm intensity and asymptomatic status. Over time, exposure to filarial antigens shifts the immune system from a T helper cell (Th) 1 towards a Th2 response. This polarization of the filarial antibody response towards Th2 enables chronic persistence of the parasites within these individuals [34]. This group serves as the primary source for LF transmission in endemic regions. There are also individuals that despite repeated exposure never become infected. One proposed hypothesis is this group is immune due to circulating antibodies against the infective $L_3$ of these parasites [37]. Additionally, there are those that can present with low to no circulating microfilariae. These individuals have a tendency towards a Th1-type hyper-responsiveness, exhibiting a significant response to the parasites [34]. Unfortunately, this has the potential to lead to subsequent damage to the lymphatic system as well. This damage has long-term consequences, as these individuals have a high probability of developing lymphangiectasia, elephantiasis, and hydrocele.

Acute filarial disease also referred to as filarial fever or adenolymphangitis (ADL) is characterized by periodic attacks of fever, chills, malaise, and enlarged lymph nodes. These acute attacks typically resolve within a week, but maybe reoccurring and are often found in those that develop the chronic form of disease. There are two recognized forms of these acute attacks. One, acute filarial lymphangitis (AFL), is caused by the death of the

adult worms either naturally or through treatment. As the worms die, the bacterial endosymbiont *Wolbachia* residing within the worms are released causing an innate immune response via macrophage activation and neutrophil recruitment [35, 36]. This immune response can lead to granulomas forming around dead parasites thereby blocking the flow of fluid within the lymphatics. This blockage can result in localized inflammation and lymphangiectasia (dilation) further damaging the lymphatic system. The second type of acute attack is acute dermatolymphangioadenitis (ADLA), caused by secondary bacterial infections due to damaged and compromised lymphatics. ADLA has been implicated as the primary pathway leading to chronic LF, lymphedema, and elephantiasis [34].

The clinical features of lymphatic filariasis are lymphedema, elephantiasis, hydrocele, chyluria and tropical pulmonary eosinophilia (TPE). Lymphedema is localized fluid retention caused by damage to the lymphatic system by the adult worms. When the adults die and block lymphatic vessels, this causes dilation and is one of the first clinical signs of disease, most commonly observed in the lower extremities (Left - Figure 1.5).

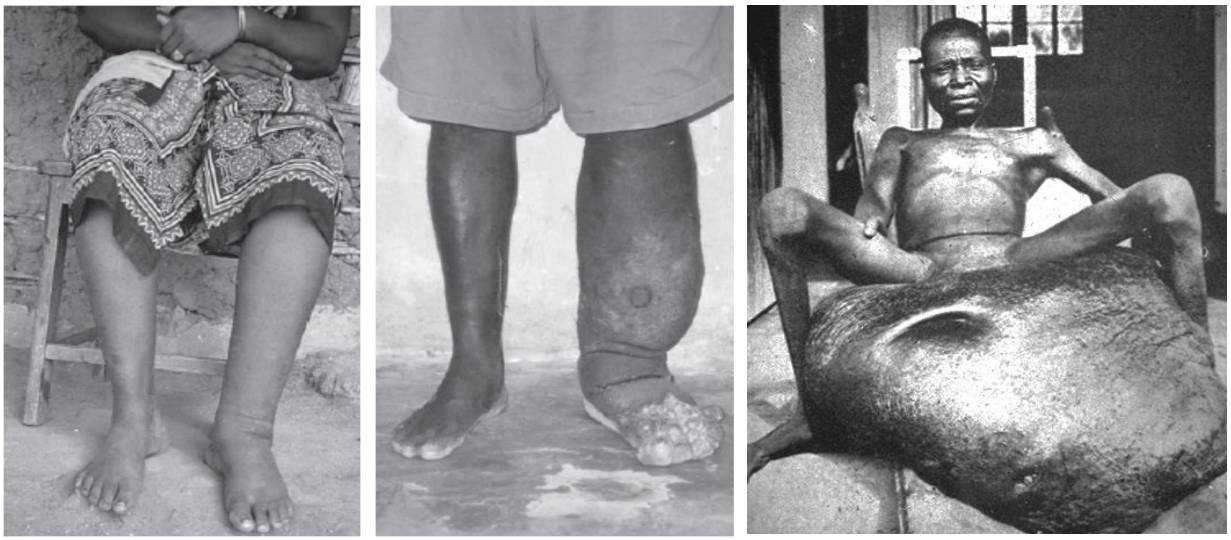


Figure 1.5: Clinical features of lymphatic filariasis. Left, lymphedema of the left limb. Middle, advanced stage elephantiasis of the left limb [34]. Right, severe hydrocele [40].

Episodic attacks of lymphedema for many years can result in excessive skin exfoliation which thickens the skin. As this skin becomes tough, recurrent erysipelas (bacterial superinfections) may cause a progression towards elephantiasis (Middle - Figure 1.5) [40]. The condition elephantiasis earns its name from the thick and elephant-like texture of the skin. Elephantiasis most commonly affects the legs; but the arms, scrotum, penis, vulva, and breasts may be affected. Following recurrent acute attacks, chronic edema lends to loss of skin elasticity and the development of fibrosis. Further progression leads to deep skin folds, dermatosclerosis, and papillomatous lesions [34]. Additionally, secondary fungal and bacterial infections may exacerbate this condition, causing difficulties in keeping the skin healthy and clean, thus further contributing to the stigma of this disease.

Unfortunately for the men in LF endemic regions, hydrocele is the most common result of infection with *W. bancrofti*. Infection with the *B. malayi* parasite typically results in milder versions of lymphedema and elephantiasis. Hydrocele occurs when there is an accumulation of fluid in the sac surrounding the testicles. The onset of hydrocele can be acute and initially unilateral. The hydrocele may resolve completely in these early stages. However, after a number of these acute attacks, the tunica vaginalis becomes thickened and progressive enlargement may occur [34]. Chyluria is an uncommon symptom of *bancroftian* filariasis where there is a rupture of damaged dilated lymphatics that result in the milky lymph fluid (chyle) to accumulate in the urinary excretory system. Chyluria is frequently recurrent with episodes lasting days or even weeks. If chyluria persists for long periods of time it may result in anemia, loss of weight, hypoproteinemia, and lymphopenia [34].

Tropical pulmonary eosinophilia (TPE) is another rare syndrome most commonly seen in India and Southeast Asia. It is caused by a hyperimmune response to the presence of microfilariae in the lungs resulting in marked eosinophilia (usually >3000 cells/$mm^3$). The predominant pulmonary symptoms are coughing, dyspnea, and wheezing. In rare cases, the lymph nodes can swell and splenomegaly can occur. Circulating microfilariae is not found in the peripheral blood, but can be detected in lung biopsies and chest x-rays identified as patchy infiltrates [40]. TPE is mainly found in males and responds well to treatment by DEC. If this syndrome is not treated, it can lead to pulmonary fibrosis and subsequent loss of lung function.

**Importance of *B. malayi* research**

These filarial parasites are transmitted by a variety of mosquito species whereas their mammalian host range is selective. *Wuchereria bancrofti* only infects humans with only sub-clinical and occult infections within non-human primates [41–43], whereas *B. malayi* infects humans, non-human primates, ferrets, multimammate rodents (*Mastomys*) and the Mongolian gerbil (*Meriones unguiculatus*) [42, 44–51]. Currently, the process of how these filarial parasites are able to invade and infect some individuals, but not others remains poorly understood. In this research, the overall goal is in elucidating what determinants, both host and parasite, play a role in filarial host-specificity. Using this comparative approach in defining how these parasites are able to survive and thrive in these selective hosts may facilitate targets for new anthelmintic compounds. Ideally, this precise approach would be applied in pharmacotherapy with the potential benefit of fewer side effects and increased parasiticidal effects than broad-spectrum drugs currently utilized.

**Animal models**

Once *W. bancrofti* and *B. malayi* were found to be the causative agents of LF, finding a suitable animal model became an important aspect for research in control and elimination of these parasites. In communication with Edeson, Wharton, and Buckley in 1955, they found in East Pahang, Malaysia that a variety of both domestic (including felines) and forest animals tested positive for filarial worms morphologically similar to *Wuchereria malayi* (Brug 1927, currently *B. malayi*). If this were to be the case, then a number of mammalian species could serve as reservoir hosts thus affecting elimination efforts. However, when the adults were carefully examined and compared with previously described filarial worms they found two distinct species *W. malayi* and *W. pahangi* (currently *B. pahangi*) [41]. Only through experiments would they be able to determine whether these parasites were the same filarial worm found in humans. Additionally, it was unknown whether these parasites would be transmissible from humans to animals. As there were not many human volunteers to become infected with filarial parasites found in animals, Edeson and Wharton (1957) instead used laboratory-bred *Mansonia uniformis* mosquitoes fed on individuals carrying *B. malayi* and infected 5 domestic felines with the subsequent $L_3$. What these scientists found was that felines could become infected with *B. malayi* $L_3$ and produce adults and circulating microfilariae. Subsequent larger studies were performed later on that confirmed these findings [43, 52].

In an effort to find less expensive and easier animal models to work with than felines, a number of different rodent species were examined. While determining whether felines could become infected, Edeson and Wharton (1957) also infected two mice and one guinea pig but were unable to establish a patent infection in these rodents. In 1961, Laing

et al. were able to infect six golden hamsters and one out of six white rats with a subperiodic *B. malayi* strain. However, the prepatent period in these animals were longer (>120 days) than that found established in the feline host [43]. Zaini et al. (1962) were able to infect four out of seven cotton rats and four out of 28 hamsters, but again, the prepatent period was longer in both species than that seen in the feline host. Guinea pigs, rabbits, white mice, and white rats were also experimentally infected with the subperiodic strain, resulting in low and inconsistent infection rates [53, 54].

In 1970, Ash and Riley infected a number of rodents that were either laboratory-bred or wild-caught from the nearby California desert outside Los Angeles [51]. Two of the laboratory-bred species were Mongolian jirds: *Meriones unguiculatus* and *M. libycus*. Both species were infected with $L_3$ subcutaneously and produced adult worms, but only *M. unguiculatus* was able to develop a patent infection in 70% of the 19 jirds inoculated [51]. Additionally, *M. unguiculatus* was found to be a susceptible host for *B. pahangi* as well [51]. Larger trials with *M. unguiculatus* found that males were far more susceptible to the filarial infection [55] and that *B. malayi* $L_3$ could be administered intraperitoneally (IP) [56]. The discovery of the IP infection route was advantageous as it allowed full development of the adult worms and progeny while being contained within the peritoneal cavity. This allowed for retrieval of the worms to be easily accessible facilitating increased production of the worms for study and drug trials.

In an effort to find a somewhat larger animal model than gerbils, several labs also experimentally infected non-human primates and ferrets. Natural non-human primate infections were first reported by Buckley and Edeson, where adult worms were recovered from the lymphatics of a Kra monkey (*Macaca irus*) in Pahang, Malaysia [41]. Other

natural infections include the Silvered leaf monkey (*Presbytis cristatus*) [57]. Over the next sixty years, scientists have experimentally infected the dusky leaf monkey (*Presbytis obscurus*) [43], Patas (*Erythrocebus patas*) and Rhesus monkeys (*Macaca mulatta*) [58], Silvered leaf and leaf monkey (*P. melalophus*) [59], and the Indian leaf monkey (*P. entellus*) [60]. Though many of these non-human primates became experimentally infected with the subperiodic strain of *B. malayi*, due to the difficulties and cost associated with these animals, they are often used in vaccine trials rather than in the maintenance of the parasite life cycle [61]. Whereas, though ferrets developed some pathologic conditions that mimicked human clinical conditions, they unfortunately only developed a transient infection overall [49, 50]. Due to decades of testing a variety of animal models, domestic felines and the Mongolian jird (*M. unguiculatus)* continue to be the primary animal models used in most clinical and research experiments.

**In vitro culture of filarial worms**

The establishment of in vivo and in vitro cultures of parasites has enabled the study their morphology, behavior, metabolism, and physiology. However, the complex life cycle of filarial parasites, having both vector and mammalian hosts, makes them tremendously difficult to study in vitro. Determining the ideal conditions for which to culture these parasites you need to take into consideration factors such as nutrients, temperature, atmospheric pressure, etc. The benefits of establishing an in vitro system are multifactorial: such as reduced cost compared to in vivo research; initial drug screening of one or many different compounds without having to run large in vivo studies; and ethical concerns of using animals in research studies. With the millions of compounds both manufactured and

natural in new and repurposed drug libraries, having an in vitro system can be crucial in finding viable drugs to combat these parasites. Even though in vitro studies have been conducted on filarial worms since the early 1900s, scientists remain unable to mimic the environment of the host. Thus, an effective in vitro system that can perpetuate the lifecycle of these parasites remains a much sought-after goal for filarial researchers today.

In vitro culturing of filarial parasites is credited to Sir Patrick Manson and his peers in the late 1800s to early 1900s, where they maintained the parasites for a few days in blood under Vaseline-ringed coverslips [62]. In the early 1900s, culturing filarial worms was conducted with *Dirofilaria immitis*, colloquially referred to as canine heartworm. These worms were cultured and maintained in whole dog blood, which was only minimally successful in culturing microfilariae and adults [64, 65]. But even during these seminal years of in vitro studies, scientists aware that cultured parasites differed physiologically than what is produced in an in vivo host [65]. By the 1950s, attempts were made in to culture *W. bancrofti* $L_3$ in capillary tubes using whole blood from human volunteers to test the efficacy of DEC [66]. In these studies, the larvae only lived for a couple of days and did not show any significant effect of the compound on the overall survival of the worms.

Thirty years later, attempts to culture any life-stage of filarial worms in a variety of conditions, mediums, and cell lines were still met with little success [68–74]. In 1983, Mak et. al attempted to culture both *B. malayi* and *B. pahangi* from the $L_3$- to $L_4$-stage. Little work had been done on *B. malayi* and *B. pahangi* prior to the 1980s, so they examined larval development in these worms using RPMI-1640 with antibiotics, 10% inactivated human serum and LLC-MK2 rhesus monkey cells as a feeder layer [74]. In this system, they were able to obtain a 20% molting success of $L_3$ to the $L_4$ stage, though molting was

delayed and morphologically the $L_4$ were smaller than what had been noted in the jird model [51]. Riberu et al., 1990 claimed to have developed a cell-free system using NI medium and 10% human serum to grow $L_3$ to sexual maturity in 60 days, with microfilariae being produced as early as 75 days [75]. However, successive labs could not reproduce these results. Instead, they saw delayed molting and stunted growth, with only approximately 1% of $L_4$ molting to the young adult stage [77–80]. Falcone et al. also used Riberu's methodology, but added human lymphatic cells and human dermal fibroblasts as feeder cells [80]. Again, the results were less than ideal. After almost a century of attempts, it had become abundantly clear that these parasites could not be cultured under standard conditions.

In the last twenty years, as culture methods in other fields have become more sophisticated, additional supplements have examined and have been found to aid in culturing *B. malayi* $L_3$. In 2003, Rajan et al. found that ascorbic acid was a critical component for $L_3$ to molt to the $L_4$-stage. Ascorbic acid acts as an antioxidant, scavenging free radicals and other reactive oxygen species (ROS) that could cause damage to the cuticles of these parasitic nematodes [81]. They cultured the $L_3$ larvae with differentiating bone marrow stromal cells, 10% FBS, 8.0 x $10^{-6}$ M P-glyceryl phosphate, $10^{-8}$ M dexamethasone, and 2.5 x $10^{-4}$ M ascorbic acid, all in RPMI-1640 media. What they found was that a concentration of 75 μM was a necessary component when added to the media and serum, and the cells and other additives did not make a difference in development overall [35]. From this point, ascorbic acid became a mainstay in labs attempting to culture these parasites. By the mid-2000s, molecular techniques became far less expensive as a research pursuit, and culturing of these parasites was largely abandoned in favor of looking

at the genome, proteome, and transcriptome of these worms. Due to concerns of drug resistance of current MDA medications to these filarial parasites, there has been a resurgence in finding a methodology in culturing these parasites. To find new drugs or classes of drugs, large-scale studies need to be pursued, with in vitro methods preferable compared to the logistics and expense of animal studies.

**Host specificity and parasite microRNAs**

Host specificity is a fundamental concept by which pathogens are able to survive and grow within a host or subset of hosts. Host specificity can be observed across the spectrum in commensalistic, mutualistic and parasitic relationships. One of the key aspects of this relationship is adaptability to either one host (specialist) or a broad range (generalist). In parasitic-host relationships, there have been a number of documented mechanisms by which parasites, specifically helminths, are able to modulate the immune system of the host to promote their own survival. Due to the evolutionary history of these relationships, hosts often become disease-tolerant or asymptomatic, even with high worm burdens, until there is a dysregulation in the host immune system that can induce the disease state [82–84]. Helminths have evolved mechanisms to modulate the host immune system by shifting the host Th1 response, to a Th2 response promoting their survival [82, 85–88]. Additionally, these parasites may also alter regulatory B cells [89], regulatory T cells (T regs) [90], and alternatively activated macrophages (M2) [91] to evade the host immune system and survive in the harsh milieu of the host.

The molecular mechanisms of how these parasites are able to modify the host immune system and the host environment as a whole are not fully understood. What is

generally accepted is parasitic nematodes release a variety of factors, mostly proteins, enabling the worms to safely penetrate and migrate through host tissues with little to no immune response from the host. These excretory/secretory (E/S) proteins released by the worms have been found to be highly complex, composed of proteases, protease inhibitors, glycolytic enzymes, venom allergen homologs and lectins [92, 93]. This composition can differ depending on the life-stage of the parasite; where one form may be circulating in the blood (e.g. microfilariae) and another in the lymphatics (adults) [93]. These differences in E/S composition have been suggested to reflect differences in immune evasion between the parasite life-stages [88, 92–94]. Besides E/S proteins, it has been suggested that post-transcriptional regulation of gene expression may also play a role in parasite establishment within the host.

MicroRNAs (miRNA) are small noncoding-RNAs (ncRNA) that regulate gene expression post-transcriptionally by binding to target messenger RNAs (mRNA). First identified in the non-parasitic helminth *Caenorhabditis elegans* [95, 96], mRNAs have been discovered in mammals, plants, viruses, and parasitic helminths [97–99]. Over the last few years, many miRNA homologs have been found in a number of parasitic nematodes such as *B. malayi* [100-101], *B. pahangi* [99], *D. immitis* [102], and *H. contortus* [99]. Additionally, many of these parasitic nematode miRNAs can only be found during specific life cycle stages of the parasites implicating a role in host establishment. MicroRNAs function by inhibition of protein-coding genes, acting as biological rheostats by transcript destabilization, translational inhibition, or both. The messenger RNA (mRNA) affected by miRNAs are degraded by two known mechanisms: direct Argonaute2-catalysed endonucleolytic cleavage of the target [103–105], or through

deadenylation and exonucleolytic attack. The latter is the primary mechanism used by miRNAs in mammals [106]. The 5' end of these small RNAs bind to a 2 to 8 nucleotide sequence of the mRNA target referred to as the seed region. This seed region is highly conserved, though perfect complementation is not necessary in order for the miRNA to bind to the mRNA and cause its degradation. In animals, miRNAs predominantly bind to the 3' untranslated regions (UTR) but have been found to bind to any region of the target mRNA [97, 107-108]. MicroRNA biogenesis begins in the nucleus of the cell they are encoded as long primary transcripts (pri-miRNA). This pri-miRNA forms a stem-loop structure containing a 5' cap and a 3' poly-A tail (Figure 1.6).

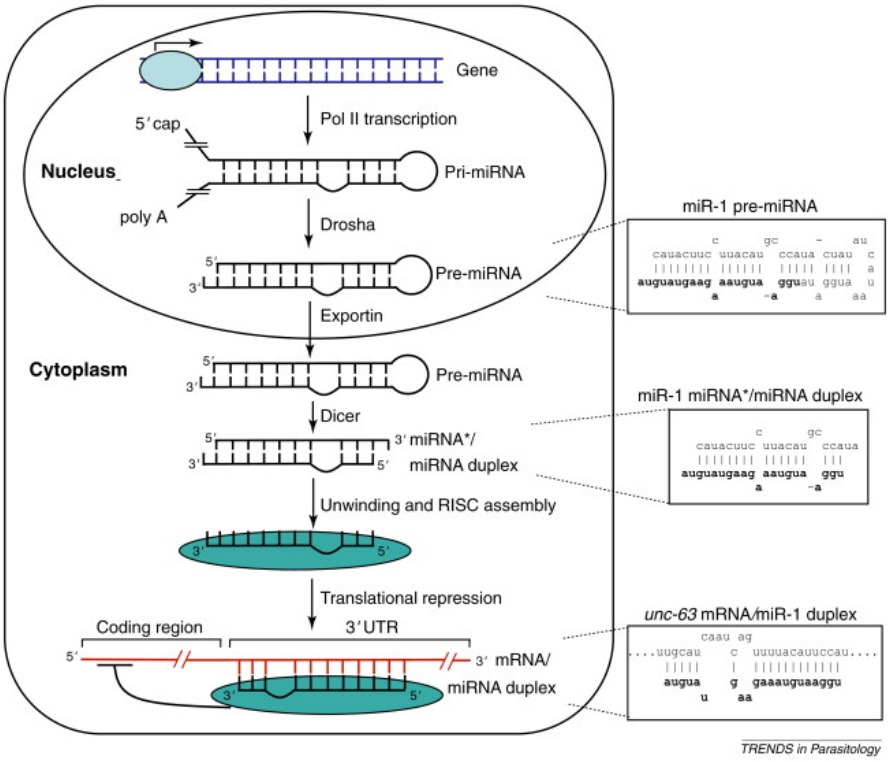



Figure 1.6: Biogenesis of miRNAs [109].

These pri-miRNAs are processed by Drosha, an RNaseII endonuclease III, that trims the 3' and 5' ends to form the pre-miRNA. This pre-miRNA is transported out of the nucleus into the cytoplasm by the protein Exportin. Once in the cytoplasm, the pre-miRNA is processed by an RNaseIII enzyme that cleaves the loop structure so that a short miRNA duplex remains. A helicase unwinds the miRNA duplex into the 20-24 nt mature miRNA which is then incorporated into a multicomponent complex known as the RNA-induced silencing complex (RISC). This RISC complex along with miRNA will bind onto the 3' UTR of the target mRNA resulting in it inhibition or destabilization.

Deep sequencing projects of filarial and other parasitic nematodes have revealed the presence of many miRNA homologs [98]. In 2010, Poole *et al*. identified 32 miRNAs belonging to 24 different families in *B. malayi*. Four years later, an additional 145 miRNAs were identified, including novel miRNAs found only in *B. malayi* when compared those found in *C. elegans*, *Capitella teleta*, *Schistosoma japonicum*, *Loa loa*, *W. bancrofti*, *Aedes aegypti*, and *Homo sapiens* [101]. Other studies have found that many miRNAs of parasitic nematodes are restricted to a specific life cycle stage, implying control of gene expression during development [99]. Recently, it has been suggested that these parasite miRNAs may play a key role in the host-pathogen interface via integration and adaptation of the parasite to the vertebrate host [98, 109]. If these nematode miRNAs are able to target and alter gene expression within the host to survive and develop, this may aid in our understanding of how the wide spectrum of disease observed in LF occurs.

## REFERENCES


[1] CDC, "Neglected Tropical Diseases," *Center for Disease Control and Prevention*, 2014. [Online]. Available: http://www.cdc.gov/globalhealth/ntd/.

[2] WHO, "Lymphatic filariasis," *World Health Organization*, 2014. [Online]. Available: http://www.who.int/mediacentre/factsheets/fs102/en/.

[3] WHO, *Global Programme to Eliminate Lymphatic Filariasis: Managing Morbidity and Preventing Disability*. Geneva, Switzerland: World Health Organization, 2013.

[4] E. Devaney, S. A. Martin, and F. J. Thompson, "Stage-specific gene expression in lymphatic filarial nematodes," *Parasitol. today*, vol. 12, no. 11, pp. 418–24, Nov. 1996.

[5] E. Michael, M. N. Malecela-Lazaro, C. Kabali, L. C. Snow, and J. W. Kazura, "Mathematical models and lymphatic filariasis control: endpoints and optimal interventions," *Trends Parasitol.*, vol. 22, no. 5, pp. 226–33, May 2006.

[6] CDC, "Lymphatic Filariasis: Elimination in the Americas." Center for Disease Control and Prevention, Atlanta, Georgia, pp. 1–4, 2011.

[7] A. Chandy, A. S. Thakur, M. P. Singh, and A. Manigauha, "A review of neglected tropical diseases: Filariasis," *Asian Pac. J. Trop. Med.*, vol. 4, no. 7, pp. 581–586, 2011.

[8] B. R. Laurence, "The Curse of Saint Thomas," *Med. Hist.*, vol. 14, no. 4, pp. 352–363, 1970.

[9] H. B. Routh and K. R. Bhowmik, “History of elephantiasis.,” *Int. J. Dermatol.*, vol. 32, no. 12, pp. 913–916, 1993.

[10] W. Prout, *On the Nature and Treatment of Stomach and Renal Diseases*, 4th revise. Philadelphia: Lea & Blanchard, 1843.

[11] D. I. Grove, *A history of human helminthology*. Wallingford, UK: CAB International, 1990.

[12] T. R. Lewis, *Haematozoon Inhabiting Human Blood: Its Relation to Chyluria and Other Diseases*. Calcutta: Office of the Superintendent of Government Printing, 1872.

[13] T. S. Cobbold, “Discovery of the adult representative of microscopic filariae,” *Lancet*, vol. ii, pp. 70–71, 1877.

[14] F. E. G. Cox, “History of human parasitology history of human parasitology,” *Clin. Microbiol. Rev.*, vol. 15, no. 4, pp. 595–612, 2002.

[15] WHO/GEPLF, *WHO/GPELF lymphatic filariasis practical entomology*. Geneva, Switzerland: World Health Organization, 2013.

[16] WHO, *Global Strategic Framework for Integrated Vector Management*. Geneva, Switzerland: World Health Organization, 2004.

[17] J. Cano *et al.*, “The global distribution and transmission limits of lymphatic filariasis: past and present,” *Parasit. Vectors*, vol. 7, no. 1, p. 466, 2014.

[18] K. D. Ramaiah, “Lymphatic filariasis elimination programme in India: progress and challenges,” *Trends Parasitol.*, vol. 25, no. 1, pp. 7–8, Jan. 2009.

[19] J. O. Gyapong *et al.*, “Integration of control of neglected tropical diseases into health-care systems: challenges and opportunities,” *Lancet*, vol. 375, no. 9709, pp. 160–5, Jan. 2010.

[20] D. Kyelem *et al.*, “Determinants of success in national programs to eliminate lymphatic filariasis: a perspective identifying essential elements and research needs,” *Am. J. Trop. Med. Hyg.*, vol. 79, no. 4, pp. 480–484, 2008.

[21] L. M. Zeldenryk, M. Gray, R. Speare, S. Gordon, and W. Melrose, “The emerging story of disability associated with lymphatic filariasis: a critical review.,” *PLoS Negl. Trop. Dis.*, vol. 5, no. 12, p. e1366, Dec. 2011.

[22] D. D. and L. Savioli, “Accelerating Work to Overcome the Global Impact of Neglected Tropical Diseases: A Roadmap for Implementation,” Geneva, Switzerland, 2012.

[23] WHO, “Global programme to eliminate lymphatic filariasis: progress report 2017,” *World Heal. Organ. Wkly. Epidemiol. Rec.*, vol. 93, no. 44, pp. 589–604, 2018.

[24] WHO, *Working to overcome the global impact of neglected tropical diseases: First WHO report on neglected tropical diseases*. Geneva, Switzerland: World Health Organization, 2010.

[25] M. J. Bockarie *et al.*, “Application of a polymerase chain reaction-ELISA to detect *Wuchereria bancrofti* in pools of wild-caught *Anopheles punctulatus* in a filariasis control area in Papua New Guinea.,” *Am. J. Trop. Med. Hyg.*, vol. 62, no. 3, pp. 363–7, Mar. 2000.

[26] C. King *et al.*, “A Trial of a Triple-Drug Treatment for Lymphatic Filariasis,” *N. Engl. J. Med.*, vol. 379, no. 19, pp. 1801–1810, 2018.

[27] T. G. Geary *et al.*, “Unresolved issues in anthelmintic pharmacology for helminthiases of humans.,” *Int. J. Parasitol.*, vol. 40, no. 1, pp. 1–13, Jan. 2010.

[28] J. Keiser and J. Utzinger, “The drugs we have and the drugs we need against major helminth infections,” *Adv. Parasitol.*, vol. 73, no. 10, pp. 197–230, Jan. 2010.

[29] E. Ghedin *et al.*, “Draft genome of the filarial nematode parasite *Brugia malayi*,” *Science (80-. ).*, vol. 317, no. 5845, pp. 1756–1760, 2007.

[30] C. K. Carlow, E. D. Franke, R. C. Lowrie, F. Partono, and M. Philipp, “Monoclonal antibody to a unique surface epitope of the human filaria *Brugia malayi* identifies infective larvae in mosquito vectors,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 84, no. 19, pp. 6914–8, Oct. 1987.

[31] E. Chanda *et al.*, “Integrated vector management: a critical strategy for combating vector-borne diseases in South Sudan,” *Malar. J.*, vol. 12, no. 1, p. 369, Jan. 2013.

[32] WHO, *Public health pesticide registration and management practices by WHO Member States*. Geneva, Switzerland: World Health Organization, 2010.

[33] WHO/GEPLF, “Lymphatic Filariasis,” World Health Organization, Geneva, Switzerland, Jan. 2013.

[34] P. E. Simonsen, “Filariasis,” in *Manson’s Tropical Diseases*, 22nd ed., G. C. Cook and A. Zumla, Eds. China: W. B. Saunders, 2008, pp. 1477–1513.

[35] T. V Rajan, N. Paciorkowski, I. Kalajzic, and C. McGuiness, “Ascorbic acid is a requirement for the morphogenesis of the human filarial parasite *Brugia malayi*,” *J. Parasitol.*, vol. 89, no. 4, pp. 868–70, Aug. 2003.

[36] T. R. Klei and T. V Rajan, *World Class Parasites: Volume 5 The Filaria*. Boston: Kluwer Academic Publishers, 2002.

[37] K. P. Day, "The endemic normal in lymphatic filariasis: A static concept," *Parasitol. Today*, vol. 7, no. 12, pp. 341–343, Jan. 1991.

[38] J. D. Turner, R. S. Langley, K. L. Johnston, G. Egerton, S. Wanji, and M. J. Taylor, "*Wolbachia* endosymbiotic bacteria of *Brugia malayi* mediate macrophage tolerance to TLR- and CD40-specific stimuli in a MyD88/TLR2-dependent manner," *J. Immunol.*, vol. 177, no. 2, pp. 1240–9, Jul. 2006.

[39] A. V Saint André *et al.*, "The role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness," *Science*, vol. 295, no. 5561, pp. 1892–5, Mar. 2002.

[40] Institute of Tropical Medicine Antwerp, "Lymphatic filariasis," 2015. [Online]. Available: http://itg.author-e.eu/Generated/pubx/173/filariasis/lymphatic_ filariasis.htm. [Accessed: 01-Jan-2016].

[41] J. J. C. Buckley and J. F. B. Edeson, "On the adult morphology of *Wuchereria sp.* (*malayi?*) From a monkey (*Macaca irus*) and from cats in malaya, and on *Wuchereria pahangi* n.sp. from a dog and a cat," *J. Helminthol.*, vol. 30, no. 1, pp. 1–20, 1956.

[42] J. F. B. Edeson and R. H. Wharton, "Transmission of *Wuchereria malayi* from man to the domestic cat," *Trans. R. Soc. Trop. Med. Hyg.*, vol. 51, no. 4, pp. 366–370, 1957.

[43] A. Laing, J. Edeson, and R. Wharton, “Studies on fiiariasis in Malaya: further experiments on the transmission of *Brugia malayi* and *Wuchereria bancrofti*,” *Ann. Trop. Med. Parasitol.*, vol. 55, no. 1, pp. 86–92, 1961.

[44] S. K. Joseph *et al.*, “Sensitization with anti-inflammatory BmAFI of *Brugia malayi* allows L3 development in the hostile peritoneal cavity of *Mastomys coucha*,” *Acta Trop.*, vol. 120, no. 3, pp. 191–205, Dec. 2011.

[45] S. Misra, M. Verma, S. K. Mishra, S. Srivastava, V. Lakshmi, and S. Misra-Bhattacharya, “Gedunin and photogedunin of *Xylocarpus granatum* possess antifilarial activity against human lymphatic filarial parasite *Brugia malayi* in experimental rodent host,” *Parasitol. Res.*, vol. 109, no. 5, pp. 1351–60, Nov. 2011.

[46] L. A. Spencer, P. Porte, C. Zetoff, and T. V Rajan, “Mice genetically deficient in immunoglobulin E are more permissive hosts than wild-type mice to a primary, but not secondary, infection with the filarial nematode *Brugia malayi*,” *Infect. Immun.*, vol. 71, no. 5, pp. 2462–2467, 2003.

[47] K. Tyagi, P. K. Murthy, and R. K. Chatterjee, “*Brugia malayi* in *Mastomys coucha*: establishment in immunosuppressed animals,” *Acta Trop.*, vol. 71, no. 2, pp. 189–94, Sep. 1998.

[48] U. Vanam, P. R. Prabhu, V. Pandey, G. Dakshinamurthy, M. V. R. Reddy, and K. Perumal, “Immune responses generated by intramuscular DNA immunization of *Brugia malayi* transglutaminase (BmTGA) in mice,” *Parasitology*, vol. 136, no. 8, pp. 887–94, Jul. 2009.

[49] R. B. Crandall, P. B. McGreevy, D. H. Connor, C. A. Crandall, J. T. Neilson, and J. W. McCall, “The ferret (*Mustela putorius furo*) as an experimental host for *Brugia malayi* and *Brugia pahangi*,” *Am. J. Trop. Med. Hyg.*, vol. 31, no. 4, pp. 752–9, Jul. 1982.

[50] S. A. Hines, R. B. Crandall, C. A. Crandall, and J. P. Thompson, “Lymphatic Filariasis: *Brugia malayi* infection in the ferret (*Mustela putorius furo*),” *Am. J. Pathol.*, vol. 134, no. 6, pp. 1373–1376, 1989.

[51] L. R. Ash and J. M. Riley, “Development of subperiodic *Brugia malayi* in the jird, *Meriones unguiculatus*, with notes on infections in other rodents,” *J. Parasitol.*, vol. 56, no. 5, pp. 969–973, 1970.

[52] A. Ewert, “Distribution of developing and mature *Brugia malayi* in cats at various times after a single inoculation,” *J. Parasitol.*, vol. 57, no. 5, pp. 1039–1042, 1971.

[53] S. S. Amhed, “Studies on the laboratory transmission of sub-periodic *Brugia malayi* and *B. pahangi*: The resistance of guinea-pigs, rabbits, and white mice to infection,” *Ann. Trop. Med. Parasitol.*, vol. 61, pp. 93–100, 1967.

[54] W. H. Cheong, S. Sivanandam, and A. H. B. Omar, “A note on an attempt at producing a rat strain of sub-periodic *Brugia malayi* from a natural simian host,” *Med. J. Malaysia*, vol. 21, p. 378, 1967.

[55] S. El-Bihari and A. Ewert, “Worm burdens and prepatent periods in jirds (*Meriones unguiculatus*) infected with *Brugia malayi*,” *Southeast Asian J. Trop. Med. Public Health*, vol. 4, no. 2, pp. 184–186, 1973.

[56] J. W. McCall, J. B. Malone, H. S. AH, and P. E. Thompson, “Mongolian jirds (*Meriones unguiculatus*) infected with *Brugia pahangi* by the intra-peritoneal

route: a rich source of developing larvae, adult filariae, and microfilariae," *J. Parasitol.*, vol. 59, no. 3, p. 436, 1973.

[57] J. R. Palmieri, Purnomo, D. T. Dennis, and H. A. Marwoto, "Filarid parasites of South Kalimantan (Borneo) Indonesia. *Wuchereria kalimantani sp.* n. (Nematoda: Filarioidea) from the silvered leaf monkey, *Presbytis cristatus* Eschscholtz 1921," *J. Parasitol.*, vol. 66, no. 4, pp. 645–651, 1980.

[58] R. B. Crandall, C. A. Crandall, and J. T. Neilson, "Antibody responses to experimental *Brugia malayi* infections in Patas and Rhesus monkeys," *Acta Trop.*, vol. 40, pp. 53–64, 1983.

[59] J. W. Mak, M. F. Choong, P. L. W. Lam, and K. Suresh, "Experimental infection of the leaf-monkeys , *Presbytis cristata* and *Presbytis melalophos* with subperiodic *Brugia malayi*," *Acta Trop.*, vol. 47, pp. 223–226, 1990.

[60] P. K. Murthy, K. Tyagi, R. P. Ghosh, P. S. Murthy, and R. K. Chatterjee, "Longitudinal humoral immune responses of Indian leaf monkey (*Presbytis entellus*) to *Brugia malayi* infection," *Parasitology*, vol. 119 ( Pt 1, pp. 53–60, 1999.

[61] C. P. Morris, H. Evans, S. E. Larsen, and E. Mitre, "A comprehensive, model-based review of vaccine and repeat infection trials for filariasis," *Clin. Microbiol. Rev.*, vol. 26, no. 3, pp. 381–421, Jul. 2013.

[62] P. R. Earl, "Filariae from the dog in vitro," in *Annals New York Academy of Sciences*, 1959, pp. 163–175.

[63] C. Wellman and F. M. Johns, "The artificial culture of filarial embryos: A preliminary note," *J. Am. Med. Assoc.*, vol. LIX, no. 17, pp. 1531–1532, 1912.

[64] F. W. Bach, “On the microfilaria cultures of Wellman and Johns with observations on the measuring of microfilariae,” *Zentralblatt fur Bakteriol. Parasitenkunde, Infekt. und Hyg.*, vol. 70, no. 1–2, pp. 50–60, 1913.

[65] G. C. Low, “The life of filarial embryos outside the body,” *J. Trop. Med. Hyg.*, vol. 15, no. 22, pp. 338–339, 1912.

[66] P. Jordan, “Action of diethylcarbamazine in vitro on infective larvae of *Wuchereria bancrofti*,” *Br. J. Pharmacol. Chemother.*, vol. 13, pp. 318–320, 1958.

[67] T. K. Sawyer and P. P. Weinstein, “Morphologic changes occurring in canine microfilariae maintained in whole blood cultures,” *Am. J. Vet. Res.*, vol. 24, no. 100, pp. 402–407, 1963.

[68] A. E. R. Taylor, “Maintenance of filarial worms in vitro,” *Exp. Parasitol.*, vol. 9, no. 2, pp. 113–120, 1960.

[69] M. Yoeli, R. S. Upmanis, and H. Most, “Studies on Filariasis III. Partial Growth of the mammalian stages of *Dirofilaria immitis* in vitro,” *Exp. Parasitol.*, vol. 15, no. 4, pp. 325–334, 1964.

[70] T. K. Sawyer, “Molting and exsheathment in vitro of third-stage *Dirofilaria immitis*,” *J. Parasitol.*, vol. 51, pp. 1016–1017, 1965.

[71] M. M. Wong, R. Knighton, J. Fidel, and M. Wada, “In vitro cultures of infective-stage larvae of *Dirofilaria immitis* and *Brugia pahangi*,” *Ann. Trop. Med. Parasitol.*, vol. 76, no. 2, pp. 239–241, 1982.

[72] M. Tanner and N. Weiss, “*Dipetalonema viteae* (Filarioidea): evidence for a serum-dependent cytotoxicity against developing third and fourth stage larvae in vitro: short communication,” *Acta Trop.*, vol. 38, pp. 325–328, 1981.

[73] S. N. Chen and R. E. Howells, “The in vitro cultivation of the infective larvae and the early mammalian stages of the filarial worm, *Brugia pahangi*,” *Ann. Trop. Med. Parasitol.*, no. 473–486, 1979.

[74] J. W. Mak, P. K. C. Lim, B. K. L. Sim, and L. M. Liew, “*Brugia malayi* and *Brugia pahangi* : Cultivation in vitro of infective larvae to the fourth and fifth Stages,” *Exp. Parasitol.*, vol. 55, pp. 243–248, 1983.

[75] W. A. Riberu, S. Atmosoedjono, Purnomo, S. Tirtokusumo, M. J. Bangs, and J. K. Baird, “Cultivation of sexually mature *Brugia malayi* in vitro,” *Am. J. Trop. Med. Hyg.*, vol. 43, no. 1, p. 3–5 (90–108), 1990.

[76] J. D. Smyth, “In vitro cultivation as a biological tool,” in *In Vitro Cultivation of Parasitic Helminths*, J. D. Smyth, Ed. Boca Raton, FL.: CRC Press, Inc., 1990, pp. 265–270.

[77] N. Wisnewski and P. P. Weinstein, “Growth and development of *Brugia pahangi* larvae under various in vitro conditions,” *J. Parasitol.*, vol. 79, no. 3, pp. 390–398, 1993.

[78] C. L. Smillie, A. C. Vickery, B. H. Kwa, J. K. Nayar, and U. R. Rao, “Evaluation of different medium supplements for in vitro cultivation of *Brugia malayi* third-stage larvae,” *J. Parasitol.*, vol. 80, no. 3, pp. 380–383, 1994.

[79] H. L. Smith, N. Paciorkowski, S. Babu, and T. V Rajan, “Development of a serum-free system for the in vitro cultivation of *Brugia malayi* infective-stage larvae,” *Exp. Parasitol.*, vol. 95, no. 4, pp. 253–64, Aug. 2000.

[80] F. H. Falcone, H. Zahner, M. Schlaak, and H. Haas, “In vitro cultivation of third-stage larvae of *Brugia malayi* to the young adult stage,” *Trop. Med. Parasitol.*, vol. 46, pp. 230–234, 1995.

[81] O. Arrigoni and M. C. De Tullio, “Ascorbic acid: much more than just an antioxidant,” *Biochim. Biophys. Acta*, vol. 1569, pp. 1–9, 2002.

[82] J. E. Allen and T. A. Wynn, “Evolution of Th2 immunity: a rapid repair response to tissue destructive pathogens,” *PLoS Pathog.*, vol. 7, no. 5, pp. 5–8, 2011.

[83] T. J. Little, D. M. Shuker, N. Colegrave, T. Day, and A. L. Graham, “The coevolution of virulence: Tolerance in perspective,” *PLoS Pathog.*, vol. 6, no. 9, pp. 1–5, 2010.

[84] R. Medzhitov, D. S. Schneider, and M. P. Soares, “Disease Tolerance as a Defense Strategy,” *Science (80-. ).*, vol. 335, no. 6071, pp. 936–941, 2012.

[85] B. Spellberg and J. E. Edwards, “Type 1/Type 2 immunity in infectious diseases,” *Clin. Infect. Dis.*, vol. 32, pp. 76–102, 2001.

[86] J. H. Schroeder *et al.*, “Live *Brugia malayi* microfilariae inhibit transendothelial migration of neutrophils and monocytes,” *PLoS Negl. Trop. Dis.*, vol. 6, no. 11, 2012.

[87] N. M. Girgis, U. M. Gundra, and P. Loke, “Immune regulation during helminth infections,” *PLoS Pathog.*, vol. 9, no. 4, pp. 11–13, 2013.

[88] J. P. Hewitson, J. R. Grainger, and R. M. Maizels, “Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity,” *Mol. Biochem. Parasitol.*, vol. 167, no. 1, pp. 1–11, 2009.

[89] L. Hussaarts, L. E. P. M. Van Der Vlugt, M. Yazdanbakhsh, and H. H. Smits, "Regulatory B-cell induction by helminths: Implications for allergic disease," *J. Allergy Clin. Immunol.*, vol. 128, no. 4, pp. 733–739, 2011.

[90] M. D. Taylor, N. van der Werf, and R. M. Maizels, "T cells in helminth infection: The regulators and the regulated," *Trends Immunol.*, vol. 33, no. 4, pp. 181–189, 2012.

[91] S. Gordon and F. O. Martinez, "Alternative activation of macrophages: Mechanism and functions," *Immunity*, vol. 32, no. 5, pp. 593–604, 2010.

[92] J. P. Hewitson *et al.*, "The secretome of the filarial parasite, *Brugia malayi* : proteomic profile of adult excretory-secretory products," *Mol. Biochem. Parasitol.*, vol. 160, no. 1, pp. 8–21, Jul. 2008.

[93] Y. Moreno and T. G. Geary, "Stage- and gender-specific proteomic analysis of *Brugia malayi* excretory-secretory products," *PLoS Negl. Trop. Dis.*, vol. 2, no. 10, p. e326, Jan. 2008.

[94] S. Bennuru, R. Semnani, Z. Meng, J. M. C. Ribeiro, T. D. Veenstra, and T. B. Nutman, "*Brugia malayi* excreted/secreted proteins at the host/parasite interface: stage- and gender-specific proteomic profiling," *PLoS Negl. Trop. Dis.*, vol. 3, no. 4, p. e410, Jan. 2009.

[95] R. C. Lee, R. L. Feinbaum, and V. Ambros, "The *Caenorhabditis elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14," *Cell*, vol. 75, no. 5, pp. 843–54, 1993.

[96] B. J. Reinhart *et al.*, "The 21-nucleotide let-7 rna regulates developmental timing in *Caenorhabditis elegans*," *Nature*, vol. 403, no. 6772, pp. 901–6, Feb. 2000.

[97] M. I. Almeida, R. M. Reis, and G. A. Calin, “MicroRNA history: Discovery, recent applications and next frontiers,” *Mutat. Res. Mol. Mech. Mutagen.*, vol. 717, no. 1–2, pp. 1–8, 2011.

[98] C. Britton, A. D. Winter, V. Gillan, and E. Devaney, “MicroRNAs of parasitic helminths-identification, characterization and potential as drug targets,” *Int. J. Parasitol. Drugs Drug Resist.*, vol. 4, no. 2, pp. 85–94, 2014.

[99] A. D. Winter *et al.*, “Diversity in parasitic nematode genomes: the microRNAs of *Brugia pahangi* and *Haemonchus contortus* are largely novel,” *BMC Genomics*, vol. 13, no. 1, p. 4, Jan. 2012.

[100] C. Poole, P. Davis, J. Jin, and L. McReynolds, “Cloning and bioinformatic identification of small RNAs in the filarial nematode, *Brugia malayi*,” *Mol. Biochem. Parasitol.*, vol. 169, no. 2, pp. 87–94, 2010.

[101] C. Poole *et al.*, “Diversity and expression of microRNAs in the filarial parasite, *Brugia malayi*,” *PLoS One*, vol. 9, no. 5, p. 1–15 (e96498), 2014.

[102] Y. Fu *et al.*, “Identification of *Dirofilaria immitis* miRNA using illumina deep sequencing,” *Vet. Res.*, vol. 44, no. 1, pp. 1–11, 2013.

[103] C. P. Bracken *et al.*, “Global analysis of the mammalian RNA degradome reveals widespread miRNA-dependent and miRNA-independent endonucleolytic cleavage,” *Nucleic Acids Res.*, vol. 39, no. 13, pp. 5658–5668, 2011.

[104] G. Meister, M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, and T. Tuschl, “Human argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs,” *Mol. Cell*, vol. 15, no. 2, pp. 185–197, 2004.

[105] K. Okamura, A. Ishizuka, H. Siomi, and M. C. Siomi, “Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways,” *Genes Dev.*, vol. 18, no. 14, pp. 1655–1666, 2004.

[106] D. P. Bartel, R. Lee, and R. Feinbaum, “MicroRNAs: Genomics, biogenesis, mechanism, and function,” *Cell*, vol. 116, pp. 281–297, 2004.

[107] R. C. Friedman, K. K. Farh, C. B. Burge, and D. P. Bartel, “Most mammalian mRNAs are conserved targets of microRNAs,” *Genome Res.*, vol. 19, pp. 92–105, 2009.

[108] J. R. Lytle, T. A. Yario, and J. A. Steitz, “Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5’ UTR as in the 3’ UTR,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 23, pp. 9667–9672, 2007.

[109] E. Devaney, A. D. Winter, and C. Britton, “microRNAs: a role in drug resistance in parasitic nematodes?,” *Trends Parasitol.*, vol. 26, no. 9, pp. 428–33, Sep. 2010.

[110] A. H. Buck *et al.*, “Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity,” *Nat. Commun.*, vol. 5, p. 5488, 2014.

# CHAPTER 2

# CLINICAL FINDINGS IN CATS AND DOGS EXPERIMENTALLY INFECTED WITH *BRUGIA MALAYI*

Erica J. Burkman, Christopher C. Evans, Michael T. Dzimianski, Bridget Garner, and Andrew R. Moorhead*

Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, Georgia, United States of America

*Corresponding author: Andrew R. Moorhead, Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, 501 D.W. Brooks Drive, Athens, Georgia, 30602, USA, email: amoorhed@uga.edu.

Burkman, Erica. To be submitted to PLOS Neglected Tropical Diseases

**ABSTRACT**

Lymphatic filariasis (LF) is a mosquito-borne disease caused by the parasitic nematodes *Wuchereria bancrofti* and *Brugia malayi*. These parasites are a major cause of morbidity globally, with an estimated 120 million people infected. Because *W. bancrofti* requires primate hosts, *B. malayi* is the preferred laboratory model and, for over 60 years, the domestic cat has been utilized as its primary non-rodent experimental host. However, a full clinical picture of the feline animal model has never been documented. In this study, the host response was examined in 10 *B. malayi*-infected cats from pre-infection to patency. In comparison, dogs, which have not been used as an experimental model, were infected with *B. malayi* and the host response was monitored from pre-infection to patency. Literature from endemic countries allude to the potential for canine reservoirs, so 11 dogs were infected with *B. malayi* to determine whether they could be definitive hosts and to examine host response. The aim of this study was to find an early clinical indicator of infection that might be present in some cats but not others, in addition to intra-host variability. Complete blood counts, lymphedema, and microfilaremia were monitored throughout the study period. Both dogs and cats became infected, though canine microfilaremia were transient. Notable differences between the two species were marked eosinophilia in the cats that was not observed in the dogs, elevated lymphocytes in dogs but not in cats, and elevated RBCs observed only in microfilaria-positive cats. These findings show a distinct difference between the infection of these two species and a difference within the blood profile in response to the establishment and development of these parasites. Confirmation that canines have the potential to become a definitive host of

*B. malayi* has implications for transmission in endemic regions potentially impacting elimination efforts and mass drug administration programs.

## INTRODUCTION

Lymphatic filariasis (LF) is a mosquito-borne disease that can result in chronic and disfiguring conditions such as lymphedema, hydrocele, and elephantiasis [1]. Human LF is primarily caused by the parasitic nematode *Wuchereria bancrofti,* with *Brugia malayi* and *B. timori* reporting 10% and <1% of cases, respectively [2]. These parasites are a major cause of morbidity throughout the tropics, with an estimated 856 million people at risk in 52 countries and 40 million disfigured and/or incapacitated by this disease [1-3]. These filarial nematodes are obligate parasites that can persist in the infected host for 5-12 years, causing permant damage to the lymphatic system and leading to chronic disease and disfigurement [4-5].

Lymphatic filarial parasites provide a unique challenge in treatment and elimination due to the complexities of their life cycles and their requirement for a mosquito intermediate host. The life cycle of these filarioids are described as a series of morphologically distinct forms in both the mosquito vector and vertebrate host comprised of five major stages separated by four molts. In each endemic area, there are local vector species belonging to the genera *Culex, Aedes*, *Anopheles,* and *Mansonia* [6-7].

The life cycle begins with the mosquito vector biting an infected host and ingesting microfilariae (mf) found in the blood. The mf enter the mid-gut with the blood meal, where they exsheath. The mf then penetrate the wall of the mid-gut and migrate to the thoracic muscles where they develop through two molts from first-stage larvae ($L_1$) to infective

third-stage larvae ($L_3$) with an approximate size of 1500 x 20 µm [8]. Once the infected mosquito bites another host, the $L_3$ enter the bite wound and migrate to lymphatic vessels. Here they molt twice to the fourth larval ($L_4$) and juvenile adult (jA) stages until developing into sexually mature adults. The adult females are ovoviviparous, producing thousands of mf after copulation with the adult males [9]. Circulating mf in the peripheral blood of the host can be observed an average of 8 months after infection with *W. bancrofti* [10] and 3-4 months for *B. malayi* [11]. These mf circulate within the host's bloodstream for up to a year, where they are ingested by another mosquito, completing the life cycle. Due to the longevity and fecundity of these worms, halting transmission remains a formidable challenge.

Lymphatic filariasis pathology consists of both chronic and acute manifestations of disease. The primary instigator of morbidity is the adult worms and their location within the lymphatics of the infected host. Movement of the worms within the lymphatic system and the resulting immunological response has the potential to elicit the chronic form of LF resulting in lymphedema, elephantiasis, and hydrocele [8, 12]. In endemic regions, the chronic form of LF occurs in approximately 10% of the infected population [12]. The remainder are symptomatic and, in these individuals, paradoxically high microfilaremia is often observed. Over time, exposure to filarial antigens shifts the immune system from a T helper cell (Th)1 towards an antibody-mediated Th2 response, and this polarization enables chronic persistence of these parasites [8]. This group serves as the primary source for LF transmission in endemic regions. There are also individuals that, despite repeated exposure, never become infected. One proposed hypothesis suggests that this immunity arises from circulating antibodies against the infective $L_3$ [13]. Additionally, there are those that can

present with little or no circulating microfilariae. These individuals have a tendency towards a Th1-type hyper-responsiveness, eliciting a significant immunological response to the parasites [8]. Unfortunately, this response has the potential to lead to significant damage to their lymphatic system. This damage has long-term consequences, with a high probability of developing lymphangiectasia, elephantiasis, and hydrocele.

While *W. bancrofti* is restricted to primate hosts, *B. malayi* has been established in both the domestic cat (*Felis catus*) and the Mongolian gerbil (*Meriones unguiculatus*) for over 40 years [10–16]. Due to the availability of both a small animal and rodent model, the *B. malayi* parasite is able to function as a suitable research model for understanding LF pathogenesis. However, a full clinical picture of the feline animal model has never been documented. The sub-periodic strain of *B. malayi* used in this study was obtained from a cat in 1969 from the laboratory of Dr. Ramachandran in Kuala Lumpur, Malaysia [11]. It was brought to the University of Georgia by Thompson and McCall where it has been cycled through cats and gerbils since [20]. It is a well-established laboratory strain with consistent infection rates in both of these models. These infections in cats can maintain a microfilaremia for as little as a few weeks, up to 6 years. We hoped to elucidate an early clinical indicator of infection that would indicate a difference in cats that become symptomatic versus cats that remain amicrofilaremic. In this study we tracked the progression of *B. malayi* infection over 1-2 years, monitoring microfilaremia levels, complete blood count (CBC) profile, and the presence or absence of lymphedema.

In addition to infecting domestic cats, dogs were infected to determine their permissivity to *B. malayi*. There are few references that note canine infection with *B. malayi* in endemic countries, but these parasites may have been mistaken for other species

(e.g. *B. pahangi*) that infect canines [17, 18]. In infecting these dogs, our aims were to first determine if patency could be established, and second, to assess host response by monitoring CBCs, microfilaremia, and lymphedema, as performed with the feline group. By measuring the host responses to *B. malayi* in two models, we hoped to better define factors affecting the ultimate pathology of infection and whether there are differences inherent to each species.

## METHODS

### Animal ethics approval

The study protocol was approved by the University of Georgia's Institutional Animal Care and Use Committee under the protocol numbers: A2010 12-005-Y3-A16 and A2013 11-009-Y3-A18. The animal care and use protocols adhered to the "Guide for the Care and Use of Laboratory Animals (the Guide)" published by the National Research Council of the National Academies, USA.

### Parasite collection

*Brugia malayi* infective $L_3$ were obtained from the Filarial Research Reagent Resource Center (FR3) in Athens, GA. $L_3$ were isolated from *Aedes aegypti* mosquitoes (Black-eyed Liverpool strain) in Hanks' balanced salt solution (HBSS) (Millipore Sigma, St. Louis, MO, USA) 15 days post-infection (dpi) as previously described [23].

**Blood sample collection and slide preparation**

Average microfilarial counts were determined for each animal by preparing standard thick blood smears in duplicate. The blood was spread on a slide with a surface area approximately 3 cm x 1.5 cm and dried at room temperature for a minimum of 24 hours prior to staining with a 10% Giemsa buffered solution (1:9 solution of Giemsa stain (VWR® Scientific, Radnor, PA) and TAE 1X buffer (2.0M Tris-Acetate + 100 mM $Na_2$ EDTA, National Diagnostics, Atlanta, GA). The average number of mf per animal at each time point was calculated.

**Complete blood count (CBC) analysis**

CBC profiles in this study were performed on samples collected prior to infection and approximately every 14 dpi in dogs, and every 30 days in cats, to assess hematological trends. A quantity of 10-12 mls of blood were collected in EDTA (K2 EDTA, BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ). Blood tubes were centrifuged at 4°C for 10 minutes at 1,000 x *g*. The CBC panel was performed on a Bayer-Advia 120 hematology analyzer (Siemens Medical Solutions USA, Inc., Malvern, PA.) at the University of Georgia Clinical Pathology Lab. The reference intervals used by these CBC panels were obtained from healthy, uninfected cats or dogs established by the Clinical Pathology Lab and are updated semi-annually. The total white blood cell count is expressed as an absolute number and is further divided into subtypes by a differential WBC count, which is expressed as a percentage and absolute number.

**Feline experimental design**

Ten domestic shorthair cats were infected with 400 *B. malayi* infective $L_3$ via subcutaneous injection (200 $L_3$ per side) in the hind limb proximity to the inguinal lymph node. Prior to infection, 10-12 ml of blood were collected in EDTA from all ten cats on day 1, 3, 6, 10, 14, 30, and subsequently every 30 days up to 265 dpi.

**Measuring lymphedema**

Both cats and dogs were checked weekly for lymphedema and any other changes to their health status. Lymphedema was defined by a swelling of the extremities, observed in either the front or back feet and marked as bilateral marked (BMA), bilateral moderate (BMO), bilateral mild (BMI), bilateral mixed (BMX), right/left rear marked (RMA/LMA), right/left rear moderate (RMO/LMO), right/left rear mild (RMI/LMI), and resolved (R).

**Canine experimental design**

A mixture of purpose-bred beagles and mongrels were used in this study. Blood was obtained from a *B. malayi*-infected feline and fed to *A. aegypti* (Black-eyed Liverpool strain) mosquitoes. Four dogs (CDP9, YZP9, 574, and 573) were infected with 1,000 *B. malayi* $L_3$ subcutaneously in the hind limbs near the inguinal node (500 $L_3$ per side). Microfilariae from one of these dogs were fed to mosquitoes to produce infective $L_3$, which in turn was used to infect a gerbil via intraperitoneal injection. These parasites developed to maturity within the gerbil, where mf were collected, fed to mosquitoes, and used to infect one additional dog (CBP) by subcutaneous injection of 1,000 $L_3$, and two additional gerbils (intraperitoneal injection of 400 $L_3$ each). Parasites from the second gerbil infection were

again passaged through the mosquitoes and used to infect an additional six dogs (CBZ, CCJ, CBN, 282, 283, 276) by subcutaneous injection of 500 $L_3$ each (250 $L_3$ per side).

Gerbils used for parasite maintenance were euthanized a minimum of 120 post-infection. Adult parasites and mf were removed and washed with Roswell Park Memorial Institute-1640 medium with L-Glutamine (RPMI-1640, Lonza, Allendale, NJ), gentamicin (0.05 mg/ml, Millipore Sigma, St. Louis, MO, USA), and penicillin-streptomycin solution (0.1 mg/ml, Gibco, Langley, OK, USA). The mf were concentrated to a 100-200 µl volume and then mixed with 4 mls of uninfected dog blood and fed to mosquitoes.

**Statistics**

A linear mixed effects model performed using SAS V 9.4 (SAS, Cary, NC) was used to test for relationships between CBC measurements and microfilaremia status by group at each time point. Additionally, changes in CBC measurements from the pre-infection baseline were analyzed. The full model included fixed factors for microfilaremia status, day, and a microfilaremia-status-by-day interaction term and a random intercept for each cat or dog. All hypothesis tests were 2-sided with significance level $\alpha = 0.05$. Multiple comparisons were adjusted using Tukey's test.

Statistical analysis to test for differences between mf− and mf+ cats and dogs was performed using a two-way ANOVA with Tukey's test to correct for multiple comparisons using GraphPad Prism version 6.02 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). These two-way ANOVAs had time as a fixed factor and were either grouped by infection status or individual averages over time for each animal. In order to

determine whether lymphedema was affected by microfilaremia status, a Cohen's kappa statistic was calculated.

## RESULTS

### Feline infection with *B. malayi*

Blood samples were taken for CBC analysis from all 10 cats pre-infection to serve as a baseline and negative control for later statistical analyses. Five out of ten cats infected with *B. malayi* $L_3$ developed a patent microfilaremia, first observed at day 128. This is similar to findings from 8 years of infections in male domestic shorthair cats, where approximately 50% become microfilaremic, with 25% resulting in a microfilarial count greater than 1,000 mf/ml (Table 2.1).

Table 2.1: Data from eight years of feline infections with *B. malayi* at the Filariasis Research Reagent Resource Center in Athens, GA.

| Year | mf + | mf - | Total |
|---|---|---|---|
| 1 | 7 | 3 | 10 |
| 2 | 6 | 4 | 10 |
| 3 | 1 | 14 | 15 |
| 4 | 8 | 2 | 10 |
| 5 | 12 | 8 | 20 |
| 6 | 6 | 4 | 10 |
| 7 | 3 | 7 | 10 |
| 8 | 4 | 5 | 9 |
| Totals: | 47 | 47 | 94 |

Several cats, C2 and C3, had detectable mf at days 156 and 240 post-infection, respectively. At this first time point, three cats (C8, C9, and C10) were found to have an mf count above 1,000 mf/ml (Figure 2.1), whereas C6 and C7 reached this threshold

approximately one year post-infection. Though C7 microfilaremia were transient, C6 continued to maintain a microfilaremia more than two years later (data not shown). Table 2.2 outlines when mf were first detected, the duration of detectable mf, the highest concentration, and date of peak microfilarial concentrations. The mean duration of detectable circulating mf was 417 days in this study. The mean time to peak mf was approximately 289 dpi, with both C9 and C10 having a peak mf of approximately 6,000 mf/ml.

Table 2.2: Microfilaremia of 10 *B. malayi* infected cats.

| Cat ID | Mf first detected (dpi*) | Duration of detectable mf (days) | Highest mf conc. detected (mf/ml) | Time to peak mf measured (dpi*) |
|---|---|---|---|---|
| C1 | 0 | 0 | 0 | 0 |
| C2 | 240 | 1 | 75 | 240 |
| C3 | 156 | 28 | 25 | 156 |
| C4 | 0 | 0 | 0 | 0 |
| C5 | 0 | 0 | 0 | 0 |
| C6 | 128 | 491 | 3,750 | 587 |
| C7 | 128 | 442 | 1,450 | 433 |
| C8 | 128 | 112 | 1,200 | 156 |
| C9 | 128 | 442 | 6,525 | 211 |
| C10 | 128 | 305 | 6,425 | 240 |

* days post-infection

**Feline pathology**

In our cats, lymphedema was first noted 51 dpi, in C4 (Figure 2.2 image, Table S2.1). Lymphedema was noted in 4 out of the 5 mf− cats (C2, C3, C4, and C5) and 3 out of the 5 mf+ cats (C6, C7, and C9; Figure 2.2). All lymphedema-positive cats presented with either mild to marked swelling in either one or multiple feet/legs. No elephantiasis and/or hydrocele was observed. Figure 2.2 indicates whether these cats presented with

lymphedema when either mf+ or mf−. A Cohen's kappa calculation was performed for these two groups, demonstrating that the observed numbers agreed less than expected by chance (kappa = -0.20).

**Feline complete blood counts**

In all cats, eosinophilia was present throughout infection regardless of microfilaremia status (Figure 2.3 a&b). Eosinophil reference interval for normal uninfected cats was 0-0.8 x $10^3$ µl. Three of these cats (C5, C9, and C10) demonstrated eosinophilia prior to infection. By week 6 post-infection, peak eosinophil count was observed in 6 out of 10 cats (Figure 2.3b). A secondary peak was noted at week 12 for 6 out of 10 cats. At week 8, there is a significant difference between the cats that became microfilaremic by 128 dpi (mf++) and those with detectable microfilaremia after 240 days (mf+). Statistically, there was no difference between these two groups and the cats that were amicrofilaremic (mf-) ($p \geq 0.05$). However, on day 45 a significant difference was observed between the mf- cats and their pre-infection baseline ($p = 0.0073$). The mf+ and mf++ cats exhibited no difference between their baseline and measurements at any time point. When compared to their baseline measurements, a significant difference was observed on day 45 with the mf- cats but not any of the mf+/mf++ cats.

Basophils, segmented neutrophils (segs), immature neutrophils (bands; data not shown), monocytes, and lymphocytes (Figure S2.1) measurements were all within the established feline reference interval for uninfected cats (baso 0.02 x $10^3$ µl, segs 2.5-12.5 x $10^3$ µl, bands 0-0.3 x $10^3$ µl, monos 0-0.9 x $10^3$ µl, lymphs 1.5-7 x $10^3$ µl). Differences in mean basophil, neutrophil, and lymphocyte measurements per animal were not observed

to be statistically significant at any time point when compared to the pre-infection baseline. There was a significant change from the baseline in monocyte count in the amicrofilaremic group at 100 dpi ($p = 0.0078$). Clinically, all three groups of cats remained well within the established monocyte reference interval for uninfected cats. There were no statistical differences in the monocyte measurements between patent and amicrofilaremic cats.

The red blood cell (RBC) measurements and RBC indices are examined in figure 2.4 a-f. The RBC figures (a&b) indicate an increase above the feline reference interval (RBC 4.9-9.8 x $10^6$ µl) in the mf+ group beginning at week 8 and persisting until week 30. The only significant difference between the mf+/mf++ and mf- groups was observed at week 20 ($p < 0.05$). Cat RBC morphology in all groups post-infection indicated few to occasional Howell-Jolly bodies, slight rouleaux, and slight anisocytosis. There were no statistically significant differences observed for mean corpuscular volume (MCV 41-57 fl) (Figure 2.4 c&d) and mean corpuscular hemoglobin (MCH 13.8-18.1 pg) (Figure 2.4 e&f) measurements compared to baseline measurements, nor between the average measurements for each group of cats ($p \geq 0.05$). However, the MCV values for the mf- cats were found to be lower than the healthy reference interval beginning week 8 and continuing for the duration of the study. All cats demonstrated MCH values below the established reference interval throughout the study, including the pre-infection baseline.

Mean corpuscular hemoglobin concentration (MCHC) remained within the reference interval for uninfected cats throughout the study (30.6-36.7 g/dl) (data not shown). However, there was a significant difference between baseline values for patent cats at 184 dpi ($p = 0.0025$), and for both amicrofilaremic ($p = 0.0104$) and patent cats at 240 dpi ($p = 0.0376$).

All other measurements on the CBC panel were not statistically significant and were within reference intervals. The white blood cells (WBC 5.8-20.5 x $10^3$ µl), hemoglobin (Hgb 10.3-16.1 g/dl), and hematocrit (Hct 30.7-46.1 %), all remained within the reference intervals established for uninfected cats.

**Canine infection with *B. malayi***

In this portion of the study, 11 dogs were infected with *B. malayi* $L_3$. Table 2.3 outlines when mf were first detected, mf duration, highest mf concentration, and time to peak mf concentration. The dogs exhibited low and transient microfilaremia. Microfilariae were first observed at 196 dpi. The mean duration of detectable microfilaremia for the 5 microfilaremic dogs was approximately 275 days. The highest microfilarial concentration observed was in dog 276 at 295 dpi. The mean time to peak mf concentration was 251 days. Figure 2.5 shows the microfilaremia of the experimentally infected dogs over the course of this study. Dogs 573 and CCJ were removed from the study at 6 and 11 months post-infection, respectively, due to unrelated illnesses. Of the nine remaining dogs, five developed detectable microfilaremia. Dog 276 was observed to have the highest peak mf count at 1,550 mf/ml. Both 276 and 282 had the longest duration of detectable microfilaremia, at 69 days. All patent infections were transient.

Table 2.3: Microfilaremia of the 11 *B. malayi* infected dogs.

| Dog ID no. | Mf first detected (dpi*) | Duration of detectable mf (days) | Highest mf conc. detected (mf/ml) | Time to peak mf measured (dpi*) |
|---|---|---|---|---|
| CDP9 | 210 | 24 | 50 | no peak |
| 574 | 210 | 24 | 350 | 210 |
| 573 | N/A | N/A | N/A | N/A |
| YZP9 | 196 | 29 | 375 | 204 |
| CBP | 0 | 0 | 0 | 0 |
| CBZ | 0 | 0 | 0 | 0 |
| CCJ | N/A | N/A | N/A | N/A |
| CBN | 0 | 0 | 0 | 0 |
| 283 | 0 | 0 | 0 | 0 |
| 282 | 274 | 69 | 575 | 295 |
| 276 | 274 | 69 | 1550 | 295 |

* days post-infection
N/A indicates dogs removed from study

**Canine pathology**

To our knowledge, no published literature currently exists on a canine model for *B. malayi*, and thus, development and pathology in this host were unknown. Where 70% of the cats in this study exhibited lymphedema, no canines exhibited any detectable pathology throughout the experiment. Figure 2.6 displays the forelimbs of one of the canines where no lymphedema was observed. The accompanying table emphasizes that, regardless of infection status, no lymphedema occurred.

**Canine complete blood counts**

Unlike the cats, no eosinophilia was observed in any dog over the course of this study ($p \geq 0.05$) (Figure 2.7 a&b). The normal uninfected reference interval for eosinophils was 0-1.3 x $10^3$ µl. Figure 7a shows the mean eosinophil count over time for each dog.

When grouped, there was no significant difference observed between patent and amicrofilaremic animals ($p \geq 0.05$) (Figure 2.7b). Similarly, no difference in eosinophil count was observed for any dog when compared to pre-infection levels.

The mean values of the RBCs remained within the established reference interval (RBC 4.98-7.92 x $10^6$ µl) for both the mf+ and mf- groups, with the exception of the mf+ group at 24 weeks (Figure 2.8b). Figure 2.8a show the average RBC values for each individual animal. RBC morphology of the dogs indicated slight poikilocytosis, polychromasia, anisocytosis, rouleaux, and few Howell-Jolly bodies 14-30 days post-infection.

We observed a significant difference in the MPV and RBC indices MCV and MCH. The MCV for two mf- dogs, CBZ and CBN, and one mf+ dog, 276, were below the established reference interval (MCV 69-80 fl) (Figure 2.8c). When averaged by group, the mf- dogs remained well below the established reference interval, whereas the mf+ dogs fell below this interval briefly at week 10, and then weeks 20-24 (Figure 2.8d). The differences between the individual measurements and their baseline values were seen at day 295. The MPV values for dogs were found to consistently remain above reference interval (MPV 6.1-10.1 fl) (Figure 2.8 e&f). The MCV and MPV values for both mf- and mf+ groups fell within the reference interval by week 24. MCH and MCHC (data not shown) remained within established reference intervals for uninfected dogs throughout the study (MCH 22-27.4 pg, MCHC 32.3-36.6 g/dL). No statistically differences in MCH values were observed between individual animals (Figure S2.2a), mf- and mf+ groups over time (Figure S2.2b), nor when compared to baseline measurements ($p \geq 0.05$). With canine MCHC measurements, there was a significant difference between the mf- and mf+ group at 253

dpi ($p < 0.0001$). When compared to baseline readings, a difference was seen in the mf+ group at 295 ($p = 0.0292$), and 329 dpi ($p = 0.0132$). The mf- dogs demonstrated a difference from baseline MCHC measurements only at 253 dpi ($p < 0.0001$).

Canine lymphocyte counts indicated no significant differences between the individual animals (Figure S2.2c) nor compared against their baseline measurements. The uninfected dog reference interval for lymphocytes were 0.4-2.9 x $10^3$ µl. The mf- group rose above the reference interval at weeks 2, 6, and 13-21. The mf+ group was elevated above the reference interval at week 14 (Figure S2.2d). There were no statistically significant differences between the mf- and mf+ groups over time.

All other measurements on the CBC panel were neither statistically nor clinically significant. The WBC, Hgb, Hct, Segs, and Bands all remained within the reference intervals established for uninfected dogs (WBC 5.5-13.9 x $10^3$ µl, Hgb 12.6-19.9 g/dL, Hct 36.6-59.6 %, Segs 2.9-12.0 x $10^3$ µl, Bands 0-0.45 x $10^3$ µl).

**DISCUSSION**

Since the establishment of the feline model for *B. malayi* in the 1960s [12, 14, 22, 23], there are no published reports on the disease progression in this host. Additionally, there are no published reports on experimental *B. malayi* infection in dogs. Ambily et al. describe finding dogs with natural *B. malayi* infections, so there is some evidence that this does occur naturally [21]. In this study, we used the same sub-periodic *B. malayi* strain propagated in the cat. We were able to achieve successful infections of both species, allowing us to compare the clinical and cellular responses between these two non-rodent non-primate mammalian hosts.

The differences in lymphedema between cats and dogs were notable. In the cats, the presence or absence of lymphedema was not found to be predictive of patency. Similar results were obtained in dogs. No dog exhibited lymphedema throughout this study. This is unexpected when compared to cats, which did become symptomatic. This lack of clinical signs may be due to the transient nature of the infection, characteristics of the canine immune system, or lymphedema may present later in the infection, beyond the scope of this study.

Though dogs do not appear to be an ideal experimental model for *B. malayi*, the information from this experiment is a value to LF researchers. It remains uncertain whether dogs become infected through repeated exposure to *B. malayi* in endemic areas. In this study, dogs were administered artificially high numbers of directly injected *B. malayi* $L_3$, which does not represent a natural infection. However, mf produced from the dog infections and cycled through mosquitoes were able to infect new dogs demonstrating that dogs are a definitive host for these parasites. It may be these parasites could adapt to such a host, maintaining a low, underlying infection, one that could easily be overlooked by blood smears when tested in endemic areas. Another possibility is that the canine immune system can easily recognize this parasite can quickly kill any invading $L_3$. Canine sentinel studies in *B. malayi*-endemic regions could provide answers to these questions.

One of the more elucidating measures of the CBC results was the eosinophilia observations. The cats maintained persistent eosinophilia throughout the experiment, whereas the dogs remained within the established reference interval. Three of the cats, both mf- and mf++, exhibited elevated eosinophil counts prior to infection. Cats are particularly prone to allergens and stress, which could have played a role in this initial response [26].

Though, 4-6 weeks post-infection there was a notable increase in measured eosinophils far above background elevation due to either stress or allergens. In a study on the developmental rate of these parasites, Edeson & Buckley described *B. malayi* infection in 54 cats from inoculation until the fourth molt. Here, they found that the third molt ($L_3$ to $L_4$) occurred 9-10 dpi, the fourth molt ($L_4$ to adults) 35-40 dpi, and circulating mf an average of 75 dpi [27]. We can speculate that this initial peak in eosinophils occurred during the fourth molt of these parasites. The second peak observed in all three groups were at 84 dpi, the earliest that mf can be detected within peripheral blood. Other helminths are known to promote eosinophilia coinciding with the events in larval development, affirming a well-studied phenomena [28–31]. In dogs, there was no eosinophilia observed throughout the study. Other helminths, such as *Dirofilaria immitis* the canine heartworm and the ascarid *Toxocara canis*, elicit an eosinophilic response in dogs, and yet these *B. malayi* does not [32-33]. A number of published studies examine the protective effect of eosinophils, suggesting they might be essential for the survival of the parasite, at least in the case of primary infection [34–37]. In a secondary infection of parasitic nematodes, such as *Trichinella spiralis* and *Nippostrongylus brasiliensis*, eosinophils act to instead clear the parasites and protect the host [31, 38]. In contrast, other helminths, such as *Schistosoma mansoni* and *Trichuris muris*, are able to survive and development within the host even when eosinophils are depleted suggesting eosinophils are not critical to overall helminth survival [28, 39]. *Brugia malayi* survives in the canine host, as evidenced by the development of circulating mf, so we postulate that, while eosinophils may provide a protective effect and promote longevity and survival within the host-niche, they may not be essential for these parasites to survive and develop to sexual maturity. Eosinophils may

still yet play a role in *B. malayi* host response in canines. There is the potential of a localized response within tissues, not measurable systemically in the blood. Further studies would be necessary to elucidate whether this immune response occurs in these hosts.

Changes in lymphocyte counts also differed between cats and dogs. The mean lymphocyte count in the cats remained within the established reference interval, while the mf- dogs exhibited moderate to marked elevation, notably in weeks 8, 12, and 22-28. There have been numerous studies with parasitic helminths which examines the role of both T and B cells in mediation of parasite establishment [38-42]. As the methods for measuring lymphocytes in this study were not able to distinguish between either a T or B cell subsets, we can only infer that this elevation in the dogs could reflect in the lower infectivity rates along with the transient microfilaremia noted in this study. Inferring from the developmental timeline in cats, this parasite may not be as proficient at evading the host immune system in the less-susceptible canine host.

At the time of this study, MPV values were not measured in cat samples. In automated laboratory results, cat RBCs and platelets tend to overlap in size. Therefore, when RBCs are excluded from the platelet count, these hidden platelets are discounted from the measured value [45]. Aggregation (or clumping) of platelets is also common in cats, resulting in inaccurate automated measurements [39-40]. For each dog, we observed a MPV above the established reference interval throughout the infection. This could suggest that immature platelets are being released from the bone marrow too early in response to this parasitic infection [46–49].

Red blood cells and RBC indices (MCV, MCH, MCHC) exhibited a marked difference between dog and cat infections. The cat RBC counts were elevated in both the

mf+ and mf++ groups, where the dog values remained within their reference interval. Interestingly, only the mf+ group demonstrated elevated levels throughout most of the experiment, compared to only weeks 20-22 in the mf++ group. Both Hct and Hgb measurements were normal in all animals. MCV values, a key parameter for anemia classification, were decreased for mf+ cats from weeks 8-34, and for both mf+ and mf- dogs throughout the experiment. Both MCH and MCHC are calculated values, though only MCH is discarded in favor of measured results for a differential diagnosis. Coalescing these results indicates that the mf+/mf++ cats presented with a form of immune-mediated anemia that was not seen in the amicrofilaremic cats nor any of the dogs [52]. Due to the nature of this study, further testing to determine a basis for anemia in iron-deficiency, thalassemia, or chronic disease was not conducted. *Brugia malayi*, like many nematodes, must acquire heme from exogenous sources due to lack of a heme biosynthetic pathway [42-43]. Though the mechanism remains unknown, we postulate that these worms are able to acquire heme from host RBCs. This study has shown that there is a distinct difference in the blood profile between mf+ cats and both mf- cats and dogs of either status.

The main purpose of this study was to present a full clinical profile of *B. malayi*-infected cats and dogs. Even though the feline animal model has been utilized for *B. malayi* studies for more than six decades, such a picture has never been documented until now. There are distinct differences between the two host species examined, and differences noted between the mf+ and mf- cats. The addition of dogs to this clinical study demonstrates their permissivity to *B. malayi* infection, though they are not ideal hosts. This does raise the question of whether dogs in *B. malayi*-endemic areas could be a source of transmission. Dogs have been found to be the unexpected source of another parasitic

nematode, the Guinea worm (*Dracunculus medinensis*), remaining at low levels until nearly eradicated in human populations [55-56]. If this is also true for *B. malayi*, this would be an unexpected source of low-level transmission, potentially affecting elimination efforts and MDA programs in these endemic regions

**ACKNOWLEDGMENTS**

This project could not have been accomplished without the expert support of those at the Filariasis Research Reagent Resource Center and the University of Georgia's animal resource center. We thank Tanya Cooper, Prasit Supakorndej (in memoriam), Kristen Mabrey, and Molly Savadelis for their technical assistance. Additionally, we thank Bob Storey for animal photographs.

**DISCLOSURES**

The authors have no financial conflict of interest.

**REFERENCES**

[1] WHO, *Global Programme to Eliminate Lymphatic Filariasis: Managing Morbidity and Preventing Disability*. Geneva, Switzerland: World Health Organization, 2013.

[2] WHO, "Lymphatic filariasis," *World Health Organization*, 2014. [Online]. Available: http://www.who.int/mediacentre/factsheets/fs102/en/.

[3] CDC, “Neglected Tropical Diseases,” *Center for Disease Control and Prevention*, 2014. [Online]. Available: http://www.cdc.gov/globalhealth/ntd/.

[4] E. Michael, M. N. Malecela-Lazaro, C. Kabali, L. C. Snow, and J. W. Kazura, “Mathematical models and lymphatic filariasis control: endpoints and optimal interventions,” *Trends Parasitol.*, vol. 22, no. 5, pp. 226–33, May 2006.

[5] E. Devaney, S. A. Martin, and F. J. Thompson, “Stage-specific gene expression in lymphatic filarial nematodes,” *Parasitol. today*, vol. 12, no. 11, pp. 418–24, Nov. 1996.

[6] R. C. Anderson, “The Superfamily Filarioidea,” in *Nematode Parasites of Vertebrates: Their Development and Transmission*, Wallingford, UK: CAB International, 1992, pp. 437–520.

[7] D. I. Grove, *A history of human helminthology*. Wallingford, UK: CAB International, 1990.

[8] P. E. Simonsen, “Filariasis,” in *Manson’s Tropical Diseases*, 22nd ed., G. C. Cook and A. Zumla, Eds. China: W. B. Saunders, 2008, pp. 1477–1513.

[9] WHO/GEPLF, “Lymphatic Filariasis,” World Health Organization, Geneva, Switzerland, Jan. 2013.

[10] J. R. Palmieri, Purnomo, D. T. Dennis, and H. A. Marwoto, “Filarid parasites of South Kalimantan (Borneo) Indonesia. *Wuchereria kalimantani sp.* n. (Nematoda: Filarioidea) from the silvered leaf monkey, *Presbytis cristatus* Eschscholtz 1921,” *J. Parasitol.*, vol. 66, no. 4, pp. 645–651, 1980.

[11] L. R. Ash and J. M. Riley, “Development of subperiodic *Brugia malayi* in the jird, *Meriones unguiculatus*, with notes on infections in other rodents,” *J. Parasitol.*, vol. 56, no. 5, pp. 969–973, 1970.

[12] T. R. Klei and T. V Rajan, *World Class Parasites: Volume 5 The Filaria*. Boston: Kluwer Academic Publishers, 2002.

[13] K. P. Day, “The endemic normal in lymphatic filariasis: A static concept,” *Parasitol. Today*, vol. 7, no. 12, pp. 341–343, Jan. 1991.

[14] L. M. Ganley-leal, “The influence of host sex on the L3 to L4 Molt of *Brugia malayi*,” *J. Parasitol.*, vol. 91, no. 5, pp. 1222–1225, 2005.

[15] S. El-Bihari and A. Ewert, “Worm burdens and prepatent periods in jirds (*Meriones unguiculatus*) infected with *Brugia malayi*,” *Southeast Asian J. Trop. Med. Public Health*, vol. 4, no. 2, pp. 184–186, 1973.

[16] J. J. C. Buckley and J. F. B. Edeson, “On the adult morphology of *Wuchereria sp.* (*malayi?*) From a monkey (*Macaca irus*) and from cats in malaya, and on *Wuchereria pahangi* n.sp. from a dog and a cat,” *J. Helminthol.*, vol. 30, no. 1, pp. 1–20, 1956.

[17] K. Kanjanopas *et al.*, “*Brugia malayi* in a naturally infected cat from Narathiwat province, Southern Thailand,” *Southeast Asian J. Trop. Med. Public Health*, vol. 32, no. 3, pp. 585–587, 2001.

[18] J. F. B. Edeson and R. H. Wharton, “Transmission of *Wuchereria malayi* from man to the domestic cat,” *Trans. R. Soc. Trop. Med. Hyg.*, vol. 51, no. 4, pp. 366–370, 1957.

[19] L. R. Ash, “Chronic *Brugia pahangi* and *Brugia malayi* infections in *Meriones unguiculatus*,” *J. Parasitol.*, vol. 59, no. 3, pp. 442–7, Jun. 1973.

[20] M. L. Michalski, K. G. Griffiths, S. a Williams, R. M. Kaplan, and A. R. Moorhead, “The NIH-NIAID Filariasis Research Reagent Resource Center.,” *PLoS Negl. Trop. Dis.*, vol. 5, no. 11, p. e1261, Nov. 2011.

[21] V. R. Ambily, U. N. Pillai, R. Arun, S. Pramod, and K. M. Jayakumar, “Detection of human filarial parasite *Brugia malayi* in dogs by histochemical staining and molecular techniques,” *Vet. Parasitol.*, vol. 181, no. 2–4, pp. 210–4, Sep. 2011.

[22] J. W. Mak, P. K. F. Yen, K. C. Lim, and N. Ramiah, “Zoonotic implications of cats and dogs in filarial transmission in peninsular Malaysia,” *Trop. Geogr. Med.*, vol. 32, pp. 259–264, 1979.

[23] J. W. McCall, “The role of arthropods in the development of animal models for filariasis research,” *J. Georg. Entomol. Soc.*, vol. 16, pp. 283–293, 1981.

[24] J. F. B. Edeson and R. H. Wharton, “The experimental transmission of *Wucheria malayi* from man to various animals in Malaya,” London, 1957.

[25] J. J. C. Edeso, J. F. B. and Buckle, “Studies on filariasis in Malaya: On the migration and rate of growth of *Wuchereria malayi* in experimentally infected cats,” *Ann. Trop. Med. Parasitol.*, vol. 53, no. 1, pp. 113–119, 1958.

[26] V. L. Corcoran, B. M., Foster, D. J., and Fuentes, “Feline asthma syndrome: A retrospective study of the clinical presentation in 29 cats,” *J. Small Anim. Pract.*, vol. 36, pp. 481–488, 1995.

[27] Y. Mutafchiev, O. Bain, Z. Williams, J. W. McCall, and M. L. Michalski, “Intraperitoneal development of the filarial nematode *Brugia malayi* in the Mongolian jird (*Meriones unguiculatus*),” *Parasitol. Res.*, vol. 113, no. 5, pp. 1827–1835, 2014.

[28] J. A. Herrick *et al.*, “Eosinophil-associated processes underlie differences in clinical presentation of loiasis between temporary residents and those indigenous to loa-endemic areas,” *Clin. Infect. Dis.*, vol. 60, no. 1, pp. 55–63, 2015.

[29] L. Huang *et al.*, “Eosinophil-derived IL-10 supports chronic nematode infection,” *J. Immunol.*, vol. 193, no. 8, pp. 4178–4187, 2014.

[30] N. G. Gebreselassie *et al.*, “Eosinophils preserve parasitic nematode larvae by regulating local immunity.,” *J. Immunol.*, vol. 188, no. 1, pp. 417–25, 2012.

[31] L. Huang *et al.*, “Eosinophils mediate protective immunity against secondary nematode infection,” *J. Immunol.*, vol. 194, no. 1, pp. 283–90, 2015.

[32] A. S. Fahrion, S. Staebler, and P. Deplazes, “Patent *Toxocara canis* infections in previously exposed and in helminth-free dogs after infection with low numbers of embryonated eggs,” *Vet. Parasitol.*, vol. 152, pp. 108–115, 2008.

[33] J. Margareth and D. A. Rodolpho, “Robust phenotypic activation of eosinophils during experimental *Toxocara canis* infection experimental animals and parasites,” *Front. Immunol.*, vol. 9, no. January, 2018.

[34] T. Ramalingam, P. Porte, J. Lee, and T. V. Rajan, “Eosinophils, but not eosinophil peroxidase or major basic protein, are important for host protection in experimental *Brugia pahangi* infection,” *Infect. Immun.*, vol. 73, no. 12, pp. 8442–8443, 2005.

[35] M. H. Shin, Y. A. Lee, and D. Y. Min, “Eosinophil-mediated tissue inflammatory responses in helminth infection,” *Korean J. Parasitol.*, vol. 47, no. SUPPL., pp. 125–132, 2009.

[36] S. Specht *et al.*, “Lack of eosinophil peroxidase or major basic protein impairs defense against murine filarial infection,” *Infect. Immun.*, vol. 74, no. 9, pp. 5236–5243, 2006.

[37] S. A. Babayan, A. F. Read, R. A. Lawrence, O. Bain, and J. E. Allen, “Filarial parasites develop faster and reproduce earlier in response to host immune effectors that determine filarial life expectancy.,” *PLoS Biol.*, vol. 8, no. 10, p. e1000525, Jan. 2010.

[38] M. L. Knott, K. I. Matthaei, P. R. Giacomin, H. Wang, P. S. Foster, and L. A. Dent, “Impaired resistance in early secondary *Nippostrongylus brasiliensis* infections in mice with defective eosinophilopoeisis,” vol. 37, pp. 1367–1378, 2007.

[39] A. Sher, R. L. Coffman, S. Hieny, and A. W. Cheever, “Ablation of eosinophil and IgE responses with anti-IL-5 or anti-IL-4 antibodies fail to affect immunity against *Schistosoma mansoni* in the mouse,” *J. Immunol.*, vol. 145, pp. 3911–3916, 2019.

[40] B. N. Paciorkowski, P. Porte, L. D. Shultz, and T. V Rajan, “B1 B Lymphocytes play a critical role in host protection against lymphatic filarial parasites,” *J. Exp. Med.*, vol. 191, no. 4, pp. 731–735, 2000.

[41] N. Harris and W. C. Gause, “To B or not to B : B cells and the Th2-type immune response to helminths,” *Trends Immunol.*, vol. 32, no. 2, pp. 80–88, 2011.

[42] R. M. Maizels and H. J. Mcsorley, “Regulation of the host immune system by helminth parasites,” *J. Allergy Clin. Immunol.*, vol. 138, no. 3, pp. 666–675, 2016.

[43] P. K. Murthy, K. Tyagi, R. P. Ghosh, P. S. Murthy, and R. K. Chatterjee, “Longitudinal humoral immune responses of Indian leaf monkey (*Presbytis entellus*) to *Brugia malayi* infection,” *Parasitology*, vol. 119 ( Pt 1, pp. 53–60, 1999.

[44] C. M. Finlay *et al.*, “Helminth products protect against autoimmunity via innate Type 2 cytokines IL-5 and IL-33, which promote eosinophilia,” *J. Immunol.*, vol. 196, no. 2, pp. 703–714, 2016.

[45] E. J. Norman, R. C. J. Barron, H. N. C. Mls, A. S. Nash, and R. B. Clampitt, “Prevalence of low automated platelet counts in cats: comparison with prevalence of thrombocytopenia based on blood smear estimation,” *Vet. Clin. Pathol.*, vol. 30, no. 3, pp. 137–140, 2001.

[46] N. C. Jain, *Schalm's Veterinary Hematology*, 4th ed. Philadelphia, Penn: Lea and Febiger, 1986.

[47] D. Zelmanovic and E. J. Hetherington, "Automated analysis of feline platelets in whole blood, including platelet count, mean platelet volume, and activation state," *Vet. Clin. Pathol.*, vol. 27, no. 1, pp. 2–9, 1998.

[48] C. J. McCoy, B. J. Reaves, S. Giguere, R. Coates, B. Rada, and A. J. Wolstenholme, "Human leukocytes kill *Brugia malayi* microfilariae independently of DNA-based extracellular trap release," *PLoS Negl. Trop. Dis.*, vol. 11, no. 1, pp. 1–20, 2017.

[49] P. Noris, C. Klersy, M. Zecca, F. Melazzini, and V. Terulla, "Platelet size distinguishes between inherited macrothrombocytopenias and immune thrombocytopenia," no. August, pp. 2131–2136, 2009.

[50] I. Thrombocytopenia, D. Schwartz, L. Sharkey, P. J. Armstrong, C. Knudson, and J. Kelley, "Platelet volume and plateletcrit in dogs with presumed primary immune-mediated thrombocytopenia," pp. 1575–1579, 2014.

[51] A. M. Vet, "Platelet indices in dogs with thrombocytopenia and dogs with normal platelet counts," *Arch. Med. Vet.*, vol. 281, pp. 277–281, 2016.

[52] A. Tefferi, C. A. Hanson, and D. J. Inwards, "Complete blood cell count in adults," *Mayo Clin. Proc.*, vol. 80, no. 7, pp. 923–936, 2005.

[53] A. N. Luck, X. Yuan, D. Voronin, B. E. Slatko, I. Hamza, and J. M. Foster, “Heme acquisition in the parasitic filarial nematode *Brugia malayi*,” *FASEB J.*, vol. 30, no. 10, pp. 3501–3514, 2016.

[54] B. Wu *et al.*, “The heme biosynthetic pathway of the obligate *Wolbachia* endosymbiont of *Brugia malayi* as a potential anti-filarial drug target,” *PLoS Negl. Trop. Dis.*, vol. 3, no. 7, pp. 1–10, 2009.

[55] E. Callaway, “Dogs thwart end to Guinea worm,” *Nat. news*, vol. 529, no. 7584, pp. 10–11, 2016.

[56] M. L. Eberhard *et al.*, “The Peculiar Epidemiology of Dracunculiasis in Chad,” *Am. J. Trop. Med. Hyg.*, vol. 90, no. 1, pp. 61–70, 2014.

**FIGURES**

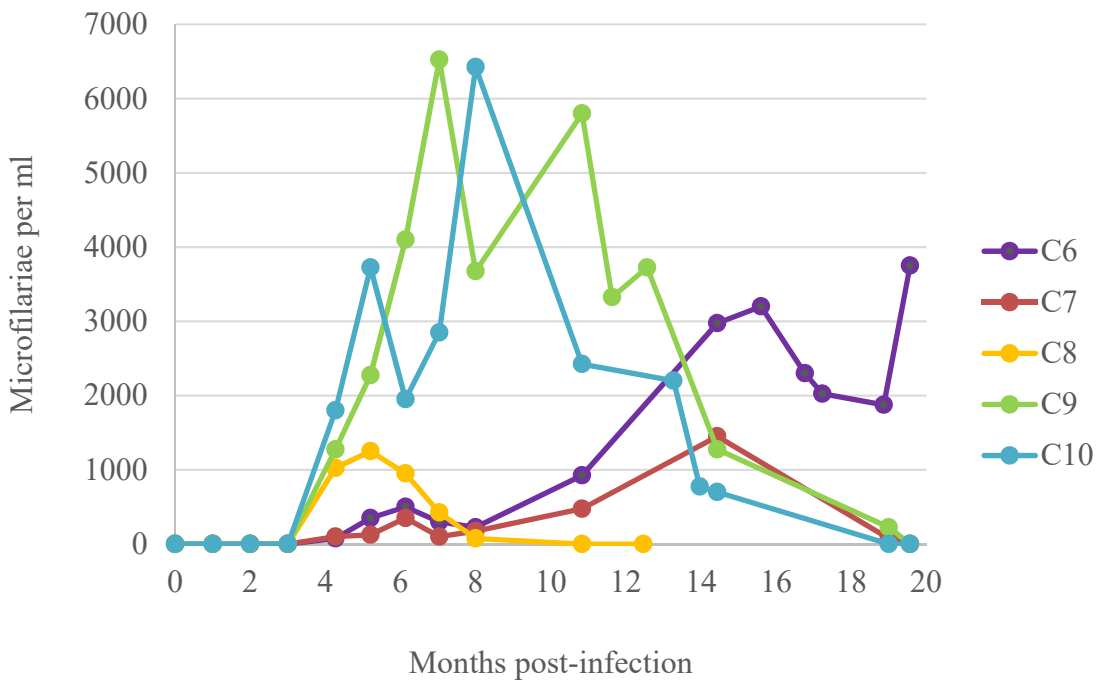



Figure 2.1: Microfilaremia of *B. malayi*-infected cats. Microfilariae were observed in 5 out of the 10 cats. Cats with no measurable microfilariae were not included in this figure.

| | Lymphedema + | Lymphedema - |
|---|---|---|
| Microfilaremic | 3 | 2 |
| Amicrofilaremic | 4 | 1 |

Figure 2.2: Feline lymphedema of *B. malayi*-infected cats. On the left is a picture of C4 presenting with acute lymphedema on the left hind limb. No lymphedema was present on the left forelimb. Photo courtesy of Bob Storey. On the right, is a contingency table indicating microfilaremia and lymphedema status.

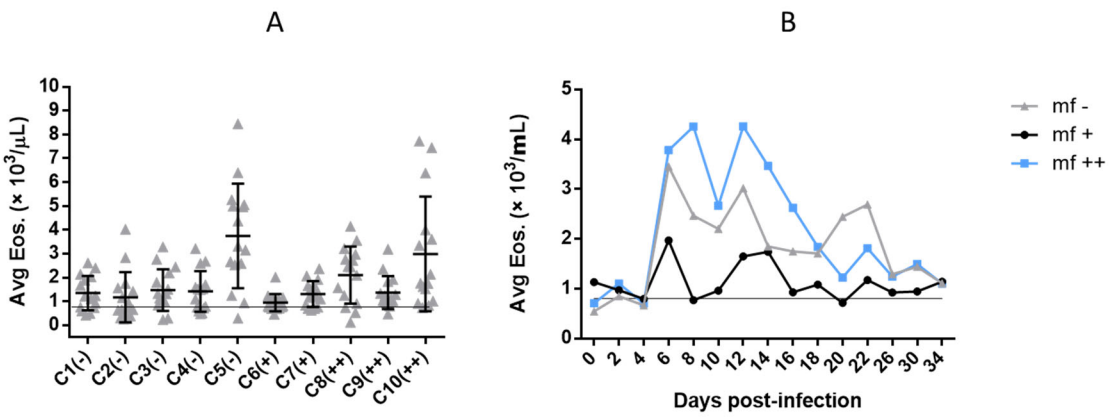



Figure 2.3: Feline CBC results for eosinophils (Eos). For both figures, the two cats with a delayed microfilaremia (C6 and C7) are indicated as mf+. The remaining microfilaremic cats are designated mf++, with the amicrofilaremic cats as mf-. The solid line indicates the uninfected reference interval for each different cell type. (A) Mean eosinophil counts for each cat over the course of study. The eos reference interval for normal uninfected cats is 0 - 0.8 x $10^3$/µl. (B) Eosinophil counts for cats grouped by microfilaremia status.

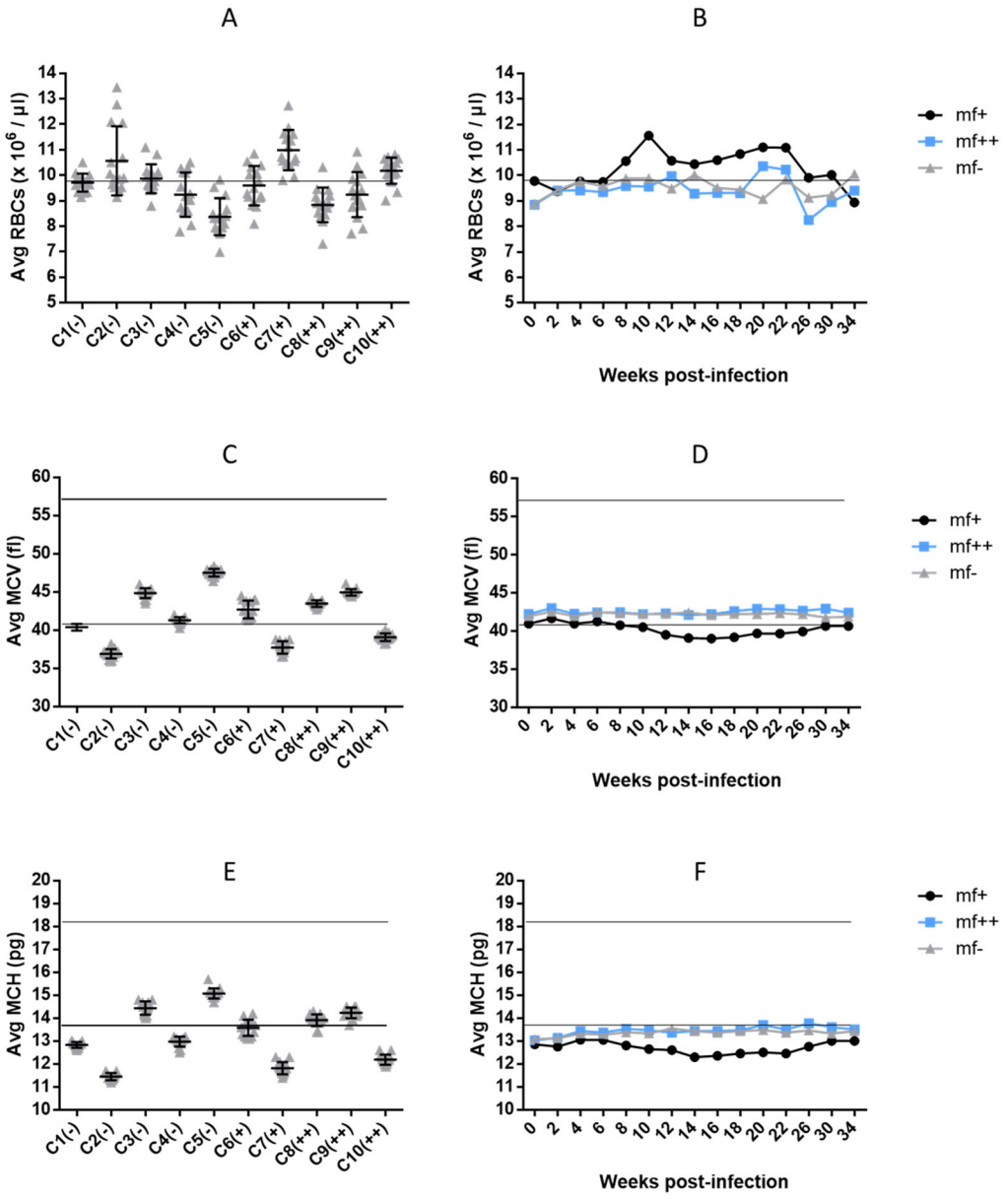



Figure 2.4: Feline CBC results for Red blood cells (RBC) and RBC indices, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). For all figures, the two cats with a delayed microfilaremia (C6 & C7) are indicated as mf+. The remaining microfilaremic cats are designated as mf++, with the amicrofilaremic cats as mf-. The solid line indicates the uninfected reference interval for each different cell type. (A) Mean RBC counts for each cat over the course of this study. The RBC reference interval for normal uninfected cats is 4.9 - 9.8 x $10^6$/µl. (B) RBC counts for cats grouped by microfilaremia status. (C) Mean MCV for each cat over the course of study. The MCV reference interval for normal uninfected cats is 41-57 fl. (D) MCV for cats grouped by microfilaremia status. There was no difference between the mf++ and mf- groups. (E) Mean MCH for each cat over the course of the study. The MCH reference interval for normal uninfected cats is 13.8-18.1 pg. (F) MCH for cats grouped by microfilaremia status.

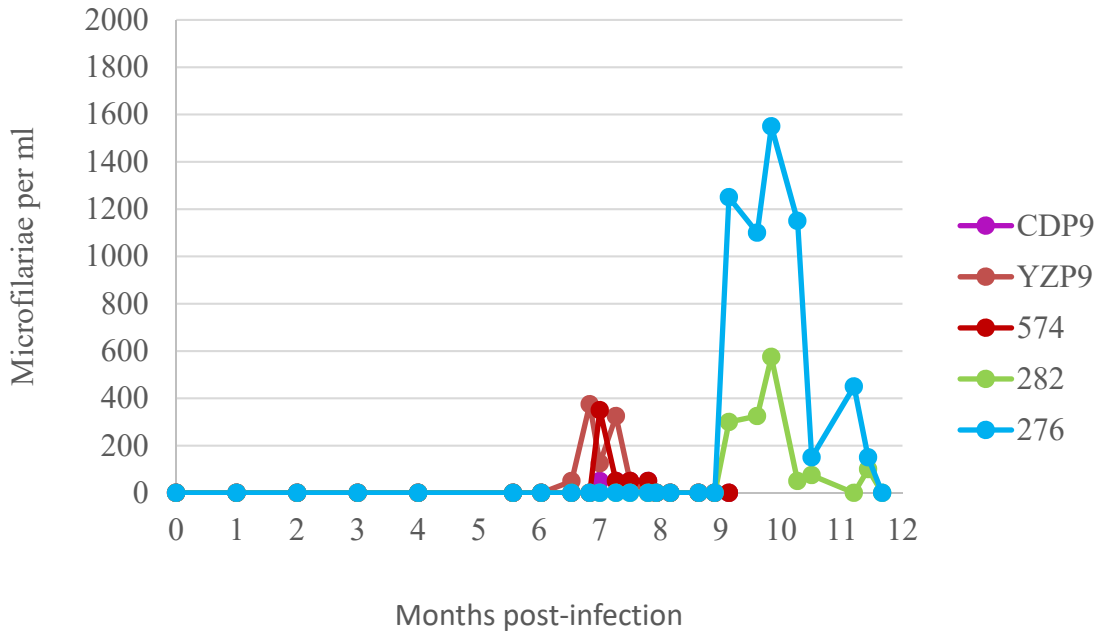
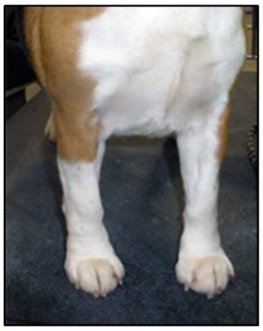



Figure 2.5: Microfilaremia of *B. malayi*-infected dogs. Amicrofilaremic dogs are not shown.



| | Lymphedema + | Lymphedema - |
|---|---|---|
| Microfilaremic | 0 | 5 |
| Amicrofilaremic | 0 | 6 |

Figure 2.6: Canine lymphedema. On the left is a picture of dog 276 taken by Bob Storey. No dogs exhibited lymphedema throughout the course of the *B. malayi* infection.

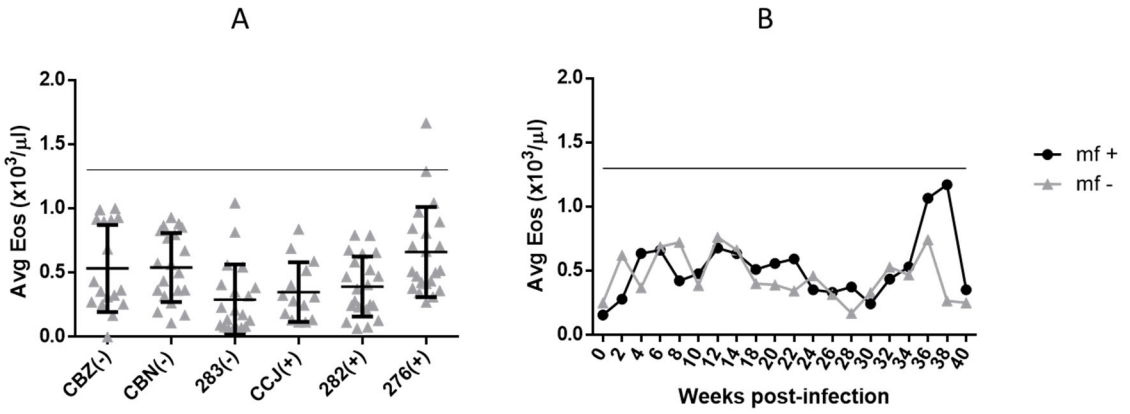



Figure 2.7: Canine CBC results for eosinophils (Eos). The amicrofilaremic dogs are designated as mf- and those with measured circulating microfilariae are mf+. The solid line indicates the uninfected reference interval for each different cell type. (A) Mean eosinophil counts for each dog over the course of study. The Eos reference interval for normal uninfected dogs is 0-1.3 x $10^3$/µl. (B) Mean number of eosinophils for dogs grouped by microfilaremia status.

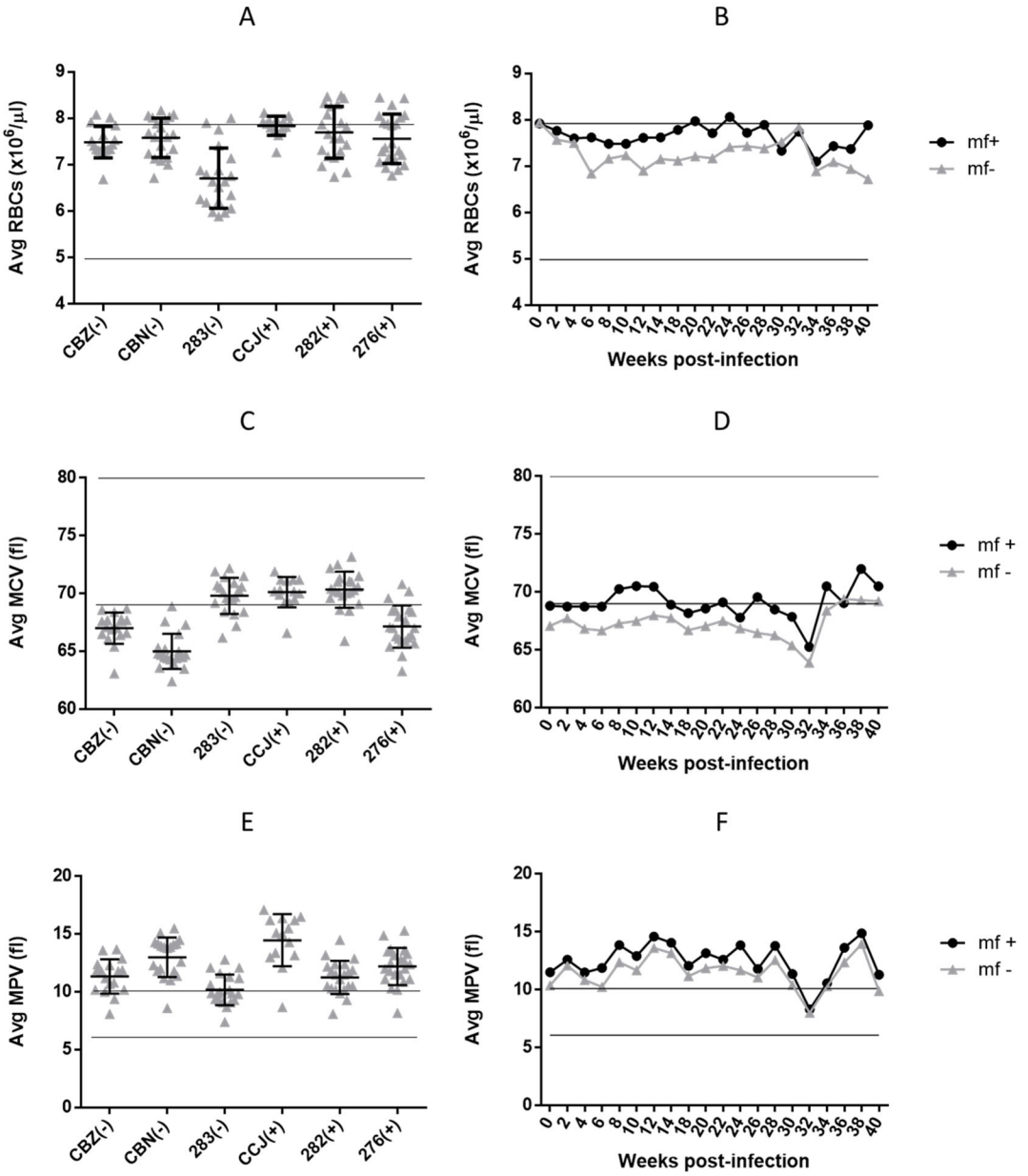



Figure 2.8: Canine CBC results for red blood counts (RBC), mean corpuscular volume (MCV), and mean platelet volume (MPV). The amicrofilaremic dogs are labeled as mf- and those with measured circulating mf are mf+. The solid line indicates the uninfected reference interval for each different cell type. (A) Mean RBC counts for each dog over the course of this study. The RBC reference interval for normal uninfected dogs is 4.98 – 7.92 x $10^6$/µl. (B) Mean number of RBCs for dogs grouped by microfilaremia status. (C) Mean MCV for each dog over the course of study. The MCV reference interval for normal uninfected dogs is 69-80 fl. (D) MCV for dogs grouped by microfilaremic status. No significant difference was found between mf- and mf+ groups. (E) Mean MPV values for each dog, with an uninfected reference interval of 6.1-10.1 fl. (F) Mean MPV in dogs grouped by microfilaremia status.

## SUPPLEMENTAL TABLES AND FIGURES

Table S2.1: Lymphedema observed in *B. malayi*-infected cats. The cats highlighted in red were amicrofilaremic, yellow designates delayed patency and green became patent within a standard timeframe. Observations were recorded weekly as bilateral marked (BMA), bilateral moderate (BMO), bilateral mild (BMI), bilateral mixed (BMX), right/left rear marked (RMA/LMA), right/left rear moderate (RMO/LMO), and right/left rear mild (RMI/LMI), and Resolved (R).

| Days p.i. | C2 | C4 | C3 | C5 | C1 | C6 | C7 | C10 | C8 | C9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | - | - | - | - | - | - | - | - | - | - |
| 51 | - | RMA | - | - | - | - | - | - | - | - |
| 54 | - | BMA | - | - | - | - | - | - | - | - |
| 59 | - | BMO | - | - | - | - | - | - | - | - |
| 66 | - | BMO | - | - | - | - | - | - | - | - |
| 77 | RMO | BMO | BMX | LMI | - | BMX | - | - | - | RMI |
| 79 | RMO | BMO | BMX | LMI | - | BMX | - | - | - | RMI |
| 85 | RMO | BMO | BMX | LMI | - | BMX | - | - | - | RMI |
| 93 | RMO | BMO | BMX | LMI | - | BMX | - | - | - | RMI |
| 100 | BMI | BMO | RMI | R | - | RMI | - | - | - | R |
| 106 | R | BMX | R | - | - | RMI | - | - | - | - |
| 114 | - | BMI | - | - | - | RMI | - | - | - | - |
| 121 | - | LMI | - | - | - | R | - | - | - | - |
| 128 | - | LMI | - | - | - | - | - | - | - | - |
| 133 | - | R | - | - | - | - | - | - | - | - |
| 455 | - | - | - | - | - | - | BMO | - | - | - |
| 461 | - | - | - | - | - | - | BMO | - | - | - |
| 479 | - | - | - | - | - | - | BMO | - | - | - |
| 491 | - | - | - | - | - | - | BMO | - | - | - |
| 498 | - | - | - | - | - | - | BMO | - | - | - |
| 508 | - | - | - | - | - | - | BMO | - | - | - |
| 517 | - | - | - | - | - | - | BMA | - | - | - |
| 528 | - | - | - | - | - | - | BMA | - | - | - |
| 540 | - | - | - | - | - | - | BMA | - | - | - |
| 548 | - | - | - | - | - | - | BMA | - | - | - |
| 553 | - | - | - | - | - | - | BMA | - | - | - |
| 561 | - | - | - | - | - | - | BMA | - | - | - |
| 568 | - | - | - | - | - | - | BMA | - | - | - |
| 575 | - | - | - | - | - | - | BMA | - | - | - |
| 584 | - | - | - | - | - | - | BMA | - | - | - |
| 589 | - | - | - | - | - | - | BMA | - | - | - |
| 595 | - | - | - | - | - | - | BMA | - | - | - |
| 598 | - | - | - | - | - | - | BMA | - | - | - |
| 603 | - | - | - | - | - | - | BMA | - | - | - |

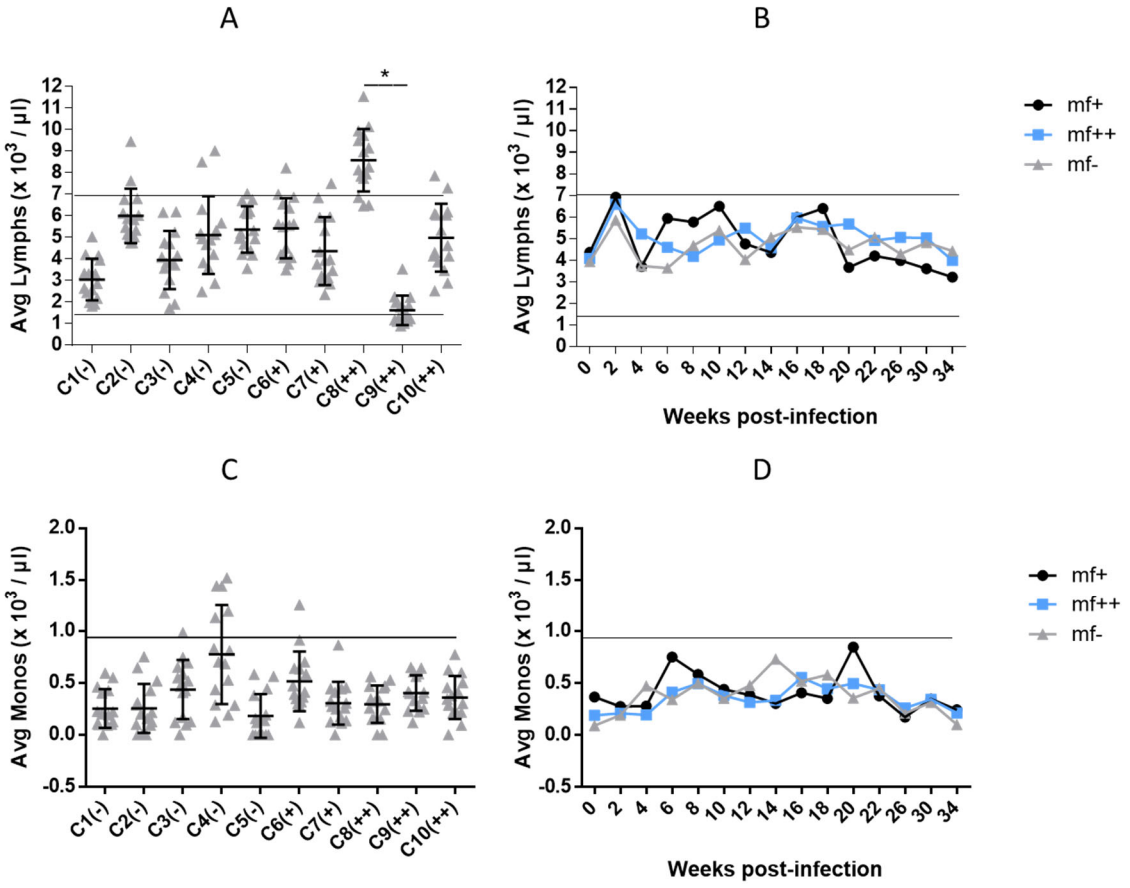



Figure S2.1: Feline CBC results for lymphocytes (lymphs) and monocytes (monos). For all figures, the two cats with a delayed microfilaremia (C6 & C7) are indicated as mf+. The remaining microfilaremic cats are noted as mf++, with the amicrofilaremic cats as mf-. The solid line indicates the uninfected reference interval for each different cell type. (A) Mean lymphocytes for each cat over the course of study. The lymphocyte reference interval for normal uninfected felines is 1.5-7 x $10^3$/µl. (B) Mean lymphocyte counts for mf-, mf+, and mf++ groups. There was no significant difference between these groups over time. (C) Mean monocyte count for each cat over the course of this study. The monocyte reference interval for normal uninfected cats is 0-0.9 x $10^6$/µl. (D) Mean monocyte counts for mf-, mf+, and mf++ groups.

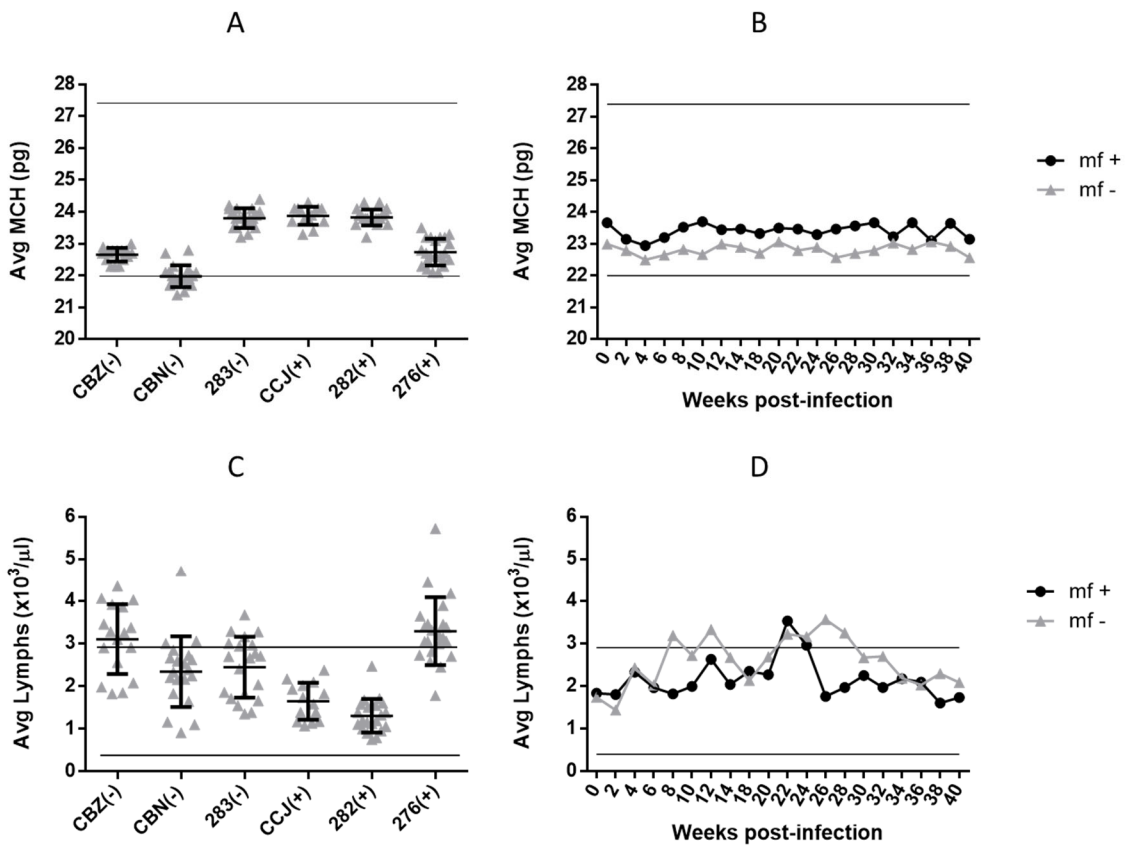



Figure S2.2: Canine CBC results mean corpuscular hemoglobin (MCH) and lymphocytes (lymphs). The amicrofilaremic dogs are designated as mf- and those with measured circulating microfilaria are mf+. The solid line indicates the uninfected reference interval for each different cell type. (A) Mean MCH for each dog throughout this study. The reference interval for canine MCH was 22-27.4 pg. (B) Mean monocyte counts for the mf+ and mf- groups. (C) Mean lymphocyte count for each dog over the course of study. The lymphocyte reference interval for normal uninfected canines was 0.4-2.9 x $10^3$/µl. There were no differences seen between these animals. (D) Mean lymphocyte counts for the mf+ and mf- groups

# CHAPTER 3

# THE EFFECT OF HEAVY METALS ON IN VITRO CULTURES WITH *BRUGIA MALAYI* THIRD-STAGE LARVAE

Erica J. Burkman, Cherica R. Richmond, Christopher C. Evans, Andrew R. Moorhead*

Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, Georgia, United States of America

*Corresponding author: Andrew R. Moorhead, Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, 501 D.W. Brooks Drive, Athens, Georgia, 30602, USA, email: amoorhed@uga.edu

Burkman, Erica. To be submitted to Parasites & Vectors

## ABSTRACT

Lymphatic filariasis (LF) is a global disease caused by parasitic nematodes transmitted by mosquitoes. In studying this disease, an effective in vitro model is vital to screening new compounds or new combinations of current anthelmintics. In this study, we used *Brugia malayi* as the laboratory model parasite for LF. Optimal in vitro conditions for the culture of these parasites have been elusive for over 40 years of study. These parasites utilize metalloproteins for development and growth. Some heavy metals are essential nutrients that are obtained either by environmental exposure or ingested. Aside from trace amounts in media and serum, however, these metals have not been previously added to in vitro cultures of these parasites. Iron (II) sulfate heptahydrate, zinc sulfate heptahydrate, and copper (II) sulfate pentahydrate were added to *B. malayi* third-stage larval ($L_3$) cultures to measure the effects of these compounds on the parasites. Copper has been used to augment anthelmintic activity against gastrointestinal parasites, particularly in areas where resistance is an ongoing problem. We found a similar killing effect with *B. malayi* $L_3$. The addition of zinc reduced survival in $L_3$ but increased overall development to the fourth larval stage ($L_4$), whereas iron increased survival but reduced development to the $L_4$. The addition of these metals was shown to augment survival and development of *B. malayi* larvae but did not provide optimal in vitro culture conditions.

## INTRODUCTION

Parasitic nematodes, in particular, the causative agents of lymphatic filariasis (LF), have a high degree of host specificity. To survive and develop to reproductive adults, they need to be able to adapt to multiple hosts, body temperatures, oxygen potential, host

immune responses, and other host-environmental conditions [1–5]. In *Wuchereria bancrofti*, the parasitic nematode that accounts for ~90% of LF cases, host range is limited to humans, some non-human primate species, and the mosquito vector [6–12]. Because *W. bancrofti* can only develop in primates, the closely related filarial nematode *Brugia malayi* has been used as a laboratory model for LF for more than 60 years [13-21].

Due to mass drug administration (MDA) efforts to cover an ever-increasing population and systemic drug resistance, there remains a growing need to develop new anthelmintics to combat LF and other neglected tropical diseases. One of the difficulties in combating LF is that drugs utilized in MDA programs, such as ivermectin and albendazole, only temporarily sterilize females to reduce circulating mf, but is not an adulticide. Thus, annual treatments in endemic areas can extend for 10-20 years to halt transmission of these parasites [22]. As such, in vitro assays are vital to screening novel anthelmintics prior to orchestrating far more costly and intricate animal model studies.

Successfully culturing filarial nematodes in an in vitro environment relies on a number of crucial factors for survival and development, including nutrients, temperature, pH, and atmospheric pressure. With the millions of compounds both manufactured and natural in new and repurposed drug libraries, a consistent and viable in vitro screening system is crucial. Even though in vitro studies have been conducted on filarial worms since the early 1900s, scientists remain unable to fully mimic the environment of the host [23–31]. Finding factors that can aid in the survival and development of these parasites have become a long sought-after goal.

The success of these parasites in their respective hosts is a direct consequence of the unique protective properties of their cuticle. The synthesis and shedding of this cuticle,

or molting, occurs four times in filarial parasites: twice in the mosquito vector and twice in the mammalian host. The process of molting in parasitic nematodes is an evolutionary function whereby the worm is allowed to grow and develop and yet remain safely contained and protected while a new cuticle forms. The cuticle is a vital barrier separating the parasite from the host environment and disruption in its synthesis can be fatal. When a new cuticle is being formed, the old cuticle separates from the new layer in a process called apolysis. Once the new cuticle is fully synthesized, the old cuticle sheds (ecdysis) and the worm continues to develop normally. In order for these nematodes to molt to the next developmental stage, apolysis, cuticle synthesis, and ecdysis must be executed accordingly to ensure healthy development [32].

Studies on the non-parasitic nematode *Caenorhabditis elegans* have implicated zinc metalloproteases to be involved in molting. Findings suggest that genes *nas-36* and *nas-37* are necessary for normal ecdysis of the outer cuticle in *C. elegans* [32, 33]. When these genes are silenced, the worms express defects in the molting process. Orthologs of the *nas-36* and *nas-37* genes have been found within the *B. malayi* genome [33, 34]. In normal cell culture media, serum serves as the source of zinc [35]. Thus, in low-concentration or serum-free cultures, the further addition of zinc may facilitate normal growth conditions.

Coinciding with molting, other cofactors must be present in the host environment in order for the filarial nematode to develop correctly before and after the molt. Iron is another essential nutrient, required for oxygen transport, heme incorporation, and as an enzymatic cofactor for biological processes such as respiration and DNA synthesis [36]. When present in excess, tissue damage and organ failure can occur, thus iron homeostasis

is tightly regulated at the cellular and systemic level. As a component of several metalloproteins, iron is crucial in oxygen sensing and transport, electron transfer, and other biological functions [36]. Free-iron has limited bioavailability in aerobic environments as ferrous iron (Fe (II)) can readily oxidize in solution to ferric iron (Fe (III)). Ferric iron is insoluble at a physiologic pH [$K_{free\ Fe(III)} = 10^{-18}$ M], thus limiting the concentration of Fe(II) systemically [37]. Iron is vital in the incorporation of heme, a tetrapyrrole with an iron cation at the center of a porphyrin subunit. Most complex organisms are able to synthesize heme through biosynthetic pathways [38], whereas free-living and parasitic nematodes are heme auxotrophs, and must acquire heme exogenously [39]. Parasitic nematodes, such as *B. malayi*, have seemingly lost the ability to synthesize heme [39, 40], relying instead on *Wolbachia* (*w*Bm), an obligate bacterial endosymbiont [41–43]. These symbiotic bacteria have lost a number of biological functions to survive independently, yet maintain key metabolic functions lacking in the nematode host, notably heme synthesis [44–47]. As heme is vital for many porphyrin-containing metalloproteins within free-living and parasitic nematodes, iron availability for these organisms is vital [48].

Filarial nematodes have evolved a number of strategies in order to evade the host's hostile environment. Both enzymatic and non-enzymatic anti-oxidant systems are utilized by these parasites to counteract reactive oxygen species (ROS) generated by host macrophages, granulocytes, and effector host cells [49, 50]. One method that filarioids use to counteract oxidative stress is by secretion of copper/zinc (Cu/Zn) superoxide dismutases (SOD) localized within the cuticle [51–53]. These SODs catalyze decomposition of superoxide into molecular oxygen and hydrogen peroxide ($H_2O_2$) to counteract free radical damage to the worm [54, 55]. These SODs have been explored in other filarial for their

protective role [56–58]. It is unknown whether the addition of exogenous ions, such as copper, will aid or hinder survival and development within *B. malayi* larvae. If these larvae are able to utilize the free copper in the media, it may increase overall survival of these helminths.

In *C. elegans*, when the $L_3$ stage is under sub-optimal environmental conditions, the larvae undergo a developmental arrest, or dauer stage, initiated by low food availability, high temperatures, and/or high population densities [59]. These dauer larvae do not feed but instead, remain in this arrested state until environmental conditions improve and subsequently can develop normally to the $L_4$ stage. The genetic machinery found in *C. elegans* linked to dauer formation includes cGMP, insulin, TGF-β pathways, and dafachronic acid signaling (DAF) and more [63, 64]. Homologs to the *C. elegans* dauer formation gene *daf-7* have been found in *B. malayi*, notably *tgh-2* [61]. This gene has been found to be highly expressed in microfilariae, which undergo a state of arrested development while waiting for a vector host. Expression levels are also high in adults, suggesting additional functionality of this gene. There is the potential that *B. malayi* $L_3$ also enter a dauer-like stage while waiting for the vector-host to feed on a mammalian host. Furthermore, the possibility that suboptimal in vitro conditions may halt $L_3$ development requires investigation. The aims of this study were to determine whether the addition of heavy metal ions would facilitate development, molting, and survival in an in vitro culture environment. We hypothesize that zinc and iron ions will aid *B. malayi* $L_3$ molting and survival, thereby facilitating development into the $L_4$ stage, whereas copper ions will hinder development.

## METHODS

### Parasite collection and assay preparation

All parasite materials used in these experiments were obtained from the Filariasis Research Reagent Resource Center (FR3) located in Athens, Georgia. The mosquitoes utilized were *Aedes aegypti* black-eyed Liverpool strain. These mosquitoes were reared in an insectary maintained at 26-28°C and 70-80% relative humidity. Heparinized blood collected from *B. malayi*-infected cats were fed to adult mosquitoes. Fresh blood was added to artificial membrane feeders as previously described [62]. After 14 days the containers of mosquitoes were placed in a standard freezer (-18°C) for approximately 75 seconds to stun the mosquitoes for $L_3$ collection. Once stunned, the mosquitoes were tapped into an 8 cm diameter glass mortar and gently crushed with a pestle. A small amount of cold Hanks Balanced Salt Solution (HBSS without sodium bicarbonate, Sigma-Aldrich, St. Louis, MO) with 0.4 µg/ml penicillin-streptomycin (Pen/Strep, Sigma-Aldrich) was used to rinse the crushed mosquitoes from the mortar into a 100 mm x 20 mm petri dish containing a 150-µm mesh sieve and warm (~37°C) HBSS with Pen/Strep. The $L_3$ migrated out of the crushed mosquitoes and through the sieve via gravity and sinusoidal motion to settle on the bottom of the petri dish. Every 15 to 20 minutes the sieve was moved to a new petri dish with warm HBSS to maximize $L_3$ recovery. The $L_3$ were then individually collected and counted into a separate petri dish. Only morphologically intact and motile larvae were collected.

**Third-stage larval preparation for motility assays**

In order to remove any remaining mosquito debris and possible bacterial contaminants, the $L_3$ was washed a minimum of three times in sterile culture media with antibiotics. Three hundred $L_3$ were collected in a single petri dish for each experiment. At room temperature, the dish was propped at a 25° angle to allow the $L_3$ to settle to the corner of the dish. After $L_3$ had settled (~15-20 mins), excess liquid was removed and any visible mosquito debris was discarded while carefully avoiding removing any worms. Once the volume was reduced, the $L_3$ was added to a sterile 1.5-mL centrifuge tube. Sterile, cold Roswell Park Memorial Institute-1640 medium with L-Glutamine (RPMI-1640, Lonza, Allendale, NJ) was added to the tube to a total volume of 1 mL. The tube was centrifuged at 1000 x *g* for 15 mins. Sterile medium with antibiotics was used to wash the worms three times to remove mosquito debris and other potential contaminants. Then, 200 µL containing the majority of the $L_3$ was added to a small petri dish containing room-temperature sterile RPMI-1640 medium and antibiotics. These $L_3$ were then prepared to be added to an assay plate.

**Preparation of culture media**

General culture media were composed of RPMI-1640 (pH 7.2, Lonza, Allendale, NJ), 10% fetal bovine serum (heat-inactivated, Hyclone, Canadian source, Lot#AAC200955), 1% penicillin (10,000 U/mL), 1% streptomycin (10,000 mg/mL), 0.4% gentamicin (10mg/mL) in sterile water (Sigma-Aldrich), and 0.1% ciprofloxacin. Preparations of iron (II) sulfate heptahydrate, zinc sulfate heptahydrate, and copper (II)

sulfate pentahydrate (Sigma-Aldrich, Saint Louis, Missouri, USA) were prepared in RPMI-1640 sterile medium at final concentrations of 20 µM (Table 3.1).

Table 3.1: Heavy metal formulations and their chemical structures.

| Chemical name | Chemical formula | Linear formula |
|---|---|---|
| Iron (II) sulfate heptahydrate | $H_{14}FeO_{11}S$ | $FeSO_4 \cdot 7H_2O$ |
| Zinc sulfate heptahydrate | $H_{14}O_{11}SZn$ | $ZnSO_4 \cdot 7H_2O$ |
| Copper (II) sulfate pentahydrate | $H_{10}CuO_9S$ | $CuSO_4 \cdot 5H_2O$ |

**Assay design**

To facilitate media changes, in vitro assays were performed in triplicate in a 12-well assay Transwell plate system (Corning-Costar, 12-mm diameter inserts, 0.4-µm pore size). Ten $L_3$ in a 200 µL volume were added to each Transwell containing 1.8 mL sterile RPMI-1640 medium. Each experiment had 6-8 replicates ($n$ = 60-80) for each treatment group. These assay plates were incubated at 37°C, 5% $CO_2$. Media was changed three times per week by preparing a new, sterile 12-well plate with warm media and transferring each Transwell to the new plate. Ascorbic acid (75 µM) was added on day 5 as previously described [63].

**Motility and survival scoring - visual recording**

Larval motility was observed daily for up to 9 days under an inverted microscope. These larvae were visually measured using a four-point scoring system as previously

described [64]. A score of 3 was noted by rapid sinusoidal thrashing movements with over 30 movements per 10 seconds (highly active), a 2 was 10-20 slow movements per 30 seconds (active), a 1 was no thrashing movements and occasional twitching at only one end of the body (less active), and 0 was immotile for at least 10 seconds (dead). Lack of motility was considered indicative of larval death.

**Motility tracking software**

In 2012, Marcellino et. al authored a paper on a new software program that can be used in tracking motility of macroscopic parasites [65]. The "WormAssay" script was made open source, which allowed for motility measurement of smaller microscopic parasites, such as *B. malayi* microfilariae and $L_3$ [66]. The source and executable code can be found at https://code.google.com/p/wormassay/. The WormAssay software was originally written for Apple computers and as such requires an Apple Mac® desktop or laptop running Mac OS X 10.9 or later (Apple, Cupertino, CA). The modifications made to the program (referred to as the Worminator) enabled individual wells with multiple organisms to be captured versus the original full-plate assay with only one organism per well. This modification offered support for HDMI input by Apple's Thunderbolt I/O port using Blackmagic software's "Intensity Extreme" video capture. A Canon Vixia HF M52 video camera (Canon, Inc., Tokyo, Japan) was employed for HD video capture for both the macro- and microscopic assays. For this version of the Worminator, an Olympus IX51 inverted microscope (Olympus, Pittsburgh, PA) was used with the Canon Vixia HF M52 attached via a Martin Microscope adapter, model MM99-58 (Easley, SC, http://www.martinmicroscope.com), and a Diagnostic Instruments DBX 1.0X C-mount

adapter (http://www.spotimaging.com). An Olympus PLAN-N 2x objective was used for $L_3$ assays and the Olympus Long Distance phase contrast condenser functioned as the primary light source.

The optimal algorithm (as suggested by Marcellino) for viewing multiple organisms per well was the Luminance Difference algorithm. In setting up a plate to be read, the WormAssay's assay analyzer option was set to "consensus voting luminance difference (dArea)" and the plate orientation was set to "no plate mode". The plate was positioned at the top first well, and each well was recorded for approximately 60 seconds.

**Fixing *B. malayi* larvae to slides and developmental assessment**

Upon the completion of each assay, all 10 larvae per well were fixed to a slide for microscopic analysis. Remaining motile worms were heat-killed in a 65°C water bath for approximately 10 minutes to straighten the larvae for slide preparation. To view the worms on the slide, they were embedded in a thin layer of glycerin jelly (Carolina Biologicals, Burlington, NC). Slides were prepared by tracing an outline using 100% petroleum jelly (Vaseline® Jelly). Small amounts of glycerin jelly were placed in 1.5-mL centrifuge tubes and heated to a liquid state at 95°C for approximately 5 minutes. The worms were individually dispensed in small droplets in the enclosed area of the slide. Once the worms were positioned on the slide, the slide was placed in a fume hood with a blower to evaporate excess solution surrounding the worms. Once evaporated, 500 µL of glycerin jelly liquid was added slowly from the center so that the solution radiated towards the edge. Once coverage was achieved, a coverslip was added quickly at a 45° angle and pressed gently

(but firmly) to seal. The slides were allowed to cure for at least 24 h before being viewed on an inverted scope.

Slides were randomized and investigators were blinded to treatment groups before developmental assessment. Larvae were examined under an Olympus IX51 inverted microscope at 400x magnification. Assessment to determine whether the larvae had molted to the $L_4$ stage prioritized anterior and posterior anatomical features described by Mak et al [23]. If the larval stage could not be determined, it was classified as "unknown". Figure 3.1 depicts these morphological features in cultured parasites. The key characteristics in defining a *B. malayi* $L_3$ is a tapered and rounded cephalic end (Fig 3.1 A), where the posterior features one terminal and two sublateral papillae (Fig 3.1 B). To identify an $L_4$, particularly in an in vitro culture where some features may not be as clearly defined as with in vivo culture, the cephalic end develops into a distinctive globular shape (C), and the terminal papillae are lost, becoming rounded and asymmetrical (D).

**Data and analysis**

For in vitro cultures, longevity and survival were measured to analyze the effects of zinc and iron on the larvae. To analyze the overall differences between the two groups, each larva in each well was visually observed and scored every 2-3 days. In these experiments, the larvae were marked as dead with a score of 0-1, and as healthy and motile with a score of 2-3. Survival analysis was performed using the log-rank Mantel-Cox and the Gehan-Breslow-Wilcoxon test. All analyses were performed in GraphPad Prism 6 (GraphPad Prism® version 6.01 for Windows, GraphPad Software, La Jolla, CA).

**Motility measurements**

Motility output was calculated by the WormAssay software by averaging mmu per individual well. Data output was written to two comma-separated value (CSV) files; one containing the average of the motion detected in the individual well for a minimum of 60 seconds for each well, and the second file contains underlying raw values used in determining the aforementioned averages. Data from the run output file were used to calculate the mean of three technical replicates to produce the motility curves for each culture condition. Motility of the treatment groups compared to the media-only control was calculated as motility % difference.

$$\text{Motility \% difference} = \left(\frac{\text{mean treatment mmu} - \text{mean control mmu}}{\text{mean control mmu}}\right) \times 100$$

Statistical analyses on the average motility units were performed using a two-way repeated-measures (mixed model) ANOVA, using time factor as a within-subjects measure in GraphPad Prism. All statistical tests were interpreted at the 5% level of significance. All data are presented as a mean $\pm$ S.D., with significance represented as $p \leq 0.05$ (*).

**RESULTS**

**Testing initial metal concentrations**

RPMI-1640 composition does not contain heavy metals, only inorganic salts, amino acids, vitamins, glucose, L-glutamine, and sodium bicarbonate. Pilot testing was performed in triplicate, 50 $L_3$ per treatment, in 15 mL of culture medium with heavy metal concentrations of 10, 20, and 30 µM. All experiments were terminated on day 8 when the

majority of the media-only control $L_3$ were non-motile/dead (84-96%). In the copper (II) sulfate pentahydrate treatment wells, 0% of $L_3$ survived to day 3. By day 8 the wells with iron (II) sulfate heptahydrate had an average survival rate of 70% (10µM), 38% (20µM), and 6% (30µM). In the zinc sulfate heptahydrate treatment wells, the average survival rate at day 8 was 20% (10µM), 4% (20µM), and 0% (20µM). The copper treatment group was excluded from additional assays due to the effect on the $L_3$ even at low concentrations. For the iron and zinc treatment groups, 20 µM concentration were selected for further analysis.

**Survival in culture**

Survival curves were constructed for each larva used in this study ($n = 610$). Figure 3.2 depicts the survival curves for the media-only control group and both the iron and zinc treatment groups over 9 days. A Mantel-Cox test (Figure 3.2 A) indicated a significant difference between the media-only controls and both zinc and iron treatment groups ($p < 0.0001$). There were significant differences in survival on day 7 between the control and iron treatment, and zinc and iron treatment groups, and on day 8 between the iron and zinc groups ($p < 0.05$) (Figure 3.2 B). All other comparisons between the treatment groups and controls were found to be non-significant (ns).

**Motility measurements**

A significant difference in motility was observed between control and iron treatment group at day 9 ($p < 0.05$). All other comparisons were not significant (ns) (Figure 3.3).

**Larval stage comparison**

At the conclusion of each experiment, larvae were mounted to assess molting and cuticular defects that may have resulted from treatment. Each larva was examined and recorded for each well for all three experiments. The average number of $L_3$, $L_4$, and "unknown" was calculated. The percentage of each developmental stage is presented in figure 3.4. A significant difference was calculated between the mean number of $L_4$ identified in the zinc treatment group and both the control and the iron treatment groups ($p \leq 0.05$). No other difference was seen between the controls and treatment groups.

## DISCUSSION

The utilization of in vitro assays with *B. malayi* has been ongoing in the continued need for anthelmintic screening. The aim of this study was to evaluate whether the addition of heavy metals would enhance survival and/or development of *B. malayi* $L_3$. In addition to motility and survival, evaluation of the morphology of all larvae was performed to determine whether these heavy metals aided development to the $L_4$ stage.

Cultivation of filarial nematodes has been a challenge in the pursuit of novel efficacious compounds or new combinations of FDA-approved anthelmintics. Particularly with decades of reports of resistance to macrocyclic lactones [71–74], imidazothiazoles/tetrahydropyrimidines [75, 76], and benzimidazoles [71, 77], exploring new methodologies to mimic the in vivo environment has been a long sought-after goal. As mentioned previously, a number of in vitro conditions have been explored, and yet, a consistent and effective culture system remains elusive. Methodologies examined have included various media (RPMI-1640, Franke's NI, endothelial basal medium, Dulbecco's

modified Eagle's medium) [31, 78, 79], 10-100% concentrations of both human and bovine serum [23, 29–31, 80, 81], fatty acids [23, 29], ascorbic acid concentrations [67, 78], and cellular co-cultures (LCC-MK2, Jurkat, fibroblasts, lymphatic endothelial cell, and human dermal fibroblasts) [23, 29, 30, 78].

Though there is a great variety of conditions examined, reproducibility has been a challenge. The inconsistency and variability in results might not be only due to sub-optimal culture conditions but also larval fitness, which can be altered by insufficient nutrient sources, individual handling, and even stress from the collection from the mosquito host [27]. Even though improvements have been made to increase survivability and development of *B. malayi* $L_3$ in the last few years [74], culturing the $L_3$ to sexually reproducing adults has not been reported. This study looked to examine the effect of heavy metals while in a minimal medium environment to determine if there is a positive effect on these larvae.

When copper was added to the culture media, the *B. malayi* larvae did not survive beyond a day or two at all concentrations tested in the pilot assay. Suttle and Jones reported on the anti-parasitical effect of trace elements (copper, selenium, and cobalt) on gastrointestinal nematodes (GINs) of ruminants [78]. When copper is exposed to acids (such as stomach acid), the copper anion forms oxygen radicals that can be harmful to parasites. Bang and colleagues reported on testing the efficacy of copper oxide wire particles (COWP) on lambs infected with *Trichostrongylus colubriformis*, *Ostertagia circumcincta*, or *Haemonchus contortus* [79]. Larval burdens with COWP treatment reduced *H. contortus* by 96% and *O. circumcincta* by 56%, but no effect was observed against *T. colubriformis*. Similar experiments with goats with a reduction in *H. contortus*

burden, but no effect on other GINs tested [80]. These metal oxide ions have also been observed to be effective adjuvants to albendazole as a potential method for overcoming benzimidazole resistance and inadequate macrofilaricidal activity [81]. This copper-albendazole combination was tested on the bovine filarial nematode *Setaria cervi* (family Onchocercidae) and was shown to disrupt parasite redox balance and induce oxidative stress. The effect on *B. malayi* third-stage larvae ($L_3$) did not improve survival or development, however, this observation could provide information on the antifilarial properties of this metal on *B. malayi* $L_3$.

Both zinc sulfate heptahydrate and iron (II) sulfate heptahydrate had an effect on survival rates of *B. malayi* $L_3$. When compared to the media-only control, a significant difference was noted, with the zinc treatment groups on average dying sooner than both the control and the iron treatment group ($p < 0.0001$). Monitoring percent survival (Fig. 3.2 B), on day 7, 55.8% of the iron treatment larvae were still alive, compared to 19.4% of the control, and 9.7% of the zinc treatment group. By day 8, both the control and zinc groups had been reduced by 62.5% and 70% respectively, compared to only a 22.6% reduction in the iron group. In serum, zinc levels can vary by as much as two-fold in lot-to-lot variations, so free zinc ion concentration can be difficult to measure when additional zinc salts are added [35]. The addition of 5-10% w/v of bovine serum containing 10-100 μM of free zinc has been found to be generally sufficient in the amount of zinc ions to be beneficial for cell cultures [35]. However, at high concentrations, zinc can induce apoptosis in cell cultures [82, 83]. Though paradoxically, the addition of zinc and iron chelators, such as TPEN (N,N,N′,N′,-tetrakis (2-pyridylmethyl) ethylenediamine) has also been shown to

induce apoptosis [84–86]. It remains unclear what physiological range would be beneficial for *B. malayi* $L_3$, at least for extending survival rates.

Alternatively, there is potential that the addition of zinc aided in the early induction of the $L_3$ to $L_4$ molt. In nematodes, astacin metalloproteases (NAS) are distinct zinc metalloendopeptidases with a catalytic active-site for binding $Zn^{2+}$ [87]. In *C. elegans*, there are at least 40 NAS enzymes, and a number of them have been found to induce defects in the cuticle and ecdysis when mutated [32, 33, 88]. If zinc ions in culture can catalyze these metalloproteases and trigger the molt in the $L_3$, we would expect to see higher molting rates in the zinc treatment groups. This is precisely what we observe in Figure 3.4, where $L_4$ were identified at significantly higher numbers than either the control or iron treatment group. We hypothesize that ecdysis is triggered by the addition of exogenous zinc and this begins the process of developmental transition for these larvae. However, if their nutrient or other environmental conditions are not properly met, the molt can occur, but the larvae would die promptly afterward.

In the iron group, larval survival was extended compared to the controls and zinc treatment group. Iron is an essential nutrient for a variety of biological processes, including involvement with many metalloproteins, electron transport, oxygen sensing, and transport, etc. [36]. However, the ferrous cation ($Fe^{2+}$) has limited bioavailability, as it readily oxidizes in solution to ferric iron ($Fe^{3+}$), becoming difficult for larvae to absorb. Because these assays are cultured in an aerobic environment, there is the possibility that the large majority of the iron added to each well became oxidized. The media for each well was changed every 2-3 days, so if there was to be a significant effect on the larvae, it would be observed in 2-3 day intervals. However, this effect was not seen in worm motility, which

remained relatively constant throughout the experiment (Figure. 3.3). We did, however, note a significantly reduced percentage of $L_4$ in control and iron treatment groups compared to the zinc treatment group. One interpretation of these data is the addition of iron to the media, while aiding motility, is an insufficient nutrient source for these larvae. These $L_3$ could have been in an arrested development/dauer-like stage, where the worms survive for an extended period without significant development. In the context of these experiments, producing conclusive evidence of arrested development was not a goal, though, further studies into this phenomenon could be useful. If a dauer-like stage can be induced in larval or even adult stages, this may provide another mechanism for halting transmission of these parasites.

In this study, we find that the addition of zinc sulfate heptahydrate and iron (II) sulfate heptahydrate, and copper (II) sulfate pentahydrate effect *B. malayi* larvae when added to in vitro assays. Copper, as seen with GI nematodes, kills the larvae quickly, even at low physiological concentrations. While survival is extended by iron supplementation, development remains stunted. The opposite is true with the addition of zinc, where survival is shortened, but development occurs. In *C. elegans*, zinc has been found to be vital in meiotic progression in oocytes and when these concentrations were reduced using the zinc chelator TPEN, it resulted in abnormalities in chromosome segregation, cell cycle progression, and oocyte reduction compared to controls [89]. The addition of specific chelators to iron and zinc may reverse the effects that were seen in these *B. malayi* cultures, including potentially mitigating the toxic effect of the copper on these larvae.

As with previous cultures studies with these parasites, optimal conditions for increasing both survival and development remain elusive. If these heavy metals could

affect these larvae in vivo without undue toxicity, they may prove to be a novel addition to augment current anthelmintics.

## CONFLICTS OF INTEREST

There were no conflicts of interest in this study.

## ACKNOWLEDGMENTS

We thank the NIAID/NIH Filariasis Research Reagent Resource Center (FR3) for supplying the *B. malayi* $L_3$. We also acknowledge the aid of Bob Storey for instruction and use of the Worminator and subsequent analysis.

## REFERENCES

[1] L. F. Silva, L. C. Alves, F. A. Brayner, C. A. Peixoto, A. Brayner, and C. A. Peixoto, "Immunocytochemical localization of antigens recognized by human antisera in infective larvae of *Wuchereria bancrofti*," *J. Parasitol.*, vol. 89, no. 3, pp. 501–506, 2003.

[2] M. K. Sahoo *et al.*, "Immunization with inflammatory proteome of *Brugia malayi* adult worm induces a Th1/Th2-immune response and confers protection against the filarial infection," *Vaccine*, vol. 27, pp. 4263–4271, 2009.

[3] M. D. Taylor, N. van der Werf, and R. M. Maizels, "T cells in helminth infection: The regulators and the regulated," *Trends Immunol.*, vol. 33, no. 4, pp. 181–189, 2012.

[4] N. M. Girgis, U. M. Gundra, and P. Loke, “Immune regulation during helminth infections,” *PLoS Pathog.*, vol. 9, no. 4, pp. 11–13, 2013.

[5] M. Crook, “The dauer hypothesis and the evolution of parasitism: 20 years on and still going strong,” *Int. J. Parasitol.*, vol. 44, pp. 1–8, 2014.

[6] J. R. Palmieri, D. H. Connor, Purnomo, and H. A. Marwoto, “*Wuchereria bancrofti* infection in the silvered leaf monkey (Presbytis cristatus),” *Am. J. Pathol.*, vol. 112, no. 3, pp. 107–108, 1983.

[7] S. Sucharit, C. Harinasuta, and W. Choochote, “Experimental transmission of subperiodic *Wuchereria bancrofti* to the leaf monkey (*Presbytis melalophos*), and its periodicity,” *Am. J. Trop. Med. Hyg.*, vol. 31, no. 3 I, pp. 599–601, 1982.

[8] J. H. Cross, F. Partono, M. Y. Hsu, L. R. Ash, and S. Oemijati, “Experimental transmission of *Wuchereria bancrofti* to monkeys,” *Am. J. Trop. Med. Hyg.*, vol. 28, no. 1, pp. 56–66, 1979.

[9] K. P. Paily, K. A. Mary, S. L. Hoti, and K. Balaraman, “Enhanced recovery of fourth stage larvae of *Wuchereria bancrofti* from mongolian gerbil, *Meriones unguiculatus* & their in vitro maintenance,” *Indian J. Med. Res.*, vol. 109, pp. 28–32, 1999.

[10] P. Jordan and K. D. Goatly, “Bancroftian filariasis in Tanganyika: A quantitative study of the uptake, fate and development of microfilariae of *Wuchereria bancrofti* in *Culex fatigans*,” *Ann. Trop. Med. Parasitol.*, vol. 56, no. 2, pp. 173–187, 1962.

[11] A. S. Dissanaike and J. W. Mak, “A description of adult *Wuchereria bancrofti* (rural strain) from an experimental infection in the long-tailed macaque, *Macaca fascicularis*,” *J. Helminthol.*, vol. 54, no. 2, pp. 117–122, 1980.

[12] S. Misra, K. Tyagi, and R. K. Chatterjee, “Research brief: Experimental transmission of nocturnally periodic *Wuchereria bancrofti* to Indian leaf monkey (*Presbytis entellus*),” *Exp. Parasitol.*, vol. 86, no. 2, pp. 155–157, 1997.

[13] J. F. B. Edeson and R. H. Wharton, “Transmission of *Wuchereria malayi* from man to the domestic cat,” *Trans. R. Soc. Trop. Med. Hyg.*, vol. 51, no. 4, pp. 366–370, 1957.

[14] A. Ewert, “Distribution of developing and mature *Brugia malayi* in cats at various times after a single inoculation,” *J. Parasitol.*, vol. 57, no. 5, pp. 1039–1042, 1971.

[15] R. B. Crandall, P. B. McGreevy, D. H. Connor, C. A. Crandall, J. T. Neilson, and J. W. McCall, “The ferret (*Mustela putorius furo*) as an experimental host for *Brugia malayi* and *Brugia pahangi*,” *Am. J. Trop. Med. Hyg.*, vol. 31, no. 4, pp. 752–9, Jul. 1982.

[16] K. Tyagi, P. K. Murthy, and R. K. Chatterjee, “*Brugia malayi* in *Mastomys coucha*: establishment in immunosuppressed animals,” *Acta Trop.*, vol. 70, no. 2, pp. 157–162, 1998.

[17] L. R. Ash, “Chronic *Brugia pahangi* and *Brugia malayi* infections in *Meriones unguiculatus*,” *J. Parasitol.*, vol. 59, no. 3, pp. 442–7, Jun. 1973.

[18] D. I. Grove, R. S. Davis, and K. S. Warren, "*Brugia malayi* microfilaraemia in mice: a model for the study of the host response to microfilariae," *Parasitology*, vol. 79, no. 3, pp. 303–316, 1979.

[19] A. Ewert and S. El-Bihari, "Rapid recovery of *Brugia malayi* larvae following experimental infection of cats," *Trans. R. Soc. Trop. Med. Hyg.*, vol. 65, no. 3, pp. 364–368, 1971.

[20] L. R. Ash and J. M. Riley, "Development of subperiodic *Brugia malayi* in the jird, *Meriones unguiculatus*, with notes on infections in other rodents," *J. Parasitol.*, vol. 56, no. 5, pp. 969–973, 1970.

[21] Y. Mutafchiev, O. Bain, Z. Williams, J. W. McCall, and M. L. Michalski, "Intraperitoneal development of the filarial nematode *Brugia malayi* in the Mongolian jird (*Meriones unguiculatus*)," *Parasitol. Res.*, vol. 113, no. 5, pp. 1827–1835, 2014.

[22] D. H. Molyneux, M. Bradley, A. Hoerauf, D. Kyelem, and M. J. Taylor, "Mass drug treatment for lymphatic filariasis and onchocerciasis," *Trends Parasitol.*, vol. 19, no. 11, pp. 516–522, 2003.

[23] J. W. Mak, P. K. C. Lim, B. K. L. Sim, and L. M. Liew, "*Brugia malayi* and *Brugia pahangi* : Cultivation in vitro of infective larvae to the fourth and fifth Stages," *Exp. Parasitol.*, vol. 55, pp. 243–248, 1983.

[24] D. Abraham, M. Mok, M. Mika-grieve, and R. B. Grieve, "In vitro Culture of *Dirofilaria immitis* Third- and Fourth-Stage Larvae under Defined Conditions," *J. Parasitol.*, vol. 73, no. 2, pp. 377–383, 1987.

[25] M. M. Wong, R. Knighton, J. Fidel, and M. Wada, “In vitro cultures of infective-stage larvae of *Dirofilaria immitis* and *Brugia pahangi*,” *Ann. Trop. Med. Parasitol.*, vol. 76, no. 2, pp. 239–241, 1982.

[26] A. U. R. Rao, C. S. Zometa, A. C. Vickery, B. H. Kwa, J. K. Nayar, and E. T. Sutton, “Effect of *Brugia malayi* on the growth and proliferation of endothelial cells in vitro,” *J. Parasitol.*, vol. 82, no. 4, pp. 550–556, 1996.

[27] C. T. Lok JB, Mika-Grieve M, Grieve RB, “In vitro development of third- and fourth-stage larvae of *Dirofilaria immitis*: comparison of basal culture media, serum levels and possible serum substitutes,” *Acta Trop.*, vol. 2, no. 41, pp. 145–54, 1984.

[28] K. Yasuraoka and H. Hata, “In vitro cultivation of parasitic helminths,” *Prog. Med. Parasitol. Japan*, vol. 7, pp. 211–226, 2003.

[29] H. L. Smith, N. Paciorkowski, S. Babu, and T. V Rajan, “Development of a serum-free system for the in vitro cultivation of *Brugia malayi* infective-stage larvae,” *Exp. Parasitol.*, vol. 95, no. 4, pp. 253–64, Aug. 2000.

[30] F. H. Falcone, H. Zahner, M. Schlaak, and H. Haas, “In vitro cultivation of third-stage larvae of *Brugia malayi* to the young adult stage,” *Trop. Med. Parasitol.*, vol. 46, pp. 230–234, 1995.

[31] W. A. Riberu, S. Atmosoedjono, Purnomo, S. Tirtokusumo, M. J. Bangs, and J. K. Baird, “Cultivation of sexually mature *Brugia malayi* in vitro,” *Am. J. Trop. Med. Hyg.*, vol. 43, no. 1, p. 3–5 (90–108), 1990.

[32] M. W. Davis, A. J. Birnie, A. C. Chan, A. P. Page, and E. M. Jorgensen, “A conserved metalloprotease mediates ecdysis in *Caenorhabditis elegans*,” *Development*, vol. 131, no. 23, pp. 6001–8, 2004.

[33] G. Stepek, G. Mccormack, A. J. Birnie, and A. P. Page, “The astacin metalloprotease moulting enzyme NAS-36 is required for normal cuticle ecdysis in free-living and parasitic nematodes,” *Parasitology*, vol. 138, pp. 237–248, 2010.

[34] A. P. Page, G. Stepek, A. D. Winter, and D. Pertab, “Enzymology of the nematode cuticle: a potential drug target?,” *Int. J. Parasitol. Drugs Drug Resist.*, vol. 4, no. July, pp. 133–141, 2014.

[35] R. A. Bozym *et al.*, “Free zinc ions outside a narrow concentration range are toxic to a variety of cells in vitro,” *Exp. Biol. Med.*, vol. 235, no. 6, pp. 741–750, 2010.

[36] P. Aisen, C. Enns, and M. Wessling-resnick, “Chemistry and biology of eukaryotic iron metabolism,” *Int. J. Biochem. Cell Biol.*, vol. 33, pp. 940–959, 2001.

[37] G. Papanikolaou and K. Pantopoulos, “Iron metabolism and toxicity,” *Toxicol. Appl. Pharmacol.*, vol. 202, no. 2, pp. 199–211, 2005.

[38] L. Koreny, J. Lukes, and M. Obornik, “Evolution of the haem synthetic pathway in kinetoplastid flagellates: An essential pathway that is not essential after all?,” *Int. J. Parasitol.*, vol. 40, pp. 149–156, 2010.

[39] A. U. Rao, L. K. Carta, E. Lesuisse, and I. Hamza, “Lack of heme synthesis in a free-living eukaryote,” *Proc. Natl. Acad. Sci.*, vol. 102, no. 12, pp. 4270–4275, 2005.

[40] S. Kumar *et al.*, “Mining predicted essential genes of *Brugia malayi* for nematode drug targets,” *PLoS One*, no. 11, pp. 1–9, 2007.

[41] A. N. Luck, X. Yuan, D. Voronin, B. E. Slatko, I. Hamza, and J. M. Foster, “Heme acquisition in the parasitic filarial nematode *Brugia malayi*,” *FASEB J.*, vol. 30, no. 10, pp. 3501–3514, 2016.

[42] B. Wu *et al.*, “The heme biosynthetic pathway of the obligate *Wolbachia* endosymbiont of *Brugia malayi* as a potential anti-filarial drug target,” *PLoS Negl. Trop. Dis.*, vol. 3, no. 7, pp. 1–10, 2009.

[43] C. Ballesteros, “Iron acquisition in the adult filarial nematode *Brugia malayi*,” McGill University, 2016.

[44] B. E. Slatko, A. N. Luck, S. L. Dobson, and J. M. Foster, “Molecular & biochemical parasitology *Wolbachia* endosymbionts and human disease control,” *Mol. Biochem. Parasitol.*, vol. 195, no. 2, pp. 88–95, 2014.

[45] F. Landmann, J. M. Foster, M. L. Michalski, B. E. Slatko, and W. Sullivan, “Co-evolution between an endosymbiont and its nematode host: *Wolbachia* asymmetric posterior localization and AP polarity establishment,” *PLoS Negl. Trop. Dis.*, vol. 8, no. 8, 2014.

[46] J. Foster *et al.*, “*Wolbachia* genome of *Brugia malayi*: Endosymbiont evolution within a human pathogenic nematode,” *PLOS Biol.*, vol. 3, no. 4, pp. 599–614, 2005.

[47] E. Ghedin *et al.*, “Draft genome of the filarial nematode parasite *Brugia malayi*,” *Science (80-. ).*, vol. 317, no. 5845, pp. 1756–1760, 2007.

[48] A. C. Gill, A. C. Darby, and B. L. Makepeace, “Iron necessity: The secret of *Wolbachia’s* success?,” *PLoS Negl. Trop. Dis.*, vol. 8, no. 10, pp. 1–8, 2014.

[49] R. M. Maizels, M. L. Blaxter, and A. L. Scott, “Immunological genomics of *Brugia malayi*: filarial genes implicated in immune evasion and protective immunity,” *Parasite Immunol.*, no. April, 2001.

[50] R. M. Maizels and H. J. Mcsorley, “Regulation of the host immune system by helminth parasites,” *J. Allergy Clin. Immunol.*, vol. 138, no. 3, pp. 666–675, 2016.

[51] C. T. Lattemann, A. Matzen, and H. Apfel, “Up-regulation of extracellular copper/zinc superoxide dismutase mRNA after transmission of the filarial parasite *Acanthocheilonema viteae* in the vertebrate host *Meriones unguiculatus*,” *Int. J. Parasitol.*, vol. 29, no. 9, pp. 1437–1446, 1999.

[52] A. W. Lee, J. Hwang, B. Na, J. Cho, and H. Lee, “Functional Expression of a Recombinant Copper/Zinc Superoxide Dismutase of Filarial Nematode, *Brugia malayi*,” *J. Parasitol.*, vol. 91, no. 1, pp. 205–208, 2005.

[53] S. Dabir, P. Dabir, K. Goswamy, and M. V. R. Reddy, “Prophylactic evaluation of recombinant extracellular superoxide dismutase of *Brugia malayi* in jird model,” *Vaccine*, vol. 26, pp. 3705–3710, 2008.

[54] J. V Bannister, W. H. Bannister, and G. Rottilio, “Aspects of the structure, function and application of superoxide dismutases,” *Crit. Rev. Biochem.*, vol. 22, pp. 111–180, 1987.

[55] I. Fridovich, “Superoxide dismutases,” *Adv. Enzymol.*, vol. 58, pp. 62–97, 1986.

[56] R. Cook, C. Carvalho-Queiroz, G. Wilding, and P. LoVerde, “Nucleic acid vaccination with *Schistosoma mansoni* antioxidant enzyme cytosolic superoxide dismutase and the structural protein filamin confers protection against the adult worm stage,” *Infect. Immun.*, vol. 72, no. October, pp. 6112–24, 2004.

[57] C. Carvalho-Queiroz, R. Cook, C. C. Wang, R. Correa-Oliveira, N. Bailey, and N. Egilmez, “Cross-reactivity of *Schistosoma mansoni* cytosolic superoxide dismutase, a protective vaccine candidate, with host superoxide dismutase and identification of parasite-specific B epitopes,” *Infect. Immun.*, vol. 72, no. May, pp. 2635–47, 2004.

[58] P. T. Loverde, C. Q. Claudia, and R. Cook, “Vaccination with antioxidant enzymes confers protective immunity against challenge infection with *Schistosoma mansoni*,” *Mem. Inst. Oswaldo Cruz*, vol. 99, pp. 1–10, 2004.

[59] P. S. Riddle, D.L., Albert, “Genetic and environmental regulation of dauer larva development,” in *C. elegans II.*, 2nd ed., J. R. Riddle, D.L., Blumenthal, T., Meyer, B.J., Priess, Ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1997, pp. 739–768.

[60] D. L. Motola *et al.*, “Identification of ligands for DAF-12 that govern dauer formation and reproduction in *C. elegans*,” *Cell*, vol. 9, pp. 1209–1223, 2006.

[61] N. Gomez-escobar, W. F. Gregory, and R. M. Maizels, “Identification of tgh-2, a filarial nematode homolog of *Caenorhabditis elegans* daf-7 and human transforming growth factor beta, expressed in microfilarial and adult stages of *Brugia malayi*,” *Infect. Immun.*, vol. 68, no. 11, pp. 6402–6410, 2000.

[62] J. W. McCall, “The Role of Arthropods in the Development of Animal Models for Filariasis Research,” *J. Georg. Entomol. Soc.*, vol. 16, pp. 283–293, 1981.

[63] T. V Rajan, N. Paciorkowski, I. Kalajzic, and C. McGuiness, “Ascorbic acid is a requirement for the morphogenesis of the human filarial parasite *Brugia malayi*,” *J. Parasitol.*, vol. 89, no. 4, pp. 868–70, Aug. 2003.

[64] W. Khunkitti, Y. Fujimaki, and Y. Aoki, “In vitro antifilarial activity of extracts of the medicinal plant *Cardiospermum halicacabum* against *Brugia pahangi*,” *J. Helminthol.*, vol. 74, pp. 241–246, 2000.

[65] C. Marcellino, J. Gut, K. C. Lim, R. Singh, J. McKerrow, and J. Sakanari, “WormAssay: a novel computer application for whole-plate motion-based screening of macroscopic parasites.,” *PLoS Negl. Trop. Dis.*, vol. 6, no. 1, p. 1–8 (e1494), Jan. 2012.

[66] B. Storey *et al.*, “Utilization of computer processed high definition video imaging for measuring motility of microscopic nematode stages on a quantitative scale: ‘The Worminator,’” *Int. J. Parasitol. Drugs Drug Resist.*, vol. 4, no. 3, pp. 233–243, 2014.

[67] E. Redman *et al.*, “The emergence of resistance to the benzimidazole anthelmintics in parasitic nematodes of livestock is characterized by multiple independent hard and soft selective sweeps,” *PLoS Negl. Trop. Dis.*, vol. 9, p. e0003494, 2015.

[68] J. S. Gilleard, “Understanding anthelmintic resistance: The need for genomics and genetics,” *Int. J. Parasitol.*, vol. 36, no. 12, pp. 1227–1239, 2006.

[69] C. Bourguinat, K. Keller, A. Bhan, A. Peregrine, T. Geary, and R. Prichard, “Macrocyclic lactone resistance in *Dirofilaria immitis*,” *Vet. Parasitol.*, vol. 181, no. 2–4, pp. 388–92, Sep. 2011.

[70] C. Bourguinat *et al.*, “Macrocyclic lactone resistance in *Dirofilaria immitis*: Failure of heartworm preventives and investigation of genetic markers for resistance,” *Vet. Parasitol.*, vol. 210, no. 3–4, pp. 167–178, 2015.

[71] J. S. Gilleard, “*Haemonchus contortus* as a paradigm and model to study anthelmintic drug resistance.,” *Parasitology*, vol. 140, no. 12, pp. 1506–22, Oct. 2013.

[72] R. M. Kaplan, “Drug resistance in nematodes of veterinary importance: a status report,” *Trends Parasitol.*, vol. 20, no. 10, pp. 477–481, 2004.

[73] R. J. Dobson, D. A. Griffiths, A. D. Donald, and P. J. Waller, “A genetic model describing the evolution of levamisole resistance in *Trichostrongylus colubriformis*, a nematode parasite of sheep,” *IMA J. Math. Appl. Med. Biol.*, vol. 4, pp. 279–293, 1987.

[74] I. M. Lu *et al.*, "Optimization of culture and analysis methods for enhancing long-term *Brugia malayi* survival, molting and motility in vitro," *Parasitol. Open*, vol. 4, no. e3, pp. 1–11, 2017.

[75] N. Wisnewski and P. P. Weinstein, "Growth and development of *Brugia pahangi* larvae under various in vitro conditions," *J. Parasitol.*, vol. 79, no. 3, pp. 390–398, 1993.

[76] E. D. Franke, W. Riberu, and I. Wiady, "In vitro cultivation of third stage larvae of *Wuchereria bancrofti* to the fourth stage," *Am. J. Trop. Med. Hyg.*, vol. 37, no. 2, pp. 370–375, 1987.

[77] C. L. Smillie, A. C. Vickery, B. H. Kwa, J. K. Nayar, and U. R. Rao, "Evaluation of different medium supplements for in vitro cultivation of *Brugia malayi* third-stage larvae," *J. Parasitol.*, vol. 80, no. 3, pp. 380–383, 1994.

[78] N. F. Suttle and D. G. Jones, "Recent developments in trace element metabolism and function: trace elements, disease resistance and immune responsiveness in ruminants," *J. Nutr.*, vol. 119, no. 7, pp. 1055–1061, 1989.

[79] K. S. Bang, A. S. Familton, and A. R. Sykes, "Effect of copper oxide wire particle treatment on establishment of major gastrointestinal nematodes in lambs," *Res. Vet. Sci.*, vol. 49, no. 2, pp. 132–137, 1990.

[80] C. Chartier, E. Etter, H. Hoste, I. Pors, C. Koch, and B. Dellac, "Efficacy of copper oxide needles for the control of nematode parasites in dairy goats," *Vet. Res. Commun.*, vol. 24, pp. 389–399, 2000.

[81] A. Zafar, I. Ahmad, A. Ahmad, and M. Ahmad, “Copper (II) oxide nanoparticles augment antifilarial activity of albendazole: In vitro synergistic apoptotic impact against filarial parasite *Setaria cervi*,” *Int. J. Pharm.*, vol. 501, no. 1–2, pp. 49–64, 2016.

[82] D. Jiang, P. Sullivan, S. Sensi, O. Steward, and J. Weiss, “Zn2+ induces permeability transition pore opening and release of pro-apoptotic peptides from neuronal mitochondria,” *J. Biol. Chem.*, vol. 276, pp. 47524–9, 2001.

[83] W. Telford and P. J. Fraker, “Preferential induction of apoptosis in mouse CD4+CD8+ alpha betaTCR10CD3e thymocytes by zinc,” *J. Cell Physiol.*, vol. 164, pp. 259–270, 1995.

[84] F. Chimienti, M. Seve, S. Richard, J. Mathieu, and A. Favier, “Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors,” *Biochem. Pharmacol.*, vol. 62, pp. 51–62, 2001.

[85] P. Zalewski, I. Forbes, and C. Giannakis, “Physiological role for zinc in prevention of apoptosis (gene-directed death),” *Biochem. Int.*, vol. 1991, pp. 1093–101, 1991.

[86] P. Zalewski, I. Forbes, and W. Betts, “Correlation of apoptosis with change in intracellular labile Zn(II) using Zinquin [(2-methyl-8-p-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II),” *Biochem. J.*, vol. 296, pp. 403–8, 1993.

[87] W. Stocker, W. Bode, and R. Zwilling, “Implications of the three-dimensional structure of astacin for the structure and function of the astacin family of zinc-endopeptidases,” *Eur. J. Biochem.*, vol. 214, pp. 215–231, 1993.

[88] G. Stepek, G. Mccormack, A. D. Winter, and A. P. Page, “A highly conserved, inhibitable astacin metalloprotease from *Teladorsagia circumcincta* is required for cuticle formation and nematode development,” *Int. J. Parasitol.*, vol. 45, no. 5, pp. 345–355, 2015.

[89] A. D. Mendoza, T. K. Woodruff, S. M. Wignall, and T. V. O’Halloran, “Zinc availability during germline development impacts embryo viability in *Caenorhabditis elegans*,” *Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol.*, vol. 191, pp. 194–202, 2017.

**FIGURES**

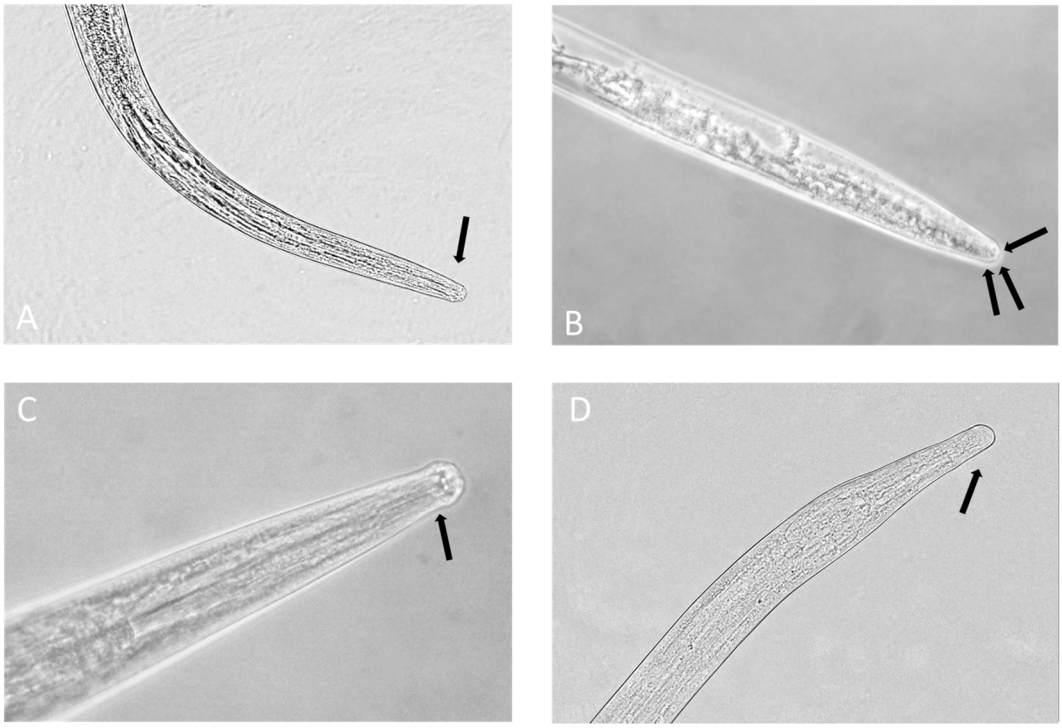



Figure 3.1. Morphological characteristics of *B. malayi* larvae at 400x magnification. Arrows indicate key features described. (A) Shows the tapered cephalic end of an $L_3$. (B) Shows the posterior end of the worm, with characteristic terminal and two sublateral papillae (C) On the $L_4$, the cephalic end is a characteristic globular shape with two indentations on each side. (D) When the $L_3$ have molted to $L_4$, the terminal papillae are lost, and the posterior end becomes rounded and asymmetrical.

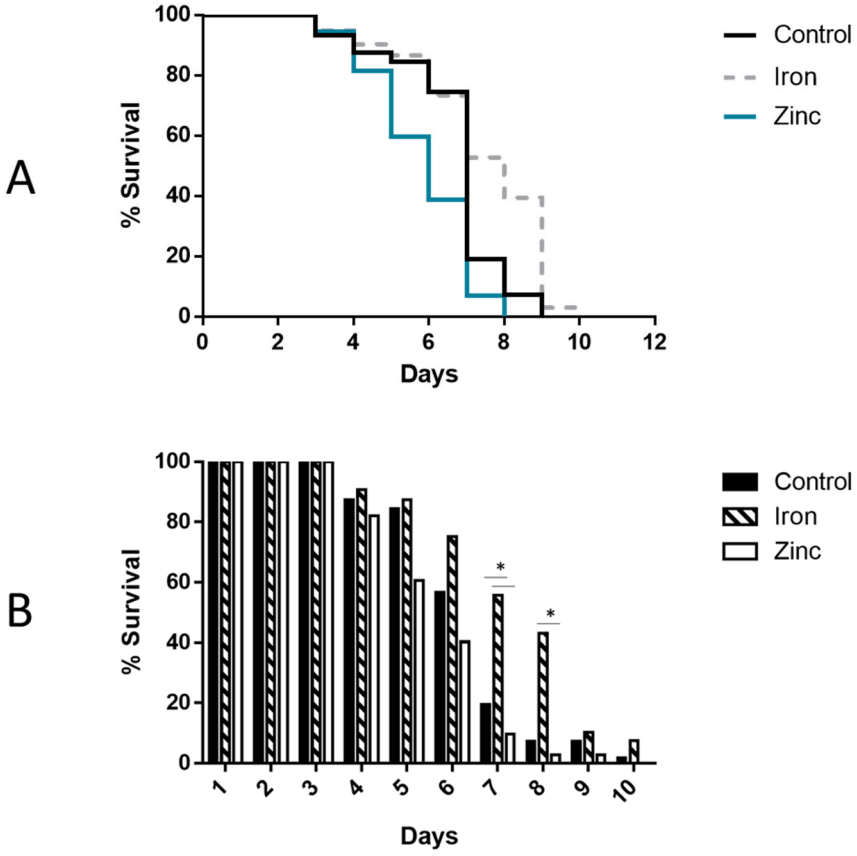



Figure 3.2. *Brugia malayi* larval survival when exposed to iron and zinc in culture medium. (A) Survival curves as determined by the Mantel-Cox log-rank for trends. (B) Displays the average percent survival rate for each treatment group over the course of the study. Significant difference marked as * is $p \leq 0.05$.

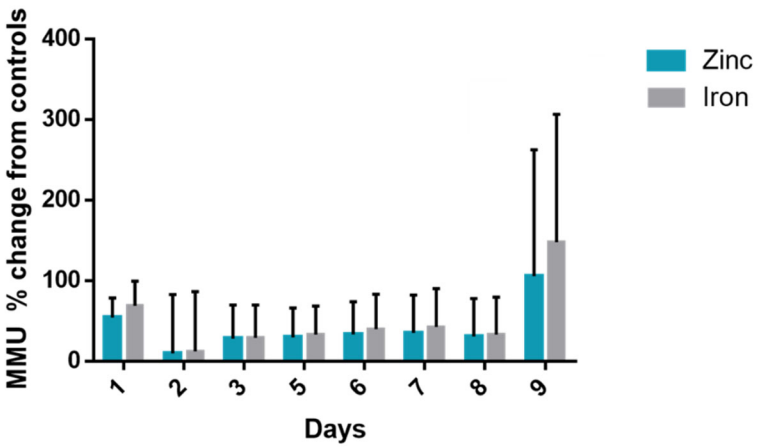



Figure 3.3. Motility % change from controls of mean motility units (mmu) for *B. malayi* larvae when cultured with iron and zinc additives. The values for the controls were set at zero.

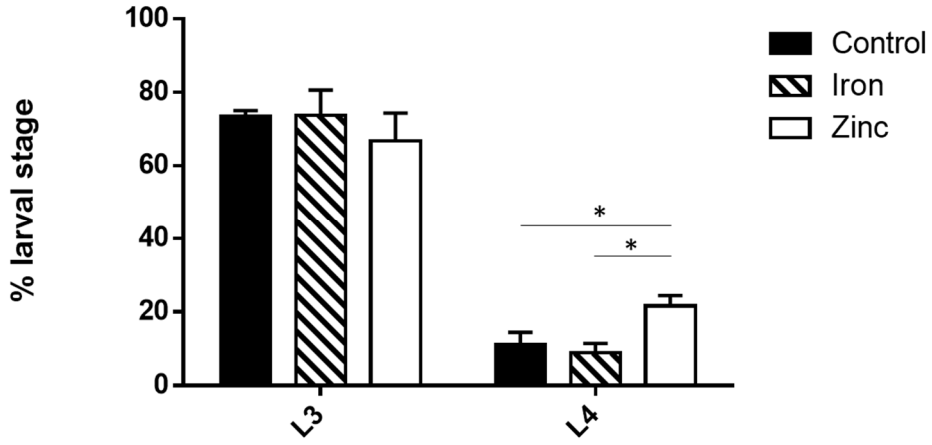



Figure 3.4. Average percentage of *Brugia malayi* larvae identified. Larval stages were identified by morphological features as described by Mak *et al.* [23]. If the larvae were unidentifiable, they were noted as "unknown". All data were calculated as mean ± S.D., significant difference marked as * is $p \leq 0.05$.

# CHAPTER 4

# *BRUGIA MALAYI* miRNAS AND POTENTIAL TARGETS WITHIN THE FELINE HOST (*FELIS CATUS*)

Erica Burkman[1], Lucienne Tritten[2], Chris Evans[1], Timothy Geary[3], Andrew R. Moorhead[1,*]

[1]University of Georgia, College of Veterinary Medicine, Department of Infectious Diseases; [2]University of Zurich, Institute of Parasitology; [3]McGill University, Institute of Parasitology

*Corresponding author: Andrew R. Moorhead, Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, 501 D.W. Brooks Drive, Athens, Georgia, 30602, USA, email: amoorhed@uga.edu.

Burkman, Erica. To be submitted to PLOS NTDs

**ABSTRACT**

Host specificity is a fundamental concept in the survival and growth of parasites within a host. In host-parasite relationships, many mechanisms have been documented by which parasites, notably helminths, modulate the immune system of their host to promote their survival. Due to the evolutionary history of these relationships, hosts often become disease-tolerant or asymptomatic, even with high worm burdens, until there is a dysregulation in the host immune system that can induce a disease state. The molecular mechanisms of how parasites modify the host immune system and the host environment as a whole are not fully understood. What is generally accepted is that parasitic nematodes release a variety of excretory/secretory (E/S) products implicated to enable these worms to safely penetrate and migrate through host tissues while eliciting little to no host immune response. These E/S secretions are composed of proteins, microvesicles, and exosomes containing small RNAs such as microRNAs (miRNA). MicroRNAs have been extensively studied and observed to induce a number of disease states in mammalian hosts along with modulation towards a tolerant environment for parasite proliferation. To investigate this relationship, we analyzed plasma of four *Brugia malayi*-infected cats with varying microfilaremias six months post-infection. From the ~32 million sequencing reads, 185 mature miRNA sequences of potential *B. malayi* origin were detected in feline plasma, with 26 miRNAs present in 10 copies or more. By in silico methods, 9 genes with potential involvement in host immunomodulation (*Celf2, SIK2*, *Ptgs1, Irf4, Irf5, Numbl, Tnfsf15, Stat3,* and *Txlnb*) were identified as high-confidence predicted targets of parasite-derived miRNAs on the feline genome. These miRNAs could be manipulating the host immune systems which would implicate a potential molecular mechanism in host immune system down-regulation.

## INTRODUCTION

In host-parasite relationships, a variety of mechanisms are employed by parasitic helminths to modulate the host immune system to promote their own survival [1]. *Wuchereria bancrofti* and *Brugia malayi* are the causative agents of lymphatic filariasis (LF) and have been documented for thousands of years in human populations [3, 4]. These helminths are able to polarize inflammatory processes within the host, shifting towards a Th2-type cellular response early during infection [5–9]. The Th2 response is characterized by elevated IgE, IgG, eosinophilia, and alternatively activated macrophages [4,9]. In the human host, Th2 associated cytokines (IL-4, IL-5, IL-9, and IL-13) are induced first by innate lymphocytes (ILC2) and later by effector CD4+ T cells [10]. As the infection persists and becomes chronic, a general shift towards tolerance occurs, resulting in reduced T cell proliferation, modification of regulatory B cells [11], regulatory T cells [12], and shifting towards an alternatively activated macrophage (M2) pathway [13]. As the parasites age and die, polarization can shift towards a pro-inflammatory Th1 and Th17 responses [10], while granulomas forming around the dead parasites may block lymphatics, potentially resulting in lymphedema, hydrocele, and elephantiasis [14].

Both innate and adaptive components of the host immune system are activated during infection with filarial parasites. The innate immune system in the vertebrate host provides the first line of defense against invading microorganisms. In a filarial infection, the first contact is with effector cells including neutrophils, eosinophils, macrophages, mononuclear phagocytes, natural killer (NK) cells, mast cells, and platelets. These cells secrete specific cytokines, chemokines, and growth factors that can enhance and recruit additional innate cells while also activating antigen presenting cells to stimulate adaptive

immunity. Some of these effector cells can also secrete cytotoxic molecules, such as reactive oxidative species and nitric oxide. These molecules have been implicated in killing *B. malayi* in both multimammate mouse (*Mastomys coucha* and *M. natalensis*) and Mongolian gerbil models (*Meriones unguiculatus*) [23–25]. Additionally, tissue macrophages, monocytes, and granulocytes can act directly against the helminths before cytokine or antibody enhancement, or when opsonized with antibodies and complement C3b [18]. The humoral components include complement, pentraxins, collectins, ficolins, and cytokines [19]. These cellular and humoral components work in concert by recognition of pathogen-associated molecular patterns (PAMPs) on the parasite. The innate immune cells can detect PAMPs by Pattern Recognition Receptors (PRRs) on their cell surface, some within the cell in endosomal vesicles, or cytoplasm if the pathogen is able to penetrate the host cell [19]. Once these cells become activated they will begin to mount a response by signaling to nearby phagocytic cells, such as by cytokine production, and begin activation of the adaptive immune system.

Natural killer cells function in innate immunity by not only targeting infected, malignant, or damaged host cells, but also activation of macrophages by the production of TNF-α, a pro-inflammatory cytokine [20]. These activated macrophages can either aid in phagocytosis of the pathogen or can interact with effector T cells for cell-mediated immunity. Once activated, cytotoxic T-cells (CD8+), NK cells, and activated macrophages attack the invading pathogen. B cells are activated by exposure to the pathogen antigen. These cells can perform a variety of functions, such as stimulation of T-helper cells (CD4+), and also by clonal expansion of memory B cells [21]. The formation of the

memory B cells is vital to the adaptive immune system, for this is how the host is able to mount a rapid secondary response if subsequently exposed to the same pathogen.

To survive in the harsh milieu of the host, these parasitic nematodes release a variety of excretory/secretory products downregulating the localized immune response, including extracellular vesicles, membrane-bound vesicles composed of microvesicles and exosomes [22]. Exosomes have been identified as important mediators of cell-to-cell communication [23]. These vesicles have also been found to be secreted throughout the parasite life cycle [24] and are able to enter host cells by the endocytic pathway or by membrane fusion. Once these vesicles enter the cytoplasm, the cargo, such as microRNAs (miRNAs), is released.

MicroRNAs are small, non-coding RNA molecules that regulate gene expression post-transcriptionally by binding to target messenger RNAs (mRNAs) within cells [25]. First discovered in the free-living nematode *C. elegans*, these miRNAs are ubiquitous among eukaryotes, found in animals, plants, and parasitic helminths [21–23]. These small RNAs function by inhibition of protein-coding genes, resulting in transcript inhibition, destabilization, or both, and subsequent degradation by either deadenylation or argonaute-2-catalyzed endonucleolytic cleavage [24, 25]. Biogenesis of these small RNAs begins in the cell nucleus as a stem-loop pri-miRNA containing a 5’ cap and a 3’ poly-A tail (Figure 4.1). The pri-miRNAs are processed by the ribonuclease III enzyme, Drosha, that trims the 5’ and 3’ ends of the stem-loop structure to form the pre-miRNA. The protein exportin transports the pre-miRNA into the cytoplasm where dicer, an RNase III enzyme, cleaves the stem-loop to form a miRNA duplex. This duplex is unwound via a helicase into the 20- to 24-nucleotide mature miRNA. These miRNAs become incorporated into an RNA-

induced silencing complex (RISC), that binds predominately onto the 3' untranslated region (UTR) of the target mRNA resulting in inhibition or destabilization [31]. The 5' end of these miRNAs bind at a highly conserved, 2- to 8-nucleotide seed region, though perfect complementation is not a requirement for binding and mRNA disruption [32].

Parasite-secreted miRNAs have been implicated in parasite establishment by down-regulation of host gene expression [1, 23]. Studies of the protozoa *Toxoplasma gondii*, *Cryptosporidium parvum*, *Plasmodium* spp., and the helminth *Heligmosomoides polygyrus* have shown that they are able to modulate the host immune environment via miRNAs [1, 29, 30]. Research with filarial nematodes, such as *Dirofilaria immitis, Loa loa*, and *Onchocerca* spp., has revealed that parasite miRNAs can be found and detected in host blood [31, 32]. In this study, our aim was to detect and identify parasite-derived miRNAs of the LF parasite *Brugia malayi* once it reached patency within the feline host. Once identified, these miRNAs were characterized via in silico analysis to determine what genes were targeted for suppression or inhibition on the feline genome. Because these miRNAs have been discovered to down-regulate components of the host immune system in other parasitic nematodes, we hypothesized that we would identify host immunity gene transcripts that could be targeted by *B. malayi* miRNAs. We identified 9 gene targets that correspond with immune genes, suggesting that miRNAs secreted by *B. malayi* effect the immune response of the host.

## METHODS

### Animal ethics approval

The study protocol was approved by the University of Georgia's Institutional Animal Care and Use Committee under the protocol numbers: A2010 12-005-Y3-A16 and A2013 11-009-Y3-A18. The animal care and use protocols adhered to the "Guide for the Care and Use of Laboratory Animals (the Guide)" published by the National Research Council of the National Academies, USA.

### Blood collection

Ten short-haired domestic cats (*Felis catus*) were used in this study. These cats were maintained at the University of Georgia (Athens, GA, USA). Each cat was inoculated subcutaneously with 400 *B. malayi* $L_3$ (200  per side) in the hind limb proximal to the inguinal lymph node. Between 10 and 20 ml of blood were collected from the internal jugular vein for all ten cats beginning with pre-infection and subsequently every 30 days for 180 days. The blood was collected in EDTA (K2 EDTA, BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ) and kept on ice or refrigerated (4°C). After 30 min, the blood tubes were centrifuged for 15 min at 1,200 x *g*. The plasma obtained from each feline sample was placed in sterile DNase/RNase-free 2-ml centrifuge tubes (Fisher Scientific, Hampton, NH, USA) and frozen at -20°C for miRNA analysis.

**Microfilarial counts of experimentally infected cats**

Out of 10 cats, 4 were selected that had the highest microfilarial (mf) counts. At 6 months post-infection, the microfilarial counts for these four cats were: cat K = 3,675 mf/ml, Q = 10,875 mf/ml, R1 = 2,475 mf/ml, R2 = 2,125 mf/ml.

**Blood sample collection and slide preparation**

Average mf counts were determined for each animal by preparing standard thick blood smears in duplicate as previously detailed (reference). Briefly, the blood was spread on a slide along with a droplet of water to lyse the red blood cells. The slides were dried at room temperature for a minimum of 24 hours prior to staining with a 10% Giemsa buffered solution (1:9 solution of Giemsa stain; Acros Organics, Fair Lawn, NJ, USA) and TAE 1X buffer (2.0M Tris-Acetate + 100 mM $Na_2$ EDTA, National Diagnostics, Atlanta, GA). Microfilariae were identified, quantified, and averaged for each cat.

**Total RNA isolation**

Plasma samples from cats with the highest mf counts at six months post-infection were selected for analysis. Total RNA, including small RNAs, were isolated using Norgen plasma/serum RNA purification midi kit (Norgen Bioteck, Canada) with a few modifications to manufacturer's instructions. Initial sample processing utilized only 500 µl, as 1.5 ml of the sample as cellular components in plasma easily obstruct isolation columns. Lysis buffer and β-mercaptoethanol (molecular grade, MilliporeSigma, Burlington, MA) were added to the plasma samples and sheared using a needle and syringe homogenization step. The columns were washed and eluted an additional 1-2x

manufacture's recommendation to reduce salt contamination and to increase RNA yields. Eluted total RNA was quantified by optical density (NanoDrop 2000, Thermo Scientific) and Agilent 2100 Bioanalyzer (Santa Clara, CA) and stored at -80°C.

RNA samples were pooled for each individual cat for Illumina deep sequencing. A minimum of 100-200 ng/µl of high-quality RNA was required for RNAseq. These pooled samples were shipped to LC Sciences (Houston, TX, USA) on dry ice for cDNA library preparation, miRNA deep sequencing, and in-depth bioinformatics analysis. Remaining isolated RNA was stored at -80°C.

**RNA-seq workflow and bioinformatics analysis**

Small RNA libraries were prepared at LC sciences for the pooled samples using the Illumina TruSeq Small RNA Preparation kit following the Illumina TruSeq Small RNA Sample Preparation Guide (Illumina Inc., San Diego, CA, USA). Clusters were generated with purified cDNA on Illumina's Cluster Station and sequenced on Illumina GAIIx following the manufacturer's instructions. Real-time sequencing image analysis and base-calling were conducted using Real-Time Analysis version 1.8.70 (Illumina Inc., San Diego, CA) and used for subsequent data analysis.

The LC Sciences proprietary pipeline script (ACGT101-miR v4.2) (Figure S4.1) was utilized for data analysis. Raw data was organized into mappable reads and mapped to the feline genome (*Felis catus*, ftp://ftp.ncbi.nlm.nih.gov/genomes/, accessed 09/2017) and mature and precursor miRNA sequences of *F. catus* and other selected mammalian species available from miRBase v21.0 (ftp://mirbase.org/pub/mirbase/CURRENT/) [1–5]. The remaining unmapped reads were filtered against the mRNA from the Rfam database

(ftp://ftp.ncbi.nlm.nih.gov/genomes/Felis_catus/RNA/ and http://rfam.janelia.org)[42] and RepBase (http://www.girinst.org/repbase/) [43]. Unmapped reads that did not map to the feline genome were aligned to the *B. malayi* genome (http://www.ncbi.nlm.nih.gov/genome/?term=brugia+malayi) [44] and select nematode miRNAs, such as *Haemonchus contortus* [28], *Ascaris suum* [45], *Strongyloides ratti* [46], *Panagrellus redivivus* [47], *Caenorhabditis elegans* [48], *C. brenneri* [49], *C. remanei, C. briggsae,* and *Pristionchus pacificus* [50]. This group of helminths contains both non-parasitic (e.g. *C. elegans*) and parasitic (e.g. *H. contortus*) worms where the genomes are known and, in some cases, highly annotated. Identified miRNAs, along with their sequences and copy numbers, were produced. Read copy counts were normalized by dividing the counts by a library size parameter.

**TargetScan analysis**

MicroRNA deep sequencing data for all four cats, along with the comparative analysis against selected genomes, were compiled in FASTQ files from LC Sciences. These data were used to identify the potential biological targets of miRNAs on the feline genome using TargetScan v7.2 [51]. The TargetScan algorithm checks for perfect Watson-Crick base-pairing between the 5' miRNA seed sequence (7nt) and either 8mer, 7mer, or 6mer conserved sites that match the miRNA seed region from 3' UTR along with additional types of seed matches found conserved within worms, but not in mammals [16, 17]. This algorithm also considers free energy of miRNA-mRNA interactions evaluating heteroduplex stability. TargetScan results include gene IDs, site types, and context++ scores (CS). The CS for each specific site is calculated as the sum of the contribution of 14

parameters as described in Agarwal et al. [51]. A conserved weighted CS of ≤ -2 were selected as the maximum threshold to designate identified genes as potential biological targets. Gene targets that either multiple miRNAs targeted or were targeted multiple times by the same miRNA were assigned a target score from 0-40. Genes targeted at least twice were selected for gene ontology (GO) analysis.

### Gene ontology analysis

Gene ontology analyses were performed using PANTHER v11.0 (Protein Analysis Through Evolutionary Relationships) classification system (http://www.pantherdb.org) [53]. In this analysis, protein-coding genes were derived from 104 organisms and classified by protein function, evolutionary relationships, and biological pathways. Phylogenetic trees made up of protein-coding gene families are used to predict orthologs, paralogs, and xenologs using a hidden Markov model. These trees are used to make inferences about gene function [54]. Selected gene IDs identified by TargetScan were compared to the *F. catus* genome to obtain statistically enriched GO terms for molecular, biological, and cellular processes. Immunological processes were isolated from biological processes and categorized. Contingency tables for each GO term were generated and *p*-values calculated.

## RESULTS

### MicroRNAs identified from *B. malayi*-infected cats

Total RNA was isolated from the four cats with the highest microfilarial counts: 168.6 ng for cat K, 241.1 ng for cat Q, 163.7 ng for cat R1, and 129.5 ng for cat R2. From these 4 samples, 41,164,433 raw reads were compiled. The workflow involved removal of

low-quality sequences (junk reads, reads where 3' ligation adapters (ADT) were not found, <15 bases, or >32 bases after the 3' ADT cut), then selected for whether these reads mapped to the feline or other mammalian reference genomes (Table S4.1). The remaining 32,584,554 mapped reads were then mapped to the *B. malayi* or selected nematodes genome. A total of 185 unique miRNA sequences of potential nematode origin were identified, with 43 known miRNAs and 142 predicted candidates. Of these 185 candidate miRNAs, 26 were found to be present in 10 copies or more (Table 4.1). As the seed regions are highly conserved within miRNA families, the remaining mature sequence can differ by species, enabling us to differentiate between host and parasite miRNAs. The workflow for the mapped reads was clustered into eight groups. 42 reads were mapped to *B. malayi* miRNAs/pre-miRNAs in miRbase (group 1a), and only 1 miRNA found from selected nematode genomes (group 1b), with the pre-miRNAs further mapped to the genome and expressed sequence tag. Of these mapped clusters, there were 5 un-mapped miRNAs to selected nematode species while forming hairpins (group 2a) and only 1 miRNA without hairpin formation (group 2b). There were 31 reads that mapped to nematodes miRNAs/pre-miRNAs but did not map to the *B. malayi* genome (group 3a), whereas, 99 miRNAs were mapped to nematode miRNAs/pre-miRNAs in miRbase but could not be classified into group 3a (group 3b). Remaining were 17 read clusters un-mapped to other nematodes but mapped to the *B. malayi* genome (groups 4a).

Table 4.1 displays the 26 miRNAs of potential nematode origin from *B. malayi*-infected cat plasma. The miRNAs that were found to be most abundant were 3 isoforms of miR100 (d, a, & c), followed by let-7, miR-40, miR-35 (a, b, e, f, g, n), lin-4, miR-92, miR-miR-39, and miR-228. A single predicted candidate PC-3p-18742 found in group 4a, which

was mapped to only the *B. malayi* genome and not the other selected nematodes, may represent a unique *B. malayi* miRNA.

**Filarial miRNA comparison in other hosts**

To examine miRNA conservation in filarial host secreted products, *B. malayi* miRNA found in cat plasma was compared to miRNAs observed in other hosts such as dogs infected with *Dirofilaria immitis*, baboons infected with *Loa loa*, humans with *Onchocerca volvulus*, and cows infected with *O. ochengi* [20, 21]. Including *B. malayi*, there were 451 miRNA candidates, with a total of 323 unique miRNAs found from all five datasets. Only 47 of these miRNA families overlapped, with the largest number overlapping between *B. malayi* and *D. immitis* groups (Figure 4.2). All of the miRNAs identified and conserved between these groups are outlined in Table S4.2.

**Predicted gene targets on feline genome**

Both the 185 unique miRNAs and the 27 highly expressed *B. malayi* potential miRNAs were used to identify potential biological targets on the feline genome with the TargetScan software. One example of these miRNA 'hits' on the feline genome is shown in figure 4.3. TargetScan algorithms identified a total of 908,959 potential gene targets by nematode miRNAs, and 116,051 targets by the most abundantly expressed *B. malayi* miRNAs. A total of 13,616 unique genes were found in these gene hits. Each potential gene target was assigned a context score (CS). The CS of < -2 reduced the potential targets for the highly expressed miRNAs down to 826 genes. These gene targets were selected for GO analysis.

Table 4.1: Twenty-six most abundant secretory miRNA candidates identified in *B. malayi* infected cats. Seed sequences (2-8 nt) are identified in bold.

| miRNA sequence | miR Name | miR family | group | Cat K copy# | Cat Q copy# | Cat R1 copy# | Cat R2 copy# | Avg copy# |
|---|---|---|---|---|---|---|---|---|
| **TACCCGTA**GCTCCGAATATGTGT | bma-miR-100d_R+1 | miR-100d | gp1a | 101.95 | 532.76 | 1,064.33 | 873.79 | 643 |
| **AACCCGTA**GTTTCGAACATGTGT | bma-miR-100a_R+1 | miR-100a | gp1a | 81.23 | 558.23 | 431.94 | 951.89 | 506 |
| **TGAGGTAG**TAGGTTGAAA | bma-let-7-p5_2ss16TA18TA | let-7 | gp3b | 765.09 | 440.63 | 202.25 | 472.77 | 470 |
| **AACCCGTA**GAACTGAAATCGTGT | bma-miR-100c_R+1 | miR-100c | gp1a | 77.08 | 310.69 | 700.15 | 456.95 | 386 |
| **TCACCGGG**TGTACATCAGCTTG | crm-miR-40_1ss21AT | miR-40 | gp3a | 240.58 | 209.00 | 245.46 | 241.75 | 234 |
| **TCACCGGG**TGTAAATTAGC | cbn-mir-35e-p3_1ss17GA | mir-35e | gp3a | 195.54 | 182.85 | 178.96 | 180.55 | 184 |
| **TCCCTGAG**ACCTCTGCTGCGA | bma-lin-4 | lin-4 | gp1a | 66.31 | 294.39 | 60.23 | 20.05 | 110 |
| **TCACCGGG**TGTAAATCTG | cbn-miR-35g_R-4_1ss9AT | miR-35g | gp3a | 111.28 | 70.63 | 90.81 | 103.68 | 94 |
| **TATTGCAC**TTGTCCCGGCCTGAAT | bma-miR-92_R+4_1ss10CT | miR-92 | gp1a | 92.83 | 89.64 | 22.59 | 45.38 | 63 |
| **TCACCGGG**TGTAAATCACTTA | cbn-miR-39_R-1_2ss11GT18GC | miR-39 | gp3b | 55.81 | 64.18 | 48.93 | 60.15 | 57 |
| **TCACCGGG**TGTAAACTTG | cbr-mir-35a-p3_1ss11AT | mir-35a | gp3a | 46.83 | 61.12 | 60.23 | 52.24 | 55 |
| **TGAAAGAC**ATGGGTAGTGAGA | bma-miR-71_R+2 | miR-71 | gp1a | 6.63 | 16.30 | 61.17 | 124.53 | 52 |
| **TCACCGGG**TGTAAAATCACCTTG | cel-miR-41-3p_R+1_2ss11AT22AT | miR-41 | gp3b | 38.96 | 35.65 | 36.70 | 27.44 | 35 |
| **AATGGCAC**TAGATGAATTCACGG | bma-miR-228 | miR-228 | gp1a | 4.14 | 16.30 | 38.58 | 49.60 | 27 |
| **TCACCGGG**TGCCAATCAGCTTG | cbr-miR-40_2ss11TC21AT | miR-40 | gp3b | 33.15 | 22.41 | 20.70 | 31.66 | 27 |
| **ACGTGTGA**AGCTTACTGGT | PC-3p-18742_26 | PC-3p-18742 | gp4a | 9.95 | 37.69 | 25.41 | 30.60 | 26 |
| **TCACCGGG**TGTAAATTCAGC | crm-mir-36-p3_2ss11AT18GA | mir-36 | gp3b | 26.52 | 21.39 | 20.70 | 22.16 | 23 |
| **TCACCGGG**TGAACACTTG | cel-miR-37-3p_R-4 | miR-37 | gp3a | 24.04 | 16.81 | 31.53 | 10.55 | 21 |
| **TGGCAGTG**TGGTTAGCTGGTTGT | bma-miR-34 | miR-34 | gp1a | 3.32 | 10.19 | 14.12 | 48.54 | 19 |
| **TGATATGT**CTGATATTCTTGGGTT | bma-miR-50 | miR-50 | gp1a | 1.66 | 4.07 | 15.06 | 44.32 | 16 |
| **TCACCGGG**TGTAAATCAAG | crm-miR-35b_R-2_1ss11AT | miR-35b | gp3b | 11.85 | 17.32 | 16.42 | 18.47 | 16 |
| **CACCGGGT**GTAAATACTG | cbn-mir-35f-1-p3_2ss10AT17GT | mir-35f | gp3b | 11.60 | 8.66 | 21.64 | 15.83 | 14 |
| **TGACTAGA**ACCATACTCAGCT | bma-miR-279_R+1 | miR-279 | gp1a | 2.49 | 2.04 | 15.06 | 34.83 | 14 |
| **TAAGTGAA**TGCTTTGCCACAGTCT | bma-miR-86 | miR-86 | gp1a | 1.66 | 0.00 | 16.94 | 21.11 | 10 |
| **TCACCGGG**TTAAATCTTG | cbn-miR-35n_R-4_1ss14AT | miR-35n | gp3a | 11.60 | 8.66 | 12.70 | 3.69 | 9 |
| **TGAGATCA**CGTTACATCCGTCT | asu-miR-81c-3p_2ss9TC20AT | miR-81c | gp2a | 1.66 | 4.07 | 5.65 | 20.05 | 8 |

**Gene ontology analysis**

Of the 826 key genes identified, 729 were process hits on PANTHER GO analysis. Top key biological processes targeted on the feline genome include biological regulation (26.2%, GO:0008283), cellular component organization (10.7%, GO:0071840), cellular processes (9.5%, GO:0009987), localization (10.4%, GO:0051179), and metabolic processes (22.8%, GO:0008152), (Figure 4.4). Of these 826 key genes identified, each miRNA that targeted these gene transcripts were scored based on the number of miRNAs hits, from either 1 to 40. Of these, the top 10 gene transcripts that were targeted were *Celf2, Sik2, Bend3, Cyp2s1, Dtnb, Fmnl3, Nfia, Oprl1, Zbtb40,* and *Golga7b*. The GO biological processes, cellular, and molecular functions of these genes are shown in tables S4.3 and S4.4. Gene transcripts found to target primarily immune system processes were *Ptgs1, Irf4, Irf5, Numbl, Tnfsf15, Stat3*, and *Txlnb* (Table 4.2).

Table 4.2: Immune genes and their biological processes identified by PANTHER GO analysis.

| Gene Symbol | Gene Name | Family/Subfamily | Protein Class |
|---|---|---|---|
| *Ptgs1* | Prostaglandin-endoperoxide synthase 1 | Prostaglandin G/H Synthase 1 | oxygenase |
| *Irf4* | Interferon regulatory factor 4 | Interferon Regulatory Factor 4 | nucleic acid binding |
| *Irf5* | Interferon regulatory factor 5 | Interferon Regulatory Factor 5 | nucleic acid binding |
| *Numbl* | NUMB like, endocytic adaptor protein | Numb-like Protein | signaling molecule |

| | | | |
|---|---|---|---|
| *Tnfsf15* | TNF superfamily member 15 | Tumor Necrosis Factor Ligand Superfamily Member 15 | tumor necrosis factor family member |
| *Stat3* | Signal transducer and activator of transcription | Signal Transducer and Activator of Transcription 3 | nucleic acid binding |
| *Txlnb* | Taxilin beta | Beta-Taxilin | antibacterial response protein |

## DISCUSSION

Identification of circulating miRNAs within biofluids has had a significant impact on disease research, from identification and gene expression of cancer to parasite-derived miRNAs [55, 56]. This study examined the parasite-derived miRNAs found in the plasma of *B. malayi*-infected cats. MicroRNAs have been found to be highly stable in blood products (plasma and serum) making them ideal candidates for their use as potential biomarkers and diagnostic tools [21, 23–26]. These small RNAs have also been found to be involved in key regulatory processes in parasite development along with establishment within a host [61]. Identification of specific host genes targeted and regulated by parasite-derived miRNAs could suggest a molecular mode of action in parasite-host interactions.

### MicroRNA comparison in other hosts

Many mammalian miRNAs are highly conserved [32], as are the miRNAs across the phylum Nematoda [56]. When comparing miRNAs found in other closely related parasitic helminths and their respective hosts (Figure 4.2), there were few common helminth miRNAs found in host serum that overlap between *B. malayi, D. immitis, L. loa,*

*O. volvulus,* and *O. ochengi.* Notably, there were no miRNAs in these datasets that that were common to all five species. This was unexpected, as miRNAs such as let-7 and lin-4 have been found to be highly conserved in helminths. Likely this is due to host miRNAs dominating these samples. As the number of miRNAs initial documented for *L. loa* and *Oncocerca* spp. was low, there is the possibility that additional sequences remain to be discovered.

As miRNAs are small RNAs, there is the chance that some sequences thought to be parasite-derived could be attributed to the host, and vice versa [62]. The seed sequence is highly conserved in miRNA families, but the remaining ~14 nucleotides can be quite different between various species. These sequence differences are how each miRNA is distinguished between mammalian and different helminth species. As bioinformatic algorithms increase in efficiency, confidence that these miRNAs are correctly attributed increase. Additionally, whether these miRNAs are being secreted by the adult worms or the circulating microfilariae is difficult to determine. The adults remain sequestered in the lymphatics, whereas the microfilariae circulate systemically throughout the blood. To truly identify miRNAs are being expressed by each developmental stage, further exploration of infected lymphatic tissues would be required. Evaluation of miRNA orthologs and homology within other parasites, rodent models, or in vitro studies gives us a glimpse into not only the potential function and purpose of these miRNAs but that they are correctly identified.

**Predicted targets on the feline genome**

All 185 unique putative miRNAs were utilized for in silico analysis. This resulted in over 900,000 gene hits. These targets include every possible variation that these miRNAs could bind to the selected genome which results in far more potential targets than the 20,285 putative genes within the cat genome [55, 56]. Focusing primarily on the 26 highly expressed miRNAs, this number was reduced to 116,000 gene hits. Targetscan algorithms calculate a context score (CS) that determines which miRNAs have a higher probability of being a legitimate target for host mRNA. The lower the score, the higher the confidence. The range of the weighted CS ranged from -2.38 to -0.156. A score of -2 as the cutoff threshold was a subjective selection to include only 5.2% (47,410 gene hits) of the entire dataset. Selecting 5% of the dataset with the highest weighted CS implicated high confidence in the gene targets identified.

Once these gene targets were identified, in order to assess the potential biological functions of these genes, GO analysis was performed. Gene targets that either multiple miRNAs targeted, or were targeted multiple times by the same miRNA, were assigned a target score from 1-40. Genes targeted at least twice were selected for GO analysis. The PANTHER GO analysis produced cellular, molecular, and protein processes related to each gene target. The majority of the target genes were involved in biological regulation, metabolic processes, cellular component organization, localization, and cellular processes. The top targeted gene transcript (40 hits) was a protein-coding gene *Celf2*, involved in RNA binding, mRNA splice site selection, alternative mRNA splicing (via spliceosome), and regulation of alternative mRNA splicing. This protein has been found to be increased in response to T-cell signaling [64–66]. The second top targeted gene transcript was Salt

Inducible Kinase 2 (*SIK2*), involved in serine/threonine-protein kinase. This protein-coding gene controls a molecular switch found to regulate macrophage polarization [68]. Inhibition of these kinases has been found to induce macrophage secretion of IL-10 and low levels of pro-inflammatory cytokines. When undergoing differentiation, the levels of IL-10 have been found to increase dramatically when SIK2 is inhibited [68]. Additional gene transcript targets in the GO analyses have been found to be involved in RNA pol II transcription, protein targeting to the cell membrane, and oxidoreductase activity, among other biological functions that could potentially aid *B. malayi* establishment and development within the mammalian host. Modulation of the T-cell response, along with elevated IL-10 is canonical in these helminth infections. If these host gene transcripts are altered by parasite miRNAs, these in silico results would support what is observed in studies of host-response to *B. malayi*.

**Immunomodulatory targets on the feline genome**

Long-term survival of these helminths within the mammalian host would necessitate sophisticated mechanisms to evade clearance by the host immune response. The top key immune genes identified as targets of highly expressed miRNAs found in *B. malayi*-infected cat blood were *Ptgs1, Irf4, Irf5, Numbl, Tnfsf15, Stat3*, and *Txlnb*. As miRNAs target mRNA within host cells, this can result in the disruption or suppression of that gene and subsequent gene products.

The gene *Ptgs1* is a prostaglandin G/H synthase 1 gene, involved in oxidoreductase activity. Prostaglandin production depends on the activity of these G/H synthases, known as cyclooxygenase (COX). These COXs are enzymes that exist as two distinct isoforms

(COX-1 & COX-2) which catalyzes the conversion of arachidonic acid to prostaglandins $H_2$ where they are metabolized to biologically active molecules such as thromboxane synthase and prostaglandin (E, F, I) synthases [23, 24]. These COX enzymes are important in facilitation of the host inflammatory response [71]. If these enzymes are improperly up- or down-regulated within the host when exposed to a pathogen, a weakened inflammatory response will result. These prostaglandins are also pharmacologically important, as the prostaglandin synthase isozymes are targets of aspirin and nonsteroidal anti-inflammatory medications [70]. The miRNA believed to target this gene transcript is let-7. These prostaglandins are found constitutively expressed in most tissues, but not all cells of a tissue in a host. When invading parasites enter into host tissues, being able to target a ubiquitous gene such as *ptgs1* would prove to be quite beneficial for the survival of this parasite.

Interferon regulatory factors (IRFs) are a family of transcription factors originally described for their role in the induction of type I interferons (IFNs) in mammals [72]. The role of IRFs in immune cell activation has been well characterized. The genes *Irf4* and *Irf5* encode for IRF-4 and IRF-5, respectively. IRF5 is one of four core molecules that has been implicated in the induction of type I IFNs [73]. IFNs are cytokines that activate in response to toll-like receptors (TLRs), PRRs, and PAMPs. TLRs are expressed by many immune cells, including dendritic cells, macrophages, lymphocytes, neutrophils, and endothelial cells. When these TLRs are activated, a cascade of signaling events occurs resulting in activation of nuclear factor-κB (NF-κB), producing a pro-inflammatory response. The miRNAs that target this gene are numerous: mir-35e, miR-40, mir-36, miR35b, miR-41, miR-39, miR-37, miR-40, mir-35a, miR-39, miR-35n, and miR-35g. If these parasite miRNAs are in fact able to suppress or reduce this pro-inflammatory signal cascade, this

would correspond to what has been documented in other parasite-host interactions [40–43].

Interferon regulatory factor 4 (IRF4) is a transcription factor only expressed in lymphocytes, dendritic cells, and macrophages [44, 45]. Only two miRNAs targeted the mRNA product of this gene: miR-228 and lin-4. Unlike IRF-5, which is induced by type I or type II IFNs, IRF-4 is induced upon activation of T and B cells serving critical functions in regulation of IL-21 production by CD4+ T-helper cells, B cell class switching, and plasma cell differentiation [40, 41]. Experiments with transgenic mice have shown that deletion of IRF-4 in B cells left them unable to differentiate memory B cells into plasma cells, resulting in inhibition of antibody production [81]. Regulation of IRF-4 expression in T cells is similar to B cells, with either T cell mitogens or anti-CD3 antibodies inducing IRF-4 expression [80]. In a set of in vitro experiments where T cells with IRF-4 knockout ($IRF4^{-/-}$) were incubated with lymph node cells with antibodies to CD3 or bacterial superantigen staphylococcal enterotoxin A (SEA), T cell proliferation was reduced [46, 48]. This underlies the importance of this transcription factor in B and T cell proliferation. Inhibition of the *Inf4* gene would impact the adaptive immune response within the infected host.

Both *Numb/Numbl* and Tumor Necrosis Factor Ligand Superfamily Member 15 (*Tnfsf15*) have been found to play a role in endothelial cell proliferation, blood, and lymphatic vessel growth. Only miR-34 was predicted to target *Numb/Numbl* mRNA, while miR-92 and lin-4 were predicted to target *Tnfsf15* products. *Numb/Numbl* genes encode proteins that have a role in cell fate determination during development [83]. These genes have been linked to their ability to bind and antagonize Notch, a family of type-1

transmembrane proteins that specify cell fate [84]. When *Numb/Numbl* genes are inactivated, this results in impaired vessel growth and a reduction in endothelial proliferation, while vascular endothelial growth factor (VEGF) receptor activation is decreased [85]. VEGF is a protein that stimulates the formation of blood vessels. TNFSF15 encodes for an endothelial cell cytokine that has been determined to inhibit endothelial cell proliferation and angiogenesis [52, 53]. TNFSF15 has also been found to be downregulated by VEGF. Therefore, if both *Tnfsf15* and *Numb/Numbl* gene products are suppressed, VEGF is decreased and endothelial cell proliferation is reduced.

The signal transducer and activator of transcription 3 (*Stat3*) gene is a member of the STAT protein family involved in transcription activation. The miRNA lin-4 has two potential binding sites on mRNA of these gene products. STAT proteins are phosphorylated by cytoplasmic Janus kinases (Jak), a part of the Jak-STAT pathway that plays a central role in both innate and adaptive immunity [88]. These proteins mediate cytokine signals during Th1 and Th2 differentiation. Studies have found that when STAT3 is suppressed, there is an increase of CD4+ T cells to induce transcription factor T-bet [89]. When naïve CD4+ T cells are STAT3-deficient, germinal center formation is impaired, and inversely, a Th1 cell phenotype has enhanced expression [90]. The STAT3 proteins are involved in many other functions aside from the ones stated above. The fact that when STAT3 is downregulated, a pro-inflammatory state occurs, appears counter-intuitive for an invading parasite. Why this gene is targeted by parasite miRNAs is a question to be further explored.

Ubiquitously expressed β-Taxilin (*Txlnb*) is found to be expressed in skeletal muscle and the heart [57, 58]. MicroRNAs let-7 and mir-35f were predicted to target this

gene. The potential functions of this gene and subsequent products are not well characterized. However, taxilin family members interact with syntaxin family members, who are involved in intracellular vesicle trafficking. Potential function that these taxilins have on vesicular trafficking remains to be elucidated.

This study is the first report of circulating miRNAs from plasma in *B. malayi* infected cats. By deep RNA sequencing, a total of 185 mature miRNAs from 4 *B. malayi*-infected cats were identified, with 26 found to be expressed as 10 copies or more. These 26 highly expressed miRNAs were then used to identify potential gene transcript targets on the feline genome. By in silico analysis using TargetScan, we identified 826 potential mRNA targets. To reduce the potential false positive rate, targets with at least 2 or more hits were used in GO analysis. The top two gene transcripts have been found in previous studies to be involved in T-cell proliferation and cytokine secretion in macrophages. An additional 7 genes were found to be involved in plasma cell and T-cell differentiation, endothelial cell proliferation, and growth, along with pro-inflammatory processes. If these targets are suppressed by this parasite-host interaction through miRNAs, this would prove to be another methodology that these worms use in host-modulation for survival within the mammalian host.

**ACKNOWLEDGMENTS**

This project could not have been accomplished without the expert support of those at the Filariasis Research Reagent Resource Center and the University of Georgia's animal resource center.

## DISCLOSURES

The authors have no financial conflict of interest.

## REFERENCES


[1] A. H. Buck *et al.*, "Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity," *Nat. Commun.*, vol. 5, p. 5488, 2014.

[2] A. Chandy, A. S. Thakur, M. P. Singh, and A. Manigauha, "A review of neglected tropical diseases: Filariasis," *Asian Pac. J. Trop. Med.*, vol. 4, no. 7, pp. 581–586, 2011.

[3] B. R. Laurence, "The Curse of Saint Thomas," *Med. Hist.*, vol. 14, no. 4, pp. 352–363, 1970.

[4] J. E. Allen and T. A. Wynn, "Evolution of Th2 immunity: a rapid repair response to tissue destructive pathogens," *PLoS Pathog.*, vol. 7, no. 5, pp. 5–8, 2011.

[5] N. M. Girgis, U. M. Gundra, and P. Loke, "Immune regulation during helminth infections," *PLoS Pathog.*, vol. 9, no. 4, pp. 11–13, 2013.

[6] J. P. Hewitson, J. R. Grainger, and R. M. Maizels, "Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity," *Mol. Biochem. Parasitol.*, vol. 167, no. 1, pp. 1–11, 2009.

[7] J. H. Schroeder *et al.*, “Live *Brugia malayi* microfilariae inhibit transendothelial migration of neutrophils and monocytes,” *PLoS Negl. Trop. Dis.*, vol. 6, no. 11, 2012.

[8] B. Spellberg and J. E. Edwards, “Type 1/Type 2 immunity in infectious diseases,” *Clin. Infect. Dis.*, vol. 32, pp. 76–102, 2001.

[9] C. a Desjardins *et al.*, “Genomics of *Loa loa*, a *Wolbachia*-free filarial parasite of humans,” *Nat. Genet.*, vol. 45, no. 5, pp. 495–500, May 2013.

[10] P. H. Gazzinelli-Guimaraes and T. B. Nutman, “Helminth parasites and immune regulation,” *F1000Research*, vol. 7, no. October, p. 2018, 2018.

[11] L. Hussaarts, L. E. P. M. Van Der Vlugt, M. Yazdanbakhsh, and H. H. Smits, “Regulatory B-cell induction by helminths: Implications for allergic disease,” *J. Allergy Clin. Immunol.*, vol. 128, no. 4, pp. 733–739, 2011.

[12] M. D. Taylor, N. van der Werf, and R. M. Maizels, “T cells in helminth infection: The regulators and the regulated,” *Trends Immunol.*, vol. 33, no. 4, pp. 181–189, 2012.

[13] S. Gordon and F. O. Martinez, “Alternative activation of macrophages: Mechanism and functions,” *Immunity*, vol. 32, no. 5, pp. 593–604, 2010.

[14] P. E. Simonsen, “Filariasis,” in *Manson’s Tropical Diseases*, 22nd ed., G. C. Cook and A. Zumla, Eds. China: W. B. Saunders, 2008, pp. 1477–1513.

[15] R. Gupta, P. Bajpai, L. M. Tripathi, V. M. L. Srivastava, S. K. Jain, and S. Misra-Bhattacharya, “Macrophages in the development of protective immunity against

experimental *Brugia malayi* infection," *Parasitology*, vol. 129, no. Pt 3, pp. 311–323, 2004.

[16] P. K. Murthy, P. S. R. Murthy, K. Tyaga, and R. K. Chatterjee, "Fate of infective larvae of *Brugia malayi* in the peritoneal cavity of *Mastomys natalensis* and *Meriones unguiculatus*," *Folia Parasitol. (Praha).*, vol. 44, pp. 302–304, 1997.

[17] S. K. Verma *et al.*, "Protection against filarial infection by 45–49kDa molecules of *Brugia malayi* via IFN-γ-mediated iNOS induction," *Vaccine*, vol. 33, no. 4, pp. 527–534, 2015.

[18] D. Male, J. Brostoff, D. Roth, and I. Roitt, *Immunology*, 7th ed. Philadelphia: Mosby Elsevier, 2006.

[19] K. Murphy, "Innate Immunity : The First Lines of Defense," in *Janeway's Immunobiology*, 8th ed., P. Travers and M. Walport, Eds. New York, NY: Garland Science, 2011, pp. 37–73.

[20] J. Kay, "Basic Principles and Concepts II: Immunology and Pathology," in *Foundations of Parasitology*, vol. 21, no. 9, 2008, pp. 23–40.

[21] A. Gunn and S. J. Pitt, *Parasitology: An Integrated Approah*, 1st ed. Oxford: Wiley-Blackwell, 2012.

[22] R. M. Johnstone, M. Adam, J. R. Hammonds, and C. Turbide, "Vesicle formation during reticulocyte maturation," *J. Biol. Chem.*, vol. 262, no. 1, pp. 9412–9420, 1987.

[23] G. Raposo and W. Stoorvogel, “Extracellular vesicles: Exosomes, microvesicles, and friends,” *J. Cell Biol.*, vol. 200, no. 4, pp. 373–383, 2013.

[24] H. Harischandra, W. Yuan, H. J. Loghry, M. Zamanian, and M. J. Kimber, “Profiling extracellular vesicle release by the filarial nematode *Brugia malayi* reveals sex- specific differences in cargo and a sensitivity to ivermectin,” *PLoS Negl. Trop. Dis.*, vol. 12, no. 4, pp. 1–25, 2018.

[25] D. P. Bartel, R. Lee, and R. Feinbaum, “MicroRNAs: Genomics, biogenesis, mechanism, and function,” *Cell*, vol. 116, pp. 281–297, 2004.

[26] M. I. Almeida, R. M. Reis, and G. A. Calin, “MicroRNA history: Discovery, recent applications and next frontiers,” *Mutat. Res. Mol. Mech. Mutagen.*, vol. 717, no. 1–2, pp. 1–8, 2011.

[27] C. Britton, A. D. Winter, V. Gillan, and E. Devaney, “MicroRNAs of parasitic helminths-identification, characterization and potential as drug targets,” *Int. J. Parasitol. Drugs Drug Resist.*, vol. 4, no. 2, pp. 85–94, 2014.

[28] A. D. Winter *et al.*, “Diversity in parasitic nematode genomes: the microRNAs of *Brugia pahangi* and *Haemonchus contortus* are largely novel,” *BMC Genomics*, vol. 13, no. 1, p. 4, Jan. 2012.

[29] K. Okamura, A. Ishizuka, H. Siomi, and M. C. Siomi, “Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways,” *Genes Dev.*, vol. 18, no. 14, pp. 1655–1666, 2004.

[30] D. W. Thomson, C. P. Bracken, and G. J. Goodall, “Experimental strategies for microRNA target identification,” *Nucleic Acids Res.*, vol. 39, no. 16, pp. 6845–6853, 2011.

[31] J. R. Lytle, T. A. Yario, and J. A. Steitz, “Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5’ UTR as in the 3’ UTR,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 23, pp. 9667–9672, 2007.

[32] R. C. Friedman, K. K. Farh, C. B. Burge, and D. P. Bartel, “Most mammalian mRNAs are conserved targets of microRNAs,” *Genome Res.*, vol. 19, pp. 92–105, 2009.

[33] M. Hakimi and D. Cannella, “Apicomplexan parasites and subversion of the host cell microRNA pathway,” *Trends Parasitol.*, vol. 27, no. 11, pp. 481–486, 2011.

[34] A. Gong *et al.*, “MicroRNA-221 controls expression of intercellular adhesion molecule-1 in epithelial cells in response to *Cryptosporidium parvum* infection,” *Int. J. Parasitol.*, vol. 41, no. 3–4, pp. 397–403, 2011.

[35] L. Tritten *et al.*, “Detection of circulating parasite-derived microRNAs in filarial infections,” *PLoS Negl. Trop. Dis.*, vol. 8, no. 7, p. e2971, Jul. 2014.

[36] L. Tritten *et al.*, “*Loa loa* and *Onchocerca ochengi* miRNAs detected in host circulation,” *Mol. Biochem. Parasitol.*, vol. 198, no. 1, pp. 14–17, 2014.

[37] A. Kozomara and S. Griffiths-jones, “miRBase: annotating high confidence microRNAs using deep sequencing data,” *Nucleic Acids Res.*, vol. 42, pp. 68–73, 2014.

[38] A. Kozomara, M. Birgaoanu, and S. Griffiths-jones, “miRBase: from microRNA sequences to function,” *Nucleic Acids Res.*, vol. 47, pp. 155–162, 2019.

[39] S. Griffiths-Jones, “The microRNA Registry,” *Nucleic Acids Res.*, vol. 32, pp. 109–111, 2004.

[40] S. Griffiths-jones, R. J. Grocock, S. Van Dongen, A. Bateman, and A. J. Enright, “miRBase: microRNA sequences, targets and gene nomenclature,” *Nucleic Acids Res.*, vol. 34, pp. 140–144, 2006.

[41] S. Griffiths-jones, H. K. Saini, S. Van Dongen, and A. J. Enright, “miRBase: tools for microRNA genomics,” *Nucleic Acids Res.*, vol. 36, pp. 154–158, 2008.

[42] P. P. Gardner *et al.*, “Rfam: Wikipedia, clans and the ‘“decimal”’ release,” *Nucleic Acids Res.*, vol. 39, no. November 2010, pp. 141–145, 2011.

[43] J. Jurka, V. V Kapitonov, A. Pavlicek, P. Klonowski, O. Kohany, and J. Walichiewicz, “Diversity of retrotransposable elements repbase update, a database of eukaryotic repetitive elements,” *Cytogenet. Genome Res.*, vol. 110, pp. 462–467, 2005.

[44] E. Ghedin *et al.*, “Draft Genome of the Filarial Nematode Parasite *Brugia malayi*,” *Science (80-. ).*, vol. 317, no. 5845, pp. 1756–1760, 2007.

[45] J. Wang *et al.*, “Deep small RNA sequencing from the nematode *Ascaris* reveals conservation, functional diversification, and novel developmental profiles,” *Genome Res.*, vol. 21, no. 9, pp. 1462–1477, 2011.

[46] R. Ahmed *et al.*, “Conserved miRNAs are candidate post-transcriptional,” *Genome Biol. Evol.*, vol. 5, no. 7, pp. 1246–1260, 2013.

[47] J. Srinivasan *et al.*, “The draft genome and transcriptome of *Panagrellus redivivus* are shaped by the harsh demands of a free-living lifestyle,” *Genetics*, vol. 193, no. April, pp. 1279–1295, 2013.

[48] S. Lall *et al.*, “A genome-wide map of conserved microrna targets in *C. elegans*,” *Curr. Biol.*, vol. 16, no. 5, pp. 460–471, 2006.

[49] Z. Shi, T. A. Montgomery, Y. Qi, and G. Ruvkun, “High-throughput sequencing reveals extraordinary fluidity of miRNA, piRNA, and siRNA pathways in nematodes,” *Genome Res.*, vol. 23, pp. 497–508, 2013.

[50] E. De Wit, S. E. V Linsen, E. Cuppen, and E. Berezikov, “Repertoire and evolution of miRNA genes in four divergent nematode species,” *Genome Res.*, vol. 19, no. 11, pp. 2064–2074, 2009.

[51] V. Agarwal, G. W. Bell, J. Nam, and D. P. Bartel, “Predicting effective microRNA target sites in mammalian mRNAs,” *Genomics Evol. Biol.*, vol. 4, pp. 1–38, 2015.

[52] B. P. Lewis, C. B. Burge, and D. P. Bartel, “Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets,” *Cell*, vol. 120, pp. 15–20, 2005.

[53] H. Mi *et al.*, “PANTHER version 11: expanded annotation data from Gene Ontology and reactome pathways, and data analysis tool enhancements,” *Nucleic Acids Res.*, vol. 45, no. November 2016, pp. 183–189, 2017.

[54] P. Gaudet, M. S. Livstone, S. E. Lewis, and P. D. Thomas, “Phylogenetic-based propagation of functional annotations within the Gene Ontology consortium,” *Brief. Bioinform.*, vol. 12, no. 5, pp. 449–462, 2011.

[55] V. De Guire *et al.*, “Circulating miRNAs as sensitive and speci fi c biomarkers for the diagnosis and monitoring of human diseases: Promises and challenges,” *Clin. Biochem.*, vol. 46, no. 10–11, pp. 846–860, 2013.

[56] L. Tritten and T. G. Geary, “MicroRNAs of filarial nematodes: A new frontier in host-pathogen interactions,” in *Non-coding RNAs and Inter-kingdom Communication*, A. L. Leitao and F. J. Enguita, Eds. Zurich, Switzerland: Springer International Publishing, 2016, pp. 207–223.

[57] L. Tritten *et al.*, “Molecular & biochemical parasitology *Loa loa* and *Onchocerca ochengi* miRNAs detected in host circulation,” *Mol. Biochem. Parasitol.*, vol. 198, no. 1, pp. 14–17, 2014.

[58] P. S. Mitchell *et al.*, “Circulating microRNAs as stable blood-based markers for cancer detection,” *Proc. Natl. Acad. Sci.*, vol. 105, no. 30, pp. 10513–10518, 2008.

[59] J. Shaffer, M. Schlumpberger, and E. Lader, “miRNA profiling from blood — challenges and recommendations,” *Qiagen*, pp. 1–10, 2012.

[60] A. M. Hoy *et al.*, “Parasite-derived microRNAs in host serum as novel biomarkers of helminth infection,” *PLoS Negl. Trop. Dis.*, vol. 8, no. 2, 2014.

[61] C. Lin, Y. Chen, C. Chen, Y. Oyang, H. Juan, and H. Huang, “Crosstalk between transcription factors and microRNAs in human protein interaction network,” *BMC*

*Syst. Biol.*, vol. 6, no. 18, pp. 1–13, 2012.

[62] M. Lagos-quintana, R. Rauhut, A. Yalcin, J. Meyer, W. Lendeckel, and T. Tuschl, "Identification of tissue-specific microRNAs from mouse," *Curr. Biol.*, vol. 12, pp. 735–739, 2002.

[63] G. Tamazian *et al.*, "Annotated features of domestic cat - *Felis catus* genome," *Gigascience*, vol. 3, p. 13, 2014.

[64] J. U. Pontius *et al.*, "Initial sequence and comparative analysis of the cat genome," *Genome Res.*, vol. 17, pp. 1675–1689, 2007.

[65] N. M. Martinez *et al.*, "Widespread JNK-dependent alternative splicing induces a positive feedback loop through CELF2-mediated regulation of MKK7 during T-cell activation," *Genes Dev.*, vol. 29, pp. 2054–2066, 2015.

[66] M. J. Mallory, J. Jackson, B. Weber, A. Chi, F. Heyd, and K. W. Lynch, "Signal- and Development-Dependent Alternative Splicing of LEF1 in T Cells Is Controlled by CELF2," *Mol. Cell. Biol.*, vol. 31, no. 11, pp. 2184–2195, 2011.

[67] M. J. Mallory, S. J. Allon, J. Qiu, M. R. Gazzara, I. Tapescu, and N. M. Martinez, "Induced transcription and stability of CELF2 mRNA drives widespread alternative splicing during T-cell signaling," *PNAS*, pp. 2139–2148, 2015.

[68] N. J. Darling, R. Toth, J. S. C. Arthur, and K. Clark, "Inhibition of SIK2 and SIK3 during differentiation enhances the anti-inflammatory phenotype of macrophages," *Biochem. J.*, vol. 474, pp. 521–537, 2017.

[69] C. R. Lee *et al.*, “Identification and functional characterization of polymorphisms in human cyclooxygenase-1 (PTGS1),” *Pharmacogenet Genomics*, vol. 17, no. 2, pp. 145–160, 2007.

[70] W. L. Smith, R. M. Garavito, and D. L. Dewitt, “Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2,” *J. Biol. Chem.*, vol. 271, no. 52, pp. 33157–33161, 1996.

[71] S. Young-Joon *et al.*, “Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation,” *Mutat. Res.*, vol. 480–481, pp. 243–268, 2001.

[72] M. Miyamoto *et al.*, “Regulated expression of a gene encoding a nuclear factor, Irf-1, that specifically binds to Ifn-beta gene regulatory elements,” *Cell*, vol. 54, pp. 903–913, 1988.

[73] B. J. Barnes, P. A. Moore, and P. M. Pitha, “Virus-specific activation of a novel interferon regulatory factor, Irf-5, results in the induction of distinct interferon alpha genes,” *J. Biol. Chem.*, vol. 276, no. 26, pp. 23382–23390, 2001.

[74] A. S. Macdonald, M. I. Araujo, J. Edward, and E. J. Pearce, “Immunology of parasitic helminth infections,” *Infect. Immun.*, vol. 70, no. 2, pp. 427–433, 2002.

[75] R. M. Maizels, M. L. blaxter, and A. L. Scott, “Immunological genomics of *Brugia malayi*: filarial genes implicated in immune evasion and protective immunity,” *Parasite Immunol.*, vol. 23, no. 7, pp. 327–344, 2001.

[76] R. Medzhitov, D. S. Schneider, and M. P. Soares, “Disease Tolerance as a Defense Strategy,” *Science (80-. ).*, vol. 335, no. 6071, pp. 936–941, 2012.

[77] C. L. King and T. B. Nutman, “Regulation of the immune response in lymphatic filariasis and onchocerciasis.,” *Immunol. Today*, vol. 12, no. 3, pp. A54–A58, 1991.

[78] A. B. Pernis, “The role of IRF-4 in B and T Cell activation and differentiation,” *J. Interf. Cytokine Res.*, vol. 22, pp. 111–120, 2002.

[79] S. Marecki and M. J. Fenton, “The role of IRF-4 in transcriptional regulation,” *J. Interf. Cytokine Res.*, vol. 133, pp. 121–133, 2002.

[80] H. Mittrucker *et al.*, “Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function,” *Science (80-. ).*, vol. 275, no. January, pp. 540–543, 1997.

[81] U. Klein *et al.*, “Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination,” *Nat. Immunol.*, vol. 7, no. 7, pp. 773–782, 2006.

[82] K. Man *et al.*, “The transcription factor IRF4 is essential for TCR affinity–mediated metabolic programming and clonal expansion of T cells,” *Nat. Immunol.*, vol. 14, no. 11, pp. 1155–1167, 2013.

[83] J. M. García-heredia and A. Carnero, “NUMB and NUMBL differences in gene regulation,” *Oncotarget*, vol. 9, no. 10, pp. 9219–9234, 2018.

[84] C. C. Nwaeburu *et al.*, “Up-regulation of microRNA let-7c by quercetin pancreatic cancer progression by activation of *Numbl* inhibits,” *Oncotarget*, vol. 7, no. 36, pp. 58367–58380, 2016.

[85] M. Van Lessen, M. Nakayama, K. Kato, J. M. Kim, K. Kaibuchi, and R. H. Adams, “Regulation of vascular endothelial growth factor receptor function in angiogenesis by Numb and Numb-Like,” *Arterioscler. Thromb. Vasc. Biol.*, pp. 1815–1825, 2015.

[86] J. Yu, S. Tian, L. Metheny-barlow, L. Chew, A. J. Hayes, and H. Pan, “Modulation of endothelial cell growth arrest and apoptosis by vascular endothelial growth inhibitor,” *Circ. Res.*, vol. 7, no. December, pp. 1161–1167, 2001.

[87] Y. Z. Hai *et al.*, “Inhibition of angiogenesis and breast cancer xenograft tumor growth by VEGI, a novel cytokine of the TNF superfamily,” *Int. J. Cancer*, vol. 136, no. January, pp. 131–136, 1999.

[88] R. Goswami and M. H. Kaplan, “STAT transcription factors in T cell control of health and disease,” in *International Review of Cell and Molecular Biology*, 1st ed., vol. 331, Elsevier Inc., 2017, pp. 123–180.

[89] X. O. Yang *et al.*, “Stat3 regulates cytokine-mediated generation of inflammatory helper t cells,” *J. Biol. Chem.*, vol. 282, no. 13, pp. 9358–9363, 2007.

[90] J. P. Ray, H. D. Marshall, B. J. Laidlaw, M. M. Staron, S. M. Kaech, and J. Craft, "Transcription factor STAT3 and Type I Interferons Are corepressive insulators for differentiation of follicular helper and T helper 1 cells," *Immunity*, vol. 40, no. 3, pp. 367–377, 2014.

[91] K. E. Fujimori, A. Uyeda, and T. Taguchi, "Regulatory expression of MDP77 protein in the skeletal and cardiac muscles," *FEBS Lett.*, vol. 529, pp. 303–308, 2002.

[92] S. Nogami *et al.*, "Identification and characterization of taxilin isoforms," *Biochem. Biophys. Res. Commun.*, vol. 319, pp. 936–943, 2004.

[93] J. Winter, S. Jung, S. Keller, R. I. Gregory, and S. Diederichs, "Many roads to maturity: microRNA biogenesis pathways and their regulation," *Nat. Cell Biol.*, vol. 11, no. 3, pp. 228–234, 2009.

[94] C. Ballesteros *et al.*, "The effect of in vitro cultivation on the transcriptome of adult *Brugia malayi*," *PLoS Negl. Trop. Dis.*, vol. 10, no. 1, p. e0004311, 2016.

**FIGURES**

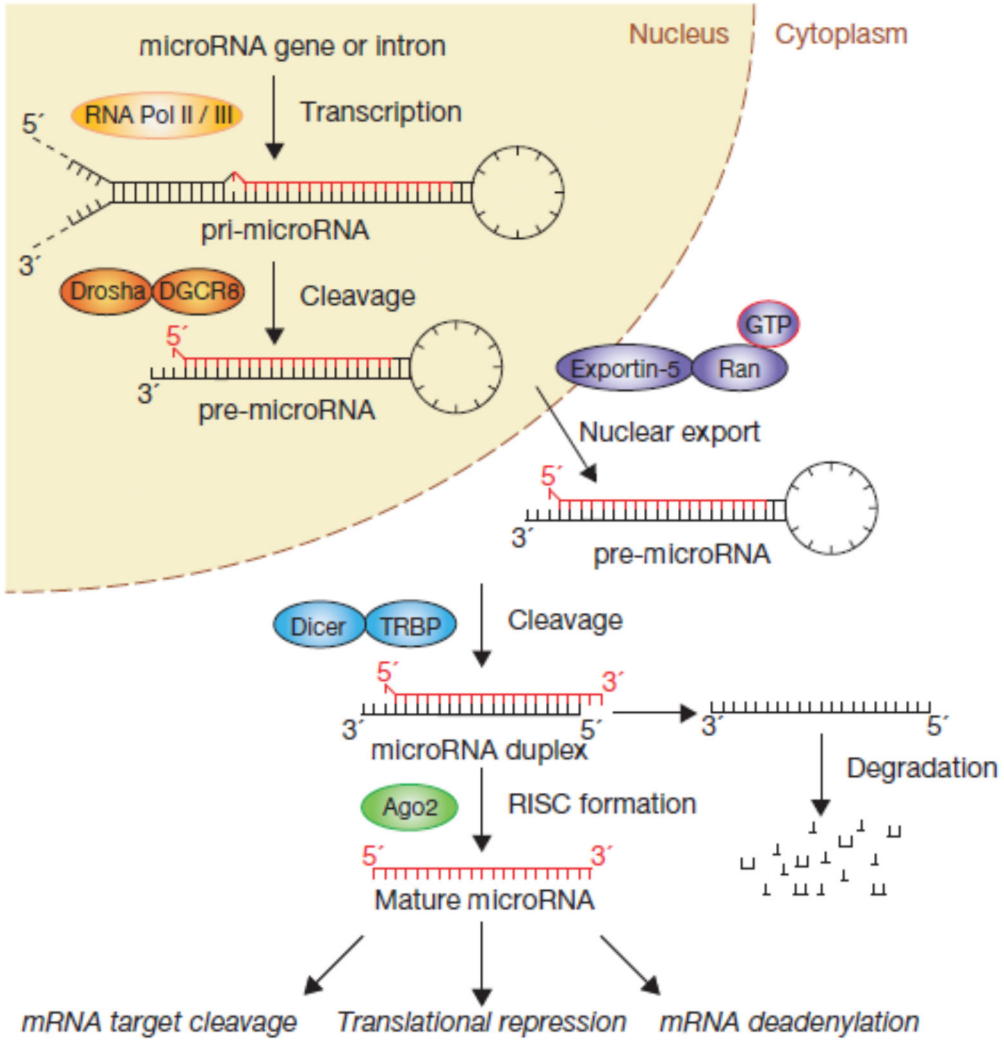



Figure 4.1: Canonical miRNA biogenesis and processing within mammals [93].

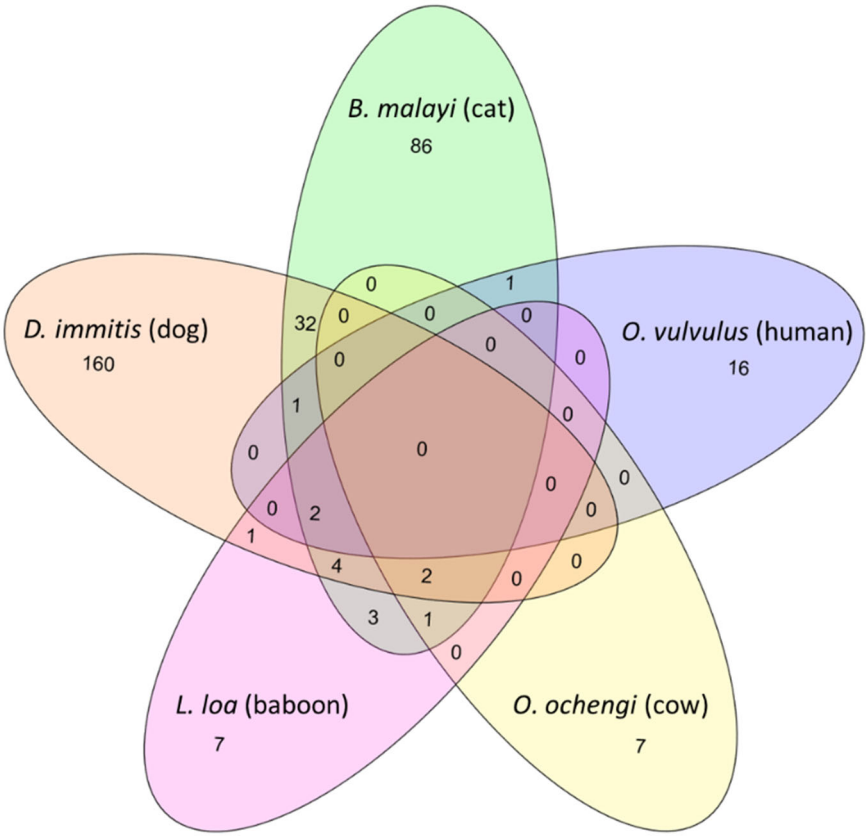



Figure 4.2: Venn diagram of selected filarioid miRNAs identified in host blood. The miRNA sequences for this diagram were from Tritten et al, 2014 [35, 94]

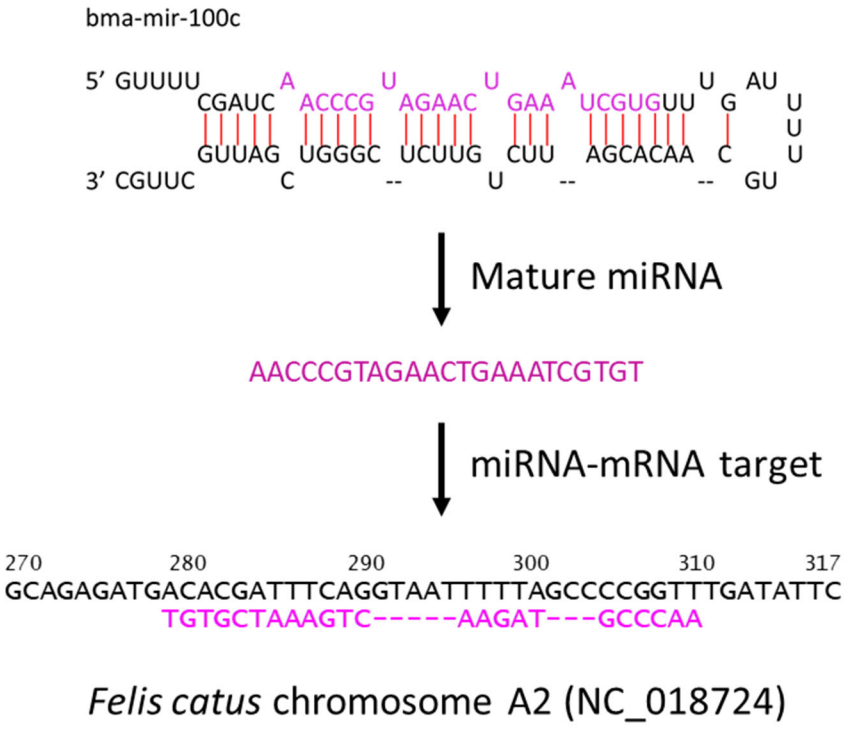



Figure 4.3: Example of *Brugia malayi* miRNA mir-100c target on *Felis catus* genome.

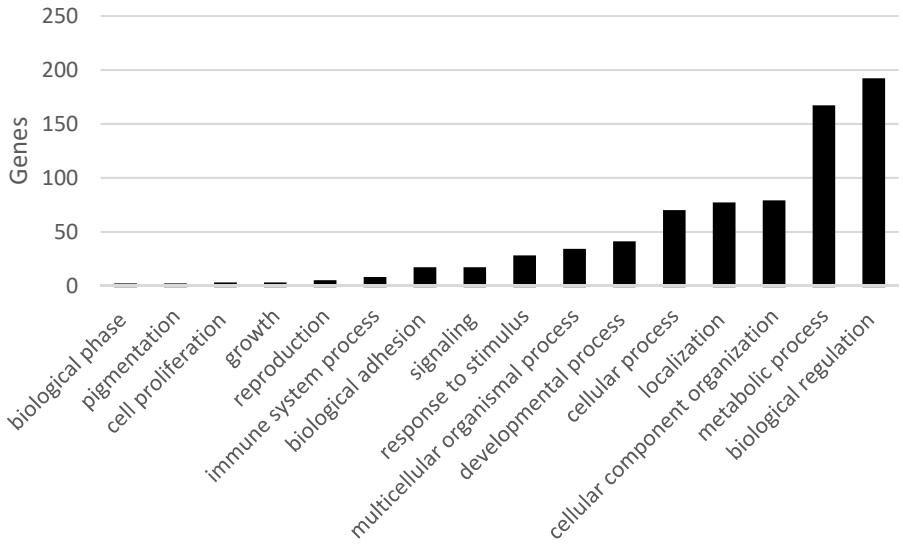



Figure 4.4: GO analysis of biological processes of key target genes.

Table S4.1: Clustering of 32,584,554 mappable reads used for the identification of *B. malayi* circulating miRNAs in cats

| Clusters | Cluster descriptions | Raw reads | % total reads | Unique sequences |
|---|---|---|---|---|
| **Raw reads** | | 41,164,433 | | |
| **Total mappable reads** | | 32,584,554 | 100 | |
| **Group 1a** | Reads map to *B. malayi* miRNAs/pre-miRNAs in miRbase and the pre-miRNAs further map to the genome & expressed sequence tag (EST). | 7,564 | 0.02 | 42 |
| **Group 1b** | Reads map to Nematoda* (except for *B. malayi*) miRNAs/pre-miRNAs in miRbase and the pre-miRNAs further map to the genome & EST. | 2 | <0.01 | 1 |
| **Group 2a** | Reads map to Nematoda miRNAs/pre-miRNAs in miRbase. The mapped pre-miRNAs do not map to the genome, but the reads (and of course the miRNAs of the pre-miRNAs) map to genome. The extended genome sequences from the genome loci may form hairpins. | 62 | <0.01 | 5 |
| **Group 2b** | Reads map to Nematoda miRNAs/pre-miRNAs in miRbase. The mapped pre-miRNAs do not map to the genome, but the reads (and of course the miRNAs of the pre-miRNAs) map to genome. The extended genome sequences from the genome loci may not form hairpins. | 16 | <0.01 | 1 |
| **Group 3a** | Reads map to Nematoda miRNAs/pre-miRNAs in miRbase. The mapped pre-miRNAs do not map to the genome, and the reads do not map to the genome | 13,568,080 | 41.64 | 31 |
| **Group 3b** | Reads map to Nematoda miRNAs/pre-miRNAs in miRbase, but cannot be classified into gp3a. | 3,766 | 0.01 | 99 |
| **Group 4a** | Reads do not map to Nematoda pre-miRNAs in miRbase. But the reads map to genome & the extended genome sequences from genome may form hairpins. | 169 | <0.01 | 17 |
| **Group 4b** | Reads do not map to nematoda pre-miRNAs in miRbase. But the reads map to genome & the extended genome sequences from genome may not form hairpins. | 27,774 | 0.09 | 0 |
| **No hit** | Sequences unlikely to be miRNAs or other RNA products | 5,310,864 | 16.30 | N/A |
| **Other RNA products** | Sequences corresponding to mRNA, rRNA, tRNA, repeatitive elements, and other RNA populations) | 237780 | 0.73 | N/A |

*** selected nematode species:** ***H. contortus, A. suum, S. ratti, P. redivivus, C. elegans, C. brenneri, C. remanei, C. briggsae, and P. pacificus***

Table S4.2: Filarioid miRNAs identified in host blood

| Filarioids in host blood* | Overlapped total | Candidate miRNAs |
|---|---|---|
| *B. malayi* (cat) *D. immitis* (dog) *L. loa* (baboon) *O. volvulus* (human) | 2 | miR-92 let-7 |
| *B. malayi* (cat) *D. immitis* (dog) *L. loa* (baboon) *O. ochengi* (cow) | 2 | lin-4 miR-100a |
| *B. malayi* (cat) *D. immitis* (dog) *L. loa* (baboon) | 4 | miR-100d miR-71 miR-100c miR-34 |
| *B. malayi* (cat) *D. immitis* (dog) *O. volvulus* (human) | 1 | miR-279 |
| *B. malayi* (cat) *L. loa* (baboon) *O. ochengi* (cow) | 1 | miR-39 |
| *B. malayi* (cat) *D. immitis* (dog) | 32 | miR-252 miR-993 miR-5866 miR-86 miR-5843 miR-5363 bantam mir-50 miR-228 miR-49 miR-50 miR-5360 miR-9 mir-92 miR-87a miR-81b mir-153 miR-2c miR-87b miR-250 miR-57 miR-1 miR-5845 mir-250 miR-5364 miR-81c miR-7 miR-240 miR-239 miR-307 miR-83 miR-100b |
| *B. malayi* (cat) *L. loa* (baboon) | 3 | mir-36 mir-35c mir-237 |
| *B. malayi* (cat) *O. volvulus* (human) | 1 | miR-750 |
| *D. immitis* (dog) *L. loa* (baboon) | 1 | miR-36 |
| *O. volvulus* (human) | 16 | PC-3p-1696273 PC-5p-31768 miR-7911c PC-5p-250919 PC-3p-250919 PC-3p-46055 miR-2266 miR-258 miR-5592 miR-63h miR-261 miR-2270 PC-5p-278615 PC-5p-464868 miR-5856a PC-5p-1545452 |
| *L. loa* (baboon) | 7 | PC-3p-21786 PC-3p-21742 PC-3p-70114 mir-5548 miR-36b PC-3p-13651 PC-3p-172942 |
| *O. ochengi* (cow) | 7 | miR-81 PC-3p-80582 mir-2264 PC-5p-151716 miR-7911a PC-3p-5495 PC-3p-41557 |

| | | |
|---|---|---|
| *B. malayi* (cat) | 86 | mir-35a mir-234 PC-3p-159115 mir-5939 miR-5849 mir-7590 mir-63c miR-37 mir-85 mir-2227 miR-35b miR-35l mir-5900 mir-8320 mir-5938 mir-281 mir-42 miR-35a mir-35k mir-100b mir-41b mir-255 mir-35e PC-3p-18742 mir-238 mir-71 miR-35n mir-90b mir-87c PC-5p-117327 mir-7918a miR-75 miR-35f mir-8325 miR-5882a mir-35m mir-2237b mir-35h mir-8216 mir-37 miR-7918a mir-1822 miR-40 mir-87 miR-5358a mir-62 mir-5358a mir-2272 mir-7579 mir-133 mir-8355 miR-5879a mir-1 mir-2263 mir-5911a mir-35d mir-8194 mir-64g miR-41 PC-3p-648320 mir-38 PC-5p-463032 mir-35g miR-38 miR-35g PC-5p-76160 mir-5991 mir-39 PC-3p-386693 mir-5971 mir-2241a PC-3p-716882 miR-5358b mir-750 PC-3p-302006 mir-58a mir-5592 mir-35f miR-5880a mir-5945 mir-5842 miR-35d mir-8192 mir-7938 mir-7926 PC-5p-988114 |
| *D. immitis* (dog) | 160 | miR-7964a PC-3p-568080 mir-2a PC-5p-285183 PC-3p-81000 mir-7690 miR-45 PC-5p-11277 PC-5p-375224 PC-3p-109773 PC-3p-290822 miR-153 PC-3p-484260 PC-3p-185335 mir-5987 miR-46 miR-7942 PC-3p-345994 PC-5p-409 PC-5p-404719 mir-5861 PC-3p-255794 mir-82 miR-2a PC-5p-86276 PC-5p-473895 PC-3p-127834 PC-5p-64126 PC-5p-34902 PC-5p-138632 PC-5p-247892 PC-3p-1208344 mir-5864 miR-5366 PC-5p-306323 PC-5p-214427 miR-60 mir-5925 mir-228 mir-86 PC-5p-333731 PC-3p-761877 PC-3p-97514 PC-3p-165941 PC-3p-397678 miR-277 PC-3p-96249 PC-5p-122212 PC-3p-11263 PC-5p-55699 PC-5p-684976 PC-5p-628017 PC-5p-329481 PC-3p-28561 PC-5p-420305 PC-3p-380603 mir-100d PC-5p-318873 PC-5p-339013 PC-3p-36872 PC-3p-60516 PC-3p-3520 PC-3p-210602 PC-3p-412500 PC-5p-1085309 PC-3p-359383 PC-3p-267479 PC-5p-1047523 mir-239 PC-3p-493780 PC-3p-258963 PC-3p-333843 PC-3p-208 PC-5p-985576 miR-1175 PC-3p-230207 PC-5p-134402 PC-3p-720916 miR-234 mir-124 PC-3p-372507 PC-3p-200816 PC-5p-223252 mir-279 PC-5p-4760 PC-3p-273888 PC-3p-718763 PC-3p-552248 PC-5p-69510 PC-3p-31697 mir-100a PC-3p-132 mir-283 mir-5363 miR-283 PC-5p-904737 PC-5p-345047 PC-3p-469670 mir-87b PC-3p-2144 PC-5p-563203 PC-5p-28007 mir-49 PC-3p-103769 PC-5p-493080 mir-5841 PC-3p-23103 miR-124 PC-3p-7315 miR-5361 PC-3p-212179 PC-3p-238008 mir-57 PC-3p-913230 PC-3p-275224 PC-5p-351138 PC-3p-806153 PC-3p-1339 PC-3p-227228 PC-3p-165115 PC-3p-327175 PC-5p-561628 PC-5p-416507 PC-5p-826524 PC-5p-930192 PC-5p-476422 PC-3p-143663 PC-5p-304241 PC-5p-30451 PC-3p-245224 PC-5p-374357 PC-3p-284644 PC-5p-764357 PC-3p-1063526 PC-3p-34138 PC-5p-807770 PC-5p-768112 PC-3p-137973 PC-3p-511787 PC-5p-775077 PC-5p-386730 miR-235 PC-3p-1000332 mir-5843 PC-5p-573542 PC-5p-268380 PC-5p-511406 PC-5p-1079097 PC-3p-375536 mir-7 PC-3p-607044 PC-3p-358504 miR-5365b PC-5p-137973 PC-3p-1092051 PC-3p-16232 PC-5p-238393 PC-3p-193291 PC-5p-229654 PC-3p-15096 |

*miRNA candidates previously described in Tritten et al, 2014 and Tritten et al, 2014(1).

Table S4.3: Top potential target transcripts of *B. malayi* miRNAs and PANTHER GO defined biological processes

| Gene ID | Family Name | Biological Process |
|---|---|---|
| Celf2 | CUGBP ELAV-like family member 2 | alternative mRNA splicing, via spliceosome(GO:0000380)<br>regulation of alternative mRNA splicing, via spliceosome(GO:0000381)<br>mRNA splice site selection(GO:0006376) |
| SIK2 | Serine/Threonine-protein kinase SIK2 | TOR signaling(GO:0031929)<br>negative regulation of TOR signaling(GO:0032007)<br>cellular response to starvation(GO:0009267) |
| Cyp2s1 | Cytochrome P450 2S1 | cellular catabolic process(GO:0044248)<br>cellular response to chemical stimulus(GO:0070887)<br>drug metabolic process(GO:0017144)<br>fatty acid metabolic process(GO:0006631)<br>response to drug(GO:0042493)<br>response to xenobiotic stimulus(GO:0009410) |
| Dtnb | Dystrobrevin Beta | cellular component morphogenesis(GO:0032989) |
| Nfia | Nuclear factor 1A-type | negative regulation of transcription by RNA polymerase II(GO:0000122)<br>positive regulation of transcription by RNA polymerase II(GO:0045944)<br>transcription by RNA polymerase II(GO:0006366) |
| Oprl1 | Nociceptin receptor | neuropeptide signaling pathway(GO:0007218)<br>sensory perception of pain(GO:0019233)<br>opioid receptor signaling pathway(GO:0038003) |
| Zbtb40 | Zinc finger and BTB domain | cellular response to DNA damage stimulus(GO:0006974) |
| Golga7b | Golgin subfamily A member 7B | peptidyl-amino acid modification(GO:0018193)<br>protein targeting to membrane(GO:0006612)<br>protein lipidation(GO:0006497)<br>protein acylation(GO:0043543) |

Table S4.4: Top potential target transcripts of *B. malayi* miRNAs and PANTHER GO cellular and molecular functions

| Gene ID | Family Name | Cellular Component |
|---|---|---|
| Celf2 | CUGBP ELAV-like family member 2 | cytoplasm(GO:0005737)<br>ribonucleoprotein complex(GO:1990904)<br>nucleus(GO:0005634) |
| SIK2 | Serine/Threonine-protein kinase SIK2 | cytoplasm(GO:0005737)<br>protein kinase complex(GO:1902911)<br>nucleus(GO:0005634) |
| Cyp2s1 | Cytochrome P450 2S1 | cytoplasm(GO:0005737)<br>intracellular membrane-bounded organelle(GO:0043231) |
| Nfia | Nuclear factor 1A-type | nuclear chromatin(GO:0000790) |
| Oprl1 | Nociceptin receptor | integral component of plasma membrane(GO:0005887) |
| Zbtb40 | Zinc finger and BTB domain | nucleus(GO:0005634) |
| Golga7b | Golgin subfamily A member 7B | intracellular(GO:0005622)<br>transferase complex(GO:1990234) |

| Gene ID | Family Name | Molecular function |
|---|---|---|
| Celf2 | CUGBP ELAV-like family member 2 | mRNA binding(GO:0003729) |
| SIK2 | Serine/Threonine-protein kinase SIK2 | protein serine/threonine kinase activity(GO:0004674) |
| Cyp2s1 | Cytochrome P450 2S1 | heme binding(GO:0020037)<br>oxidoreductase activity(GO:0016705)<br>steroid hydroxylase activity(GO:0008395) |
| Nfia | Nuclear factor 1A-type | RNA polymerase II transcription factor activity (GO:0000981) |
| Oprl1 | Nociceptin receptor | G-protein coupled peptide receptor activity(GO:0008528)<br>neuropeptide binding(GO:0042923) |
| Golga7b | Golgin subfamily A member 7B | catalytic activity (GO:0140096)<br>transferase activity (GO:0016747) |

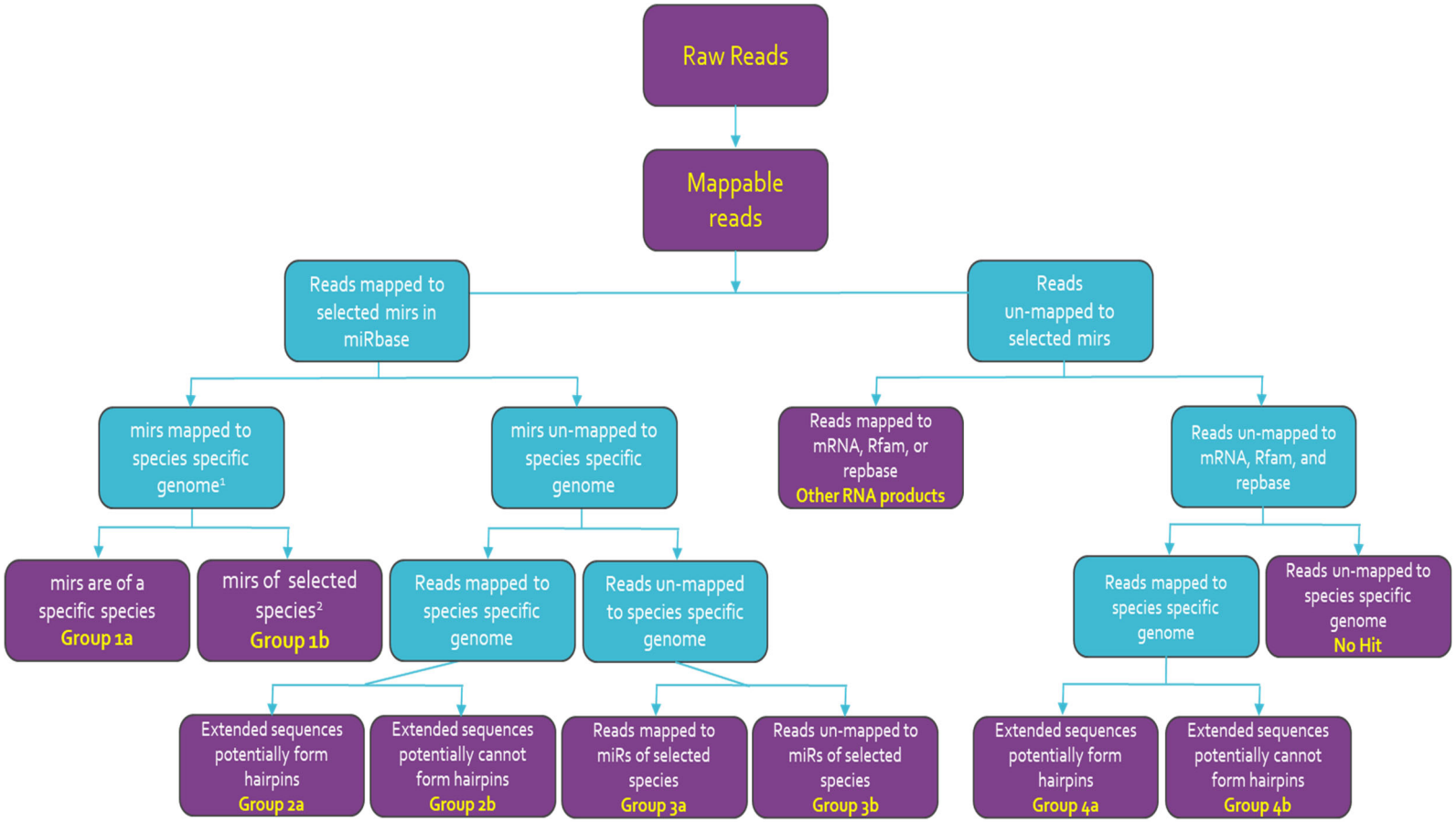



Figure S4.1: LC Sciences workflow for miRNA discovery. To filter out cat and other mammalian miRNAs, the selected species genome was *Felis catus*[1] and selected mammalian genomes[2]. The second workflow distinguished helminth miRNAs with the selected species genome *B. malayi*[1] and selected nematode[2] genomes.

# CHAPTER 5

## *SUMMARY AND CONCLUSIONS*

The works presented in this thesis aimed to investigate a holistic view of host-pathogen interactions of the filarial nematode *Brugia malayi*. Processes that affect the development and survival of these parasites were studied in both an in vitro environment and within infected mammalian hosts. The life stages evaluated were $L_3$ in vitro and adults and microfilariae within the host. At each developmental stage, there are unique challenges that these parasites must overcome in order to survive and develop to reproductive adults. These studies explored how *B. malayi* $L_3$ react when conditions are favorable for survival and/or development. We also examined host-response to these parasites, along with parasite response to the host. These works aim to advance our understanding of these complex organisms.

### Clinical findings of experimentally infected dogs and cats with *B. malayi*

Since the establishment of the *B. malayi* feline model over 60 years ago [1–4], no clinical reports on the disease progression in this animal model have been published. There are limited clinical data on human and non-human primates [5–8]. Limitations include short-term analysis, and in humans, knowing the exact date of infection is often impossible. In non-human primate studies, monitoring clinical data for the life span of the parasite has either been not within the scope of the research project or not financially reasonable. Due

to the long-term maintenance of *B. malayi* infected cats at the NIA/NIAID FR3, long-term data collection on these hosts were possible.

In addition to the cats, 11 dogs were infected. We used the same sub-periodic *B. malayi* strain propagated in the cat to infect these dogs. Initially, it was unknown whether dogs could become infected with this parasite, nor whether they would mature to reproductive adults. Surprisingly, the first round of infections resulted in a single dog producing circulating microfilariae (mf) and we were able to use the subsequent mf to infect additional dogs. For each round, mf counts improved, with the highest mf counts found in two dogs in round three. Due to the small sample size of this study, it is difficult to conclude whether this parasite was acclimating to the canine host or mere random chance. This manuscript provides the first evidence that in controlled infections canines can be a definitive host for *B. malayi*.

One of the more informative measures of the CBC results was eosinophil count. The cats maintained persistent eosinophilia throughout the experiment, whereas the dogs remained within the established reference range. Other helminths such as *Loa loa*, and *Trichinella spiralis*, have been observed to elevate eosinophilia in response to a primary infection [9–12]. However, in secondary infection with parasitic nematodes, eosinophils act to clear the worms and protect the host [12, 13]. In dogs, no eosinophilia was observed throughout the study. Other helminths, such as *Dirofilaria immitis* the canine heartworm and the ascarid *Toxocara canis*, elicit an eosinophilic response in dogs, and yet *B. malayi* did not [13, 14]. A number of published studies examine the protective effect of eosinophils for the parasites, suggesting they might be essential for the survival of these helminths [15–18]. *Brugia malayi* survives in the canine host, as evidenced by the development of

circulating mf, so we postulate that, while eosinophils may provide a protective effect and promote longevity and survival within the host-niche, they may not be essential for these parasites to develop to sexual maturity. Eosinophils may still yet play a role in the host response to *B. malayi* host response in domestic dogs, potentially in regards to a secondary infection with these parasites. Additionally, there is the potential for a localized response within tissues, not measurable systemically in the blood.

The other CBC measurements that differed between dogs and cats were lymphocytes, MPV, RBCs, and RBC indices (MCV, MCH, and MCHC). The average breakdown of these differences is shown in Table 5.1. In cats, the only differences noted from the CBC results within the mf-, mf+, and mf++ groups were elevated RBC and reduced MCV values only in the mf+ group indicating anemia as a response to these parasites. In the dogs, lymphocytes were elevated in the mf- group only, which may indicate lymphocyte-mediated clearance of these nematodes. No other differences were observed. Comparing intraspecies differences, eosinophils are elevated in the cats but not in the dogs. The dogs exhibited elevated MPV and reduced MCV values, where the cats remained within the established reference ranges. Lastly, the cats had values below the reference range for MCH, while dogs remained within the reference range.

The main purpose of this study was to present a full clinical profile of *B. malayi*-infected cats and dogs. Though the feline animal model has been utilized for *B. malayi* studies for more than sixty years, the clinical profile has not been documented until now. There are distinct differences between microfilaremia and amicrofilaremia, along with intraspecies differences. The addition of dogs to this clinical study demonstrates their permissivity, though they are not an ideal laboratory model. This does raise the question

of whether dogs in *B. malayi*-endemic areas could be a source of transmission, impacting elimination efforts and MDA programs in these regions. Dogs have been discovered to be the unexpected source of the parasitic nematode, the Guinea worm (*Dracunculus medinensis*), remaining at low levels until nearly eradicated in human populations [20, 21]. There is the potential that this also occurs in *B. malayi* endemic areas, where local dogs and cats would be a ubiquitous source for transmission.

Table 5.1: Summary of CBC data in dogs and cats infected with *B. malayi*.

| | Cat | | | Dog | |
|---|---|---|---|---|---|
| Cell type | mf- | mf+ | mf++ | mf- | mf+ |
| Eosinophils | ↑ | ↑ | ↑ | - | - |
| Lymphocytes | - | - | - | ↑ | - |
| Red blood cells | - | ↑ | - | - | - |
| MPV | nm | nm | nm | ↑ | ↑ |
| MCV | - | ↓ | - | ↓ | ↓ |
| MCH | ↓ | ↓ | ↓ | - | - |
| MCHC | - | - | - | - | - |
| Monocytes | - | - | - | - | - |
| Basophils | - | - | - | - | - |
| White blood cells | - | - | - | - | - |
| Hgb | - | - | - | - | - |
| Hct | - | - | - | - | - |
| Segs | - | - | - | - | - |
| Bands | - | - | - | - | - |

| | |
|---|---|
| ↑ | Elevated above reference range |
| ↓ | Below reference range |
| - | Within reference range |
| nm | Not measured |

**The effect of heavy metals on *B. malayi* $L_3$ in in vitro assays**

The utilization of in vitro assays on *B. malayi* has been ongoing in the in providing a platform to study these parasites outside the mammalian host. In chapter 3, the aim was to evaluate whether the addition of heavy metals would enhance survival, motility, and development within *B. malayi* $L_3$. We hypothesized that zinc and iron ions will facilitate *B. malayi* $L_3$ molting and survival, whereas copper ions will hinder development. In addition to motility and survival, evaluation of the morphology of all the larvae was performed to determine whether these heavy metals exhibited an effect on the development to the $L_4$ stage. Though both iron and zinc additives did improve overall survival and development respectively, the $L_3$ did not fully develop past the early $L_4$ stage. As such, the additions of these metals to in vitro cultures are insufficient to enable these $L_3$ to fully mature to reproductive adults.

Successfully culture of filarial nematodes in an in vitro relies on a number of crucial factors for parasite survival and development. Determining the ideal conditions to culture these parasites must take into consideration factors such as nutrients, temperature, pH, atmospheric pressure, etc. Studies on the non-parasitic nematode *C. elegans* have revealed that the addition of heavy metals in vitro is beneficial [22]. Heavy metals, such as iron and zinc, are essential nutrients acquired either via environmental exposure or ingestion through a food source. However, these metals, aside from trace amounts in media and serum, have not been previously added to in vitro cultures of *B. malayi*. Iron (II) sulfate heptahydrate, zinc sulfate heptahydrate, and copper (II) sulfate pentahydrate were added to *B. malayi* third-stage ($L_3$) larval cultures to measure the effects of these compounds on the parasites.

Coinciding with molting, other cofactors must be present in the host environment in order for the filarial nematode to develop correctly before and after the molt. Iron (Fe) is an essential nutrient, required for oxygen transport, heme incorporation, and an enzymatic cofactor for biological processes such as energy production and DNA synthesis [23]. Most complex organisms are able to synthesize heme through biosynthetic pathways [24] whereas free-living and parasitic nematodes must acquire heme exogenously [25]. Parasitic nematodes, such as *B. malayi*, have seemingly lost the ability to synthesize heme [22, 23], relying instead on *Wolbachia* (*w*Bm), an obligate bacterial endosymbiont [24–26]. These symbiotic bacteria have lost a number of biological functions to survive independently, yet maintain key metabolic functions lacking in the nematode host, notably heme synthesis [27–30]. In chapter 2, microfilaremic cats exhibited elevated RBC values throughout infection with *B. malayi*, suggesting a form of immune-mediated anemia not seen in amicrofilaremic cats nor any of the dogs [34]. *Brugia malayi*, like many nematodes, must acquire heme from exogenous sources due to lack of a heme biosynthetic pathway [42-43]. Though the mechanism remains unknown, we postulate that these worms were either able to acquire heme from host RBCs or cause a disruption of erythropoiesis. As heme is vital for many porphyrin-containing metalloproteins within free-living and parasitic nematodes, iron availability for these organisms could be vital [35].

In this study, we do find that the addition of zinc sulfate heptahydrate and iron (II) sulfate heptahydrate, and copper (II) sulfate pentahydrate effect *B. malayi* larvae when added to in vitro assays. The copper, as seen with STH and GI nematodes, kills the larvae quickly, even at low physiological concentrations. The addition of iron was found to extend survival, but development was stunted. The opposite was true with the addition of zinc,

where survival was shortened, but development progressed at a higher percentage than was seen in the iron and control groups. As with previous cultures with these parasites, optimal conditions in increasing both the survival and development of *B. malayi* $L_3$ remain elusive. If these heavy metals could affect these larvae within an in vivo host without undue toxicity, they may prove to be a novel addition to augment current anthelmintics. If a drug or additive can induce the molt resulting in a shortened life-span and developmental stunting, resulting in slow killing versus instant death, this may provide an additional method for parasite control.

### ***Brugia malayi*** **miRNAs and their immune gene targets in cats**

Chapter 4 examined parasite-derived miRNAs found in *B. malayi* plasma of *B. malayi* infected cats. This study is the first to identify *B. malayi* miRNAs within this mammalian host. MicroRNAs have been previously found to be highly stable in blood products (plasma and serum) making them ideal candidates for their use as potential biomarkers and diagnostic tools [33–37].

In this study, 26 highly expressed miRNAs were predicted to be parasite-derived. These miRNAs sequences were then mined against the feline genome to determine what genes were being targeted by these filarioids. This study found seven immune genes targeted by these parasite-derived miRNA. Parasite-secreted miRNAs have been implicated to play a key role in parasite establishment by down-regulation of host gene expression [34, 38]. Additionally, other parasitic nematodes such as *Schistosoma spp*., *Haemonchus contortus*, and *Dirofilaria immitis* have also been shown to secrete small RNA species with potential roles in host-modulation [33, 39–43]. Therefore, miRNA

manipulation by the parasite is a powerful tool utilized by these helminths in order to survive and thrive within a suitable host.

A total of 185 miRNAs were identified in *B. malayi* infected cat blood. Of these, 26 miRNAs were highly expressed. These miRNAs were then processed through a bioinformatics analysis using TargetScan, to identify potential gene targets on the feline genome. A large dataset of gene hits was composed and sorted by TargetScan weighted context scores. A total of 5% of the dataset were selected for GO analysis. As a result, nine gene transcripts that could potentially modulate host immunity were identified, *Celf2, Sik2, Ptgs1, Irf4, Irf5, Numbl, Tnfsf15, Stat3*, and *Txlnb*. The two most highly targeted genes were *Celf2 and Sik2,* involved in T-cell proliferation and IL-10 secretion by macrophages. Additional gene transcription targets have been characterized in numerous studies to be involved in oxidoreductase activity, pro-inflammatory activation, lymphocyte and endothelial cell proliferation, and cytokine mediation. These immune functions have been found in previous studies to be downregulated by helminths [43–49]. However, because gene transcript targets were identified by in silico, experimental confirmation is necessary to determine if they are true parasite-derived targets. If these targets are suppressed by this parasite-host interaction through miRNAs, this would prove to be another methodology that these worms use in host-modulation for survival within the mammalian host.

**Final remarks**

The first study, chapter 2, aimed at finding early clinical indicators of infection that would indicate a difference in hosts resulting in a patent infection versus those that remained amicrofilaremic. Within the cats, we primarily note differences between cats with

a delayed microfilaremia versus those that obtained patency during a typical timeframe or those that remained amicrofilaremic. Why and how are these infections delayed? Possibilities include immune suppression by the host, arrested development, or even larval migration to tissues outside the lymphatic system. In our in vitro experiments, we noted that when the molt was delayed, survival was extended in the $L_3$. Other parasites, such as *C. elegans*, *Onchocerca gutturose*, *O. lienalis*, and *B. pahangi,* enter an arrested developmental stage when environmental or host conditions are not ideal [50, 51]. There is the possibility that *B. malayi* $L_3$ development can be inhibited in order to increase their chance of survival when conditions are favorable.

Parasite-secreted miRNAs have been implicated to play a key role in parasite establishment by down-regulation of host gene expression [34, 38]. MicroRNA predicted targets on the feline genome conveys the complex manner by which these helminths are able to alter the host environment to ensure their own survival. However, immune genes can differ significantly between even closely related hosts. Though miRNAs do not have to match identically to host sequences to cause destabilization or repression, some conservation is essential. We propose that this could be a manner by which host-specificity occurs. These findings suggest a molecular mechanism by which these parasites are able to directly alter host cells to provide an environment for survival. Overall, these findings add to the understanding of how these parasitic helminths are able to alter, manipulate, and ultimately survive within the harsh immunological environment of the mammalian host.

## REFERENCES


[1] T. R. Klei and T. V Rajan, *World Class Parasites: Volume 5 The Filaria*. Boston: Kluwer Academic Publishers, 2002.

[2] L. M. Ganley-leal, "The influence of host sex on the L3 to L4 Molt of *Brugia malayi*," *J. Parasitol.*, vol. 91, no. 5, pp. 1222–1225, 2005.

[3] J. W. Mak, P. K. F. Yen, K. C. Lim, and N. Ramiah, "Zoonotic implications of cats and dogs in filarial transmission in peninsular Malaysia," *Trop. Geogr. Med.*, vol. 32, pp. 259–264, 1979.

[4] J. W. McCall, "The Role of Arthropods in the Development of Animal Models for Filariasis Research," *J. Georg. Entomol. Soc.*, vol. 16, pp. 283–293, 1981.

[5] J. R. Palmieri, D. H. Connor, Purnomo, and H. A. Marwoto, "*Wuchereria bancrofti* infection in the silvered leaf monkey (*Presbytis cristatus*)," *Am. J. Pathol.*, vol. 112, no. 3, pp. 107–108, 1983.

[6] R. P. Ghosh, P. K. Murthy, K. Tyagi, P. S. R. Murthy, and R. K. Chatterjee, "Longitudinal cellular immune responses in asymptomatic and symptomatic *Brugia malayi*-infected Indian leaf monkey *Presbytis entellus*," *J. Parasitol.*, vol. 85, no. 5, pp. 861–866, 1999.

[7] R. B. Crandall, C. A. Crandall, and J. T. Neilson, "Antibody responses to experimental *Brugia malayi* infections in Patas and Rhesus monkeys," *Acta Trop.*, vol. 40, pp. 53–64, 1983.

[8] S. Bonne-Annee and T. B. Nutman, "Human innate lymphoid cells (ILCs) in filarial infections," *Parasite Immunol.*, vol. 40, no. February, pp. 1–9, 2017.

[9] J. A. Herrick *et al.*, “Eosinophil-associated processes underlie differences in clinical presentation of loiasis between temporary residents and those indigenous to loa-endemic areas,” *Clin. Infect. Dis.*, vol. 60, no. 1, pp. 55–63, 2015.

[10] L. Huang *et al.*, “Eosinophil-derived IL-10 supports chronic nematode infection,” *J. Immunol.*, vol. 193, no. 8, pp. 4178–4187, 2014.

[11] N. G. Gebreselassie *et al.*, “Eosinophils preserve parasitic nematode larvae by regulating local immunity.,” *J. Immunol.*, vol. 188, no. 1, pp. 417–25, 2012.

[12] L. Huang *et al.*, “Eosinophils mediate protective immunity against secondary nematode infection,” *J. Immunol.*, vol. 194, no. 1, pp. 283–90, 2015.

[13] M. L. Knott, K. I. Matthaei, P. R. Giacomin, H. Wang, P. S. Foster, and L. A. Dent, “Impaired resistance in early secondary *Nippostrongylus brasiliensis* infections in mice with defective eosinophilopoeisis,” *Int. J. Parasitol.*, vol. 37, pp. 1367–1378, 2007.

[14] A. S. Fahrion, S. Staebler, and P. Deplazes, “Patent *Toxocara canis* infections in previously exposed and in helminth-free dogs after infection with low numbers of embryonated eggs,” *Vet. Parasitol.*, vol. 152, pp. 108–115, 2008.

[15] J. Margareth and D. A. Rodolpho, “Robust phenotypic activation of eosinophils during experimental *Toxocara canis* infection experimental animals and parasites,” *Front. Immunol.*, vol. 9, no. January, 2018.

[16] T. Ramalingam, P. Porte, J. Lee, and T. V. Rajan, “Eosinophils, but not eosinophil peroxidase or major basic protein, are important for host protection in experimental *Brugia pahangi* infection,” *Infect. Immun.*, vol. 73, no. 12, pp. 8442–8443, 2005.

[17] M. H. Shin, Y. A. Lee, and D. Y. Min, “Eosinophil-mediated tissue inflammatory responses in helminth infection,” *Korean J. Parasitol.*, vol. 47, no. SUPPL., pp. 125–132, 2009.

[18] S. Specht *et al.*, “Lack of eosinophil peroxidase or major basic protein impairs defense against murine filarial infection,” *Infect. Immun.*, vol. 74, no. 9, pp. 5236–5243, 2006.

[19] S. A. Babayan, A. F. Read, R. A. Lawrence, O. Bain, and J. E. Allen, “Filarial parasites develop faster and reproduce earlier in response to host immune effectors that determine filarial life expectancy.,” *PLoS Biol.*, vol. 8, no. 10, p. e1000525, Jan. 2010.

[20] E. Callaway, “Dogs thwart end to Guinea worm,” *Nat. news*, vol. 529, no. 7584, pp. 10–11, 2016.

[21] M. L. Eberhard *et al.*, “The Peculiar Epidemiology of Dracunculiasis in Chad,” *Am. J. Trop. Med. Hyg.*, vol. 90, no. 1, pp. 61–70, 2014.

[22] M. W. Davis, A. J. Birnie, A. C. Chan, A. P. Page, and E. M. Jorgensen, “A conserved metalloprotease mediates ecdysis in *Caenorhabditis elegans*,” *Development*, vol. 131, no. 23, pp. 6001–8, 2004.

[23] P. Aisen, C. Enns, and M. Wessling-resnick, “Chemistry and biology of eukaryotic iron metabolism,” *Int. J. Biochem. Cell Biol.*, vol. 33, pp. 940–959, 2001.

[24] L. Koreny, J. Lukes, and M. Obornik, “Evolution of the haem synthetic pathway in kinetoplastid flagellates: An essential pathway that is not essential after all?,” *Int. J. Parasitol.*, vol. 40, pp. 149–156, 2010.

[25] A. U. Rao, L. K. Carta, E. Lesuisse, and I. Hamza, “Lack of heme synthesis in a free-living eukaryote,” *Proc. Natl. Acad. Sci.*, vol. 102, no. 12, pp. 4270–4275, 2005.

[26] S. Kumar *et al.*, “Mining predicted essential genes of *Brugia malayi* for nematode drug targets,” *PLoS One*, no. 11, pp. 1–9, 2007.

[27] A. N. Luck, X. Yuan, D. Voronin, B. E. Slatko, I. Hamza, and J. M. Foster, “Heme acquisition in the parasitic filarial nematode *Brugia malayi*,” *FASEB J.*, vol. 30, no. 10, pp. 3501–3514, 2016.

[28] B. Wu *et al.*, “The heme biosynthetic pathway of the obligate *Wolbachia* endosymbiont of *Brugia malayi* as a potential anti-filarial drug target,” *PLoS Negl. Trop. Dis.*, vol. 3, no. 7, pp. 1–10, 2009.

[29] C. Ballesteros, “Iron acquisition in the adult filarial nematode *Brugia malayi*,” McGill University, 2016.

[30] B. E. Slatko, A. N. Luck, S. L. Dobson, and J. M. Foster, “Molecular & biochemical parasitology *Wolbachia* endosymbionts and human disease control,” *Mol. Biochem. Parasitol.*, vol. 195, no. 2, pp. 88–95, 2014.

[31] F. Landmann, J. M. Foster, M. L. Michalski, B. E. Slatko, and W. Sullivan, “Co-evolution between an endosymbiont and its nematode host: *Wolbachia* asymmetric posterior localization and AP polarity establishment,” *PLoS Negl. Trop. Dis.*, vol. 8, no. 8, 2014.

[32] J. Foster *et al.*, “*Wolbachia* genome of *Brugia malayi*: Endosymbiont evolution within a human pathogenic nematode,” *PLOS Biol.*, vol. 3, no. 4, pp. 599–614, 2005.

[33] E. Ghedin *et al.*, “Draft genome of the filarial nematode parasite *Brugia malayi*,” *Science (80-. ).*, vol. 317, no. 5845, pp. 1756–1760, 2007.

[34] A. Tefferi, C. A. Hanson, and D. J. Inwards, “Complete blood cell count in adults,” *Mayo Clin. Proc.*, vol. 80, no. 7, pp. 923–936, 2005.

[35] A. C. Gill, A. C. Darby, and B. L. Makepeace, “Iron necessity: The secret of *Wolbachia*’s success?,” *PLoS Negl. Trop. Dis.*, vol. 8, no. 10, pp. 1–8, 2014.

[36] A. D. Winter *et al.*, “Diversity in parasitic nematode genomes: the microRNAs of *Brugia pahangi* and *Haemonchus contortus* are largely novel,” *BMC Genomics*, vol. 13, no. 1, p. 4, Jan. 2012.

[37] D. W. Thomson, C. P. Bracken, and G. J. Goodall, “Experimental strategies for microRNA target identification,” *Nucleic Acids Res.*, vol. 39, no. 16, pp. 6845–6853, 2011.

[38] J. R. Lytle, T. A. Yario, and J. A. Steitz, “Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5’ UTR as in the 3’ UTR,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 23, pp. 9667–9672, 2007.

[39] R. C. Friedman, K. K. Farh, C. B. Burge, and D. P. Bartel, “Most mammalian mRNAs are conserved targets of microRNAs,” *Genome Res.*, vol. 19, pp. 92–105, 2009.

[40] M. Hakimi and D. Cannella, “Apicomplexan parasites and subversion of the host cell microRNA pathway,” *Trends Parasitol.*, vol. 27, no. 11, pp. 481–486, 2011.

[41] A. H. Buck *et al.*, “Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity,” *Nat. Commun.*, vol. 5, p. 5488, 2014.

[42] L. Tritten, D. Clarke, S. Timmins, T. Mctier, and T. G. Geary, "*Dirofilaria immitis* exhibits sex- and stage-specific differences in excretory/secretory miRNA and protein profiles," *Vet. Parasitol.*, vol. 232, pp. 1–7, 2016.

[43] L. Tritten *et al.*, "Detection of circulating parasite-derived microRNAs in filarial infections," *PLoS Negl. Trop. Dis.*, vol. 8, no. 7, p. e2971, Jul. 2014.

[44] M. Zamanian *et al.*, "Release of small RNA-containing exosome-like vesicles from the human filarial parasite *Brugia malayi*," *PLoS Negl. Trop. Dis.*, vol. 9, no. 9, pp. 1–23, 2015.

[45] H. Han *et al.*, "MicroRNA expression profile in different tissues of BALB/c mice in the early phase of *Schistosoma japonicum* infection," *Mol. Biochem. Parasitol.*, vol. 188, no. 1, pp. 1–9, Mar. 2013.

[46] M. de Souza Gomes, M. K. Muniyappa, S. G. Carvalho, R. Guerra-Sá, and C. Spillane, "Genome-wide identification of novel microRNAs and their target genes in the human parasite *Schistosoma mansoni*," *Genomics*, vol. 98, no. 2, pp. 96–111, 2011.

[47] E. Cretney *et al.*, "The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells," *Nat. Immunol.*, vol. 12, no. 4, pp. 304–313, 2011.

[48] C. C. Motran and L. Cervi, "Helminth infections: Recognition and modulation of the immune response by innate immune Cells," *Front. Immunol.*, vol. 9, no. April, pp. 1–12, 2018.

[49] J. P. Hewitson, J. R. Grainger, and R. M. Maizels, "Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity," *Mol.*

*Biochem. Parasitol.*, vol. 167, no. 1, pp. 1–11, 2009.

[50] H. J. McSorley, J. P. Hewitson, and R. M. Maizels, "Immunomodulation by helminth parasites: Defining mechanisms and mediators," *Int. J. Parasitol.*, vol. 43, no. 3–4, pp. 301–310, 2013.

[51] N. M. Girgis, U. M. Gundra, and P. Loke, "Immune regulation during helminth infections," *PLoS Pathog.*, vol. 9, no. 4, p. e1003250, Apr. 2013.

[52] R. M. Maizels, N. Gomez-escobar, W. F. Gregory, J. Murray, and X. Zang, "Immune evasion genes from filarial nematodes," *Int. J. Parasitol.*, vol. 31, pp. 889–898, 2001.

[53] R. T. Semnani *et al.*, "Filaria-induced monocyte dysfunction and its reversal following treatment," *Infect. Immun.*, vol. 74, no. 8, pp. 4409–17, Aug. 2006.

[54] S. Townson *et al.*, "Antibiotics and *Wolbachia* in filarial nematodes: antifilarial activity of rifampicin, oxytetracycline and chloramphenicol against *Onchocerca gutturosa, Onchocerca lienalis* and *Brugia pahangi*," *Ann. Trop. Med. Parasitol.*, vol. 94, no. 8, pp. 801–816, 2000.

[55] P. S. Riddle, D.L., Albert, "Genetic and environmental regulation of dauer larva development," in *C. elegans II.*, 2nd ed., J. R. Riddle, D.L., Blumenthal, T., Meyer, B.J., Priess, Ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1997, pp. 739–768.