THE IMPACT OF *IN VIVO* DOXYCYCLINE ADMINISTRATION ON *WOLBACHIA* AT DIFFERENT LIFECYCLE STAGES IN CANINE HEARTWORM (*DIROFILARIA IMMITIS*)

by

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(Under the Direction of Andrew R. Moorhead)

ABSTRACT

Canine heartworm disease is caused by the filarial nematode *Dirofilaria immitis*, which has been found on all continents except Antarctica. The endosymbiont of several filarial nematode species, *Wolbachia*, is crucial in the reproduction, development, and survival of the filarial nematode. *Wolbachia* also contributes to immunomodulation in the vertebrate hosts of the nematodes, supporting the establishment of infection. The investigation of the characteristics of this endosymbiont provided novel insights into drug targets for filarial disease control and treatment. In the process of treating filarial nematodes, the acute or chronic release of *Wolbachia* can be concerning as *Wolbachia* induces inflammatory reactions, which lead to pathologic lesions. Doxycycline targets *Wolbachia* and has been utilized in the treatment of onchocerciasis and dirofilariasis. In heartworm treatment, the American Heartworm Society (AHS) recommends a doxycycline treatment at 10 mg/kg twice daily for 28 days before adulticidal treatment. However, the phenotypes and mechanisms of the impact of doxycycline on *Wolbachia* and, consequently, *D. immitis* require further investigation. In this dissertation, chapter 2 discusses a possible mechanism behind the phenotype that doxycycline-treated microfilariae (mf) cannot

complete a new lifecycle in subsequent hosts. Chapter 3 presents the *Wolbachia* levels in adult *D. immitis* with doxycycline treatment at different dosages. Chapter 4 explores gene expression alterations in *D. immitis* mf in the presence or absence of doxycycline treatment. This dissertation's research investigated the impact of doxycycline on *Wolbachia* and *D. immitis* at different life cycle stages (mf, infective stage larvae, and adult), providing insights into the basic science of heartworm research and clinical practice in heartworm treatment.

INDEX WORDS: *Dirofilaria immitis*, canine heartworm, doxycycline, *Wolbachia*, quantitative PCR, gene expression

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DEDICATION

To my loving husband, Ruochong Wang, thank you for your unwavering support, patience, and belief in me. This achievement is ours.

To my wonderful parents, Lixin Li and Wensheng Chu, thank you for your unconditional love and support for me. I hope this achievement makes you proud, as I am forever grateful to you. This work is dedicated to you both with the deepest gratitude and love.

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ABBREVIATION

AHS	American Heartworm Society
BALB/c	Bagg Albino mice with the genotype at the color locus of c/c
BID	Twice daily
CDC	Centers for Disease Control and Prevention
cDNA	Complimentary DNA
COX	Cytochrome C oxidase
FAD	Flavin Adenine Dinucleotide
FBS	Fetal Bovin Serum
FDA	U.S. Food and Drug Administration
FR3	Filariasis Research Reagent Resource Center
GABA	Gamma-aminobutyric acid
GI	Gastrointestinal
GO	Gene Ontology
HRP	Horseradish Peroxidase
HSP60	Heat-Shock Protein 60
HWD	Heartworm Disease
IFN	Interferons
IL	Interleukins
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	Inducible Nitric Oxide Synthase
IVM	Ivermectin
L1	First-stage Larvae
L2	Second-stage Larvae
L3	Third-stage Larvae
L4	Fourth-stage Larvae
LMM	Linear Mixed Model
LPS	Lipopolysaccharide
MEL	Melarsomine
mf	Microfilariae
ML	Macrocyclic Lactones
MO	Missouri strain
mtDNA	Mitochondria DNA
NCBI	National Center for Biotechnology Information
NO	Nitric Oxide
OD	Optical Density
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
PMN	Polymorphonuclear leukocyte

*w*Di	*Wolbachia* of *Dirofilaria immitis*
WSP	*Wolbachia* surface protein
Th	T helper cells
TLR	Toll-like Receptors
THS	Toronto Humane Society
TNF	Tumor Necrosis Factor
rRNA	Ribosome RNA

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

History and biology of canine heartworm

1) Discovery and life cycle of *Dirofilaria immitis*

Heartworm disease is caused by the nematode *Dirofilaria immitis*. This thread-like roundworm usually resides in the pulmonary artery of the host. The first case of canine heartworm in the United States was recorded in 1847, with the description of "large blood-vessels were found filled with worms" [1]. In the following one hundred years, studies focused

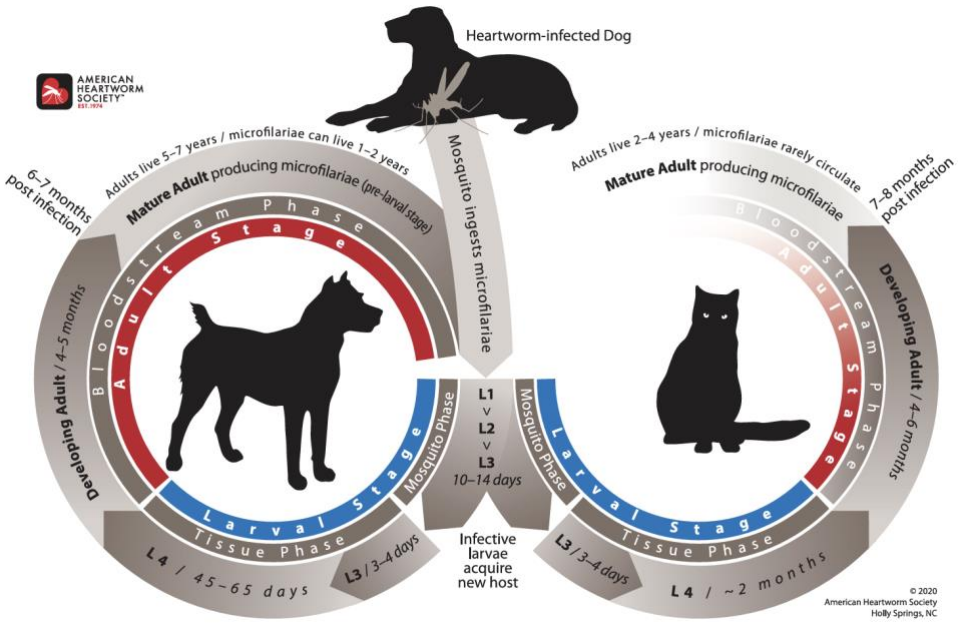

Figure 1.

Dirofilaria immitis life cycle. Picture by American Heartworm Society. URL: https://www.heartwormsociety.org/pet-owner-resources/2014-03-24-22-40-20

on the completion of the life cycle of this parasite, including the relationship between microfilariae (mf) and adults, the development of mf in the intermediate host, and the route of migration of the larvae in the dog [2].

Canids (including domestic dogs, coyotes, foxes, and wolves) are considered the definitive hosts for this parasite. However, cases can be found in other species, such as domestic cats, bears, ferrets, seals, sea lions, and humans [3, 4]. Adult heartworms have a life span of 5 – 7 years; the mf can live for 1 – 2 years (Fig. 1). The life cycle of *D. immitis* requires both intermediate and definitive hosts. The cycle starts as the susceptible mosquito, the intermediate host, takes a blood meal from a microfilaremic vertebrate host. The mf remains in the midgut of the mosquito before migrating to the large cells of the Malpighian tubes. The larvae will go through a "sausage" stage, in which they develop an esophagus, intestine, and rectum. Six or seven days after the blood meal, the larvae enter the lumen of the tubules, where they finish the first molt. The second molt occurs at around thirteen days. After two molts, the now infective stage larvae (L3) migrate to the head and mouthparts of mosquitoes [4, 5]. Vectors that carry the L3 can then infect the subsequent host by taking another blood meal, and the L3 enter the host via bite wounds on the skin. The molt from L3 to L4 begins as early as three days post infection, and the L4 start to migrate in the subcutaneous tissues and muscle. The worms reach the heart 67-120 days post infection as immature adults. The immature adult reaches the pulmonary artery, where it increases in size and becomes sexually mature. Microfilariae can be detected as early as 180 days post infection [3, 4, 6, 7] (Table 1.1; Figure 1).

Table 1.1

Development of *D. immitis* in hosts.

Day	Stage	Length (cm)	Host	Location	Event

0	mf	0.03	Canine	Blood	Mosquito ingests microfilaremic blood
1	mf	0.03	Mosquito	Mid-gut	mf stay in mid-gut.
2 – 5	mf	0.03 – 0.015	Mosquito	Malpighian tubules, cytoplasm of the primary cells	Grow into "sausage" stage (L1). Development of gut starts on day 5.
6 – 7	L1	0.015	Mosquito	Malpighian tubules lumen	L1 move out of cell, enter the lumen of Malpighian tubules
8 – 10	L2	0.05	Mosquito	Malpighian tubules lumen	First molt
12 – 13	L3	0.07 – 0.13	Mosquito	Malpighian tubules lumen	Second molt
14 – 15	L3	0.07 – 0.13	Mosquito	Malpighian tubules lumen	L3 migrate to the head and mouth parts.
15 (0*)	L3	0.07 – 0.13	Canine	Subcutaneous tissue near the entry site	L3 enter the host via puncture wound caused by mosquito taking a blood meal.
18 – 27 (3 – 12*)	L3 – L4	0.1 – 0.15	Canine	Subcutaneous tissue near the entry site	Third molt
36 (21*)	L4	0.2 – 0.3	Canine	Abdomen	L3 and L4 migrate in subcutaneous and muscle tissues.
65 – 85 (50 – 70*)	L4 – immature adult	1 – 2 [a] 2 – 4 [b]	Canine	Thorax, abdomen, head, neck, forelimbs	Final molt. Immature adults migrate in subcutaneous and muscle tissues.
85 – 125 (67 – 110*)	Immature adult	2 – 4 [b] 10 – 12 [c]	Canine	Head, neck, forelimbs, thorax, abdomen; Right side of heart, pulmonary artery	First worm enters the blood vessels on day 67. The worms grow in size intensively until they become sexually mature.
135 (120*)	Sexually mature adult	10 – 12	Canine	Right side of heart, pulmonary artery	
195 (180*)	Sexually mature adult	M: 12 – 20 F: 25 - 31	Canine	Right side of heart, pulmonary artery	Adults start to produce mf. Adults can live 5 – 7 years.

195 (180*)	mf	0.03	Canine	Blood	Mf can live 1 – 2 years.

Notes:

mf: microfilariae; L1: First-stage larvae; L2: Second-stage larvae; L3: Third-stage larvae; L4: Fourth-stage larvae; M: male; F: female. a: around day 65; b: around day 85; c: around day 125.

*: number in parentheses are days in canine host.

Complied and adapted from [4-6, 8, 9].

2) The third-stage to fourth-stage larval molt

As stated above, *D. immitis* requires multiple molts to finish its lifecycle. The first molt that occurs in vertebrate hosts is the L3 to L4 molt. In the definitive hosts, this molt occurs between 3 – 12 days post infection. The larvae grow from 0.7 – 1.3 mm in width to 2 – 3 mm in length [5, 6, 9]. However, tracking this molt *in vivo* is not feasible other than by necropsy of canine hosts as in Kotani and Powers (1982), a study that is unlikely to be repeated due to current pressures on terminal experimentation on canines. Scientists have examined the *in vitro* culture and molting system of *D. immitis*, as well as other filarial nematodes. The *in vitro* approach has allowed us to investigate features of the first molt after the larvae enter their definitive hosts.

Various conditions have been tested for *in vitro D. immitis* culture. The results indicated that *D. immitis* L3 could molt into L4 without cellular co-culture, though co-culture may improve the long-term viability of the L3 [10-12]. The molt occurred after 48 – 72 hours of culture. The larvae can be cultured under a wide range of conditions, including different basal culture media types, fetal bovine serum (FBS) or albumin concentration, and gas phases [11, 13-18]. Abraham et al. (1988) and Devaney (1985) studied cuticular morphology during the *in vitro* molting process using electron microscopy [11, 17]. Devaney (1985) cultured *D. immitis* L3 in medium ML-15 with a feeder layer of canine sarcoma cells, maintained at 37^{o}C in air (0.03% to 0.04%

CO_2). The molting started with the loosening of the cuticle at the head or at the tail end of the L3. The L3 gradually broke through the old cuticle, starting from the head. Numerous multivesicular bodies in the hypodermis were present during the molting process, indicating an active metabolic process for cuticle synthesis [11].

Abraham et al. (1988) carried out the *in vitro* molting experiment without co-cultured cells using a 1:1 mixture of NCTC-135 and IMDM (Iscove's modified Dulbecco's Medium), supplied with 20 % FBS and cultured at 37°C with 5% CO_2 . They examined the ultrastructure of the larvae at 0, 1, 2, 3, and 8 days of culture. Multivesicular bodies were observed at 0 hr in culture and in larger quantities in the hypodermis after 24 hr of culture. By 48 hr of culture, the trilaminate epicuticle of the L4 became evident, and multivesicular bodies remained. By 72 hr of culture, ecdysis occurred with a marked decrease of multivesicular bodies, and the multivesicular bodies became difficult to observe after 8 days in culture [17]. They concluded that the molting process consists of three steps: 1) the development of the L4 cuticle, 2) ecdysis, and 3) the thickening of the L4 cuticle. Their observation of the multivesicular bodies and the changes in the larval surface structure aligns well with the previous findings, indicating that the L3 to L4 *in vitro* molt is a true molt instead of an atypical reaction to the culture system [11, 17, 19].

3) *Dirofilaria immitis* gene expression features in different life cycle stages

Dirofilaria immitis exhibits distinct features at different stages. The features include metabolic patterns, immunogenic antigens, gene expression levels, and reactions to antihelminth treatments [18, 20, 21]. The length of larvae ranges from less than 0.03 cm (mf) to more than 25 cm (adult females). The hosts, nutrition sources, and immune response change as the life cycle stage environment changes: 1) peripheral blood (mf), 2) mosquitoes (mf to L3), 3) subcutaneous

tissue and 4) muscle tissue of the vertebrate hosts (L3 to immature adults), and 5) the right heart and pulmonary artery of definitive hosts (immature adults and adults) [5, 6]. Each environment has particular immune components. The mf may induce an immune reaction by hemocytes or hemocyte-derived microvesicles in the mosquito when mf are in the Malpighian tubes [22]. The tissue immune response in dogs after the L3 invades the hosts and the circulating white blood cells where mf survive also apply stress on the parasite. The larvae need to adjust accordingly to survive within different hosts in response to their immune systems.

Transcriptomic studies can provide insight into how larvae behave at different stages. Luck et al. (2014) investigated the transcriptome of *D. immitis* at different stages (mf, L3, L4, adult male, adult female) and found that a large number of *D. immitis* genes were expressed significantly differently among stages and sexes [18]. They found that L3 had the most distinct transcriptional profile, adult females and L4 had the most similar profiles; the similarity of transcriptional profiles of adult males vs. mf, adult males vs. adult females, and mf vs. adult females lies in between. The gene ontology (GO) terms of overrepresented genes in mf, L3, and L4 are shown in Table 1.2. The upregulation of certain genes can correspond with certain stage-specific activities (i.e., the development of reproductive organs in L4).

Table 1.2

Upregulated/overexpressed GO terms at different stages

Stage	GO terms over-expression
Mf	Transportation, including monovalent cations, oxygen and metal ions; Nucleic acid binding and transcription; Eukaryotic cilium, including ciliated structures formation and synaptic structures development; Gamma-aminobutyric acid (GABA) signaling; Tetrapyrrole/heme binding; DNA integration.
L3	Genes associated with extracellular matrix and structural components of the collagen/cuticles;

	Energy metabolism, glycogen/carbohydrate synthesis, transport, muscle development, collagens and structural constituents of the cuticle and molting.
L4	Cellular differentiation, reproduction and reproductive development; Transcription/translation, splicing, muscle cell development and locomotion, cellular components and organization, cellular localization and migration, binding, transport, apoptosis, pyrophosphatase activity, cellular protein complex disassembly, multi-organism processes. (overexpressed as compared to L3 stage) reproductive tract development, meiosis, protein binding/complexes folding, endocytosis, nervous system development, cellular component organization.

Note: adapted from [18]

4) Pathogenesis of heartworm in canine hosts

When immature adults arrive in the pulmonary artery branches, lesions in the distal pulmonary artery and caudal lung lobe start to develop [7]. These lesions include: endothelial proliferation, tunica intima thickening, and endoarteritis [7, 23]. As the disease progresses, the worms become larger, mature adults; occasional natural worm death may occur. Dying worms produce more lesions as they decompose and are forced by the blood flow into smaller branches of the arteries while releasing *Wolbachia* [24, 25]. The granulomatous reaction in the arterial wall, platelet aggregation, and fibrin deposition, along with the dead worm, lead to the formation of thromboembolic disease [24, 25]. The pulmonary arteries become dilated and more tortuous due to the increased arterial pressure to compensate for the obstruction of blood flow caused by vascular wall lesions and thromboembolism [7, 26]. Severe blockage of blood flow and pulmonary hypertension can lead to congestive heart failure. Under certain circumstances, wherein a large number of heartworms cause congestion in the right atrium and vena cava, Caval Syndrome, the acute and usually deadly syndrome of heartworm infection, may develop [7].

Significance of *Dirofilaria immitis*

1) Clinical impacts of heartworm disease

Heartworm disease can be asymptomatic or symptomatic and can be lethal if left untreated [27]. Heartworm disease is treatable; however, the damage to the respiratory system is irreversible. The diagnostic methods for heartworm disease include mf detection and antigen testing. Negative results on both tests do not rule out the possibility of heartworm infection, as the antigen test targets antigens from female adults, and the mf test can only be positive if male and female adult worms are both present [27]. The test timing for optimal results is 5 – 6 months and 7 months post-infection for antigen and mf testing, respectively. At the same time, extensive pathological changes to the entire pulmonary arterial system may have occurred before any signs or a positive diagnosis result [27].

The treatment process is relatively long (120 days), and strict activity restriction is strongly recommended for the patients [27]. Dying heartworms produce the most severe pulmonary changes during the disease progression. Natural or drug-induced heartworm death can trigger granulomatous inflammation, which is associated with thromboembolic disease [26]. Pulmonary thromboembolism will occur as a result of successful adulticidal treatment [27]. According to the American Heartworm Society (AHS), the best recommendation to counter the threat of heartworm infection, especially in endemic areas, is to prevent dogs from getting infected [27].

2) Epidemiology of heartworm

Heartworm disease exists on all continents except for Antarctica. It is endemic in North, Central, and South America, including the United States [3]. A study focused on the incidence

rates of HWD in the USA between 2013 – 2016 indicated an overall increase of 21.7 %, with relatively no change in the proportion of dogs receiving heartworm prevention [28]. Multiple factors may contribute to the difficulty of disease control, including the relocation of infected animals, climate change, insufficient owner compliance, and the accompanying spread of mosquito vectors [3, 29].

In 2023, in the United States, 195,078 out of 19,295,283 dogs were diagnosed positive for heartworm disease, which indicates that one dog would be heartworm positive for every 100 dogs that were tested [30]. This number is probably underestimated since the data was collected by clinical case reports, and not every pet owner will perform a heartworm test. Wild canids in North America, including coyotes, foxes, and wolves, can serve as reservoirs for heartworm disease transmission [3].

The mf to L3 development of heartworm can be completed in more than 70 species of mosquitoes, with 25 species proven to be major intermediate hosts [3, 4]. Studies have shown that the minimum temperature for L3 to develop is 14ºC; this can explain the lower prevalence rates in colder regions [4, 29]. However, the heat island effect keeps urban temperatures relatively higher than rural environments, allowing vectors such as *Aedes albopictus* and *Culex* spp. to survive and spread heartworm disease year-round [3].

3) Rise of macrocyclic lactone (ML) resistance

Year-round preventive administration is strongly recommended to reduce the risk of heartworm infection. The US Food and Drug Administration (FDA) has approved heartworm preventives belonging to the ML class of drugs, which include ivermectin, milbemycin oxime, moxidectin, and selamectin [27]. Although the exact mechanism of ML against filarial

nematodes is unclear, the binding of ML to glutamate-gated chloride ion channels, which function in hyperpolarization, paralysis, and death of heartworms, has been observed [31]. The resistance to MLs presents as the loss of effectiveness against infection when the preventives have been given correctly, as well as the loss of microfilaricidal activity [32].

The mechanism by which *D. immitis* becomes ML-resistant requires further investigation. Multiple studies have observed the genetic changes (such as ABC transporter genes) in ML-resistant strains [33]. Several factors, such as ML selection pressure, resistant strain inbreeding, and transmission intensity, may accelerate the emergence of *D. immitis* ML-resistant strains [32].

ML-resistant *D. immitis* strains in the United States have mainly been found in the lower Mississippi River Valley region [34, 35]. However, the prevalence and progression of ML-resistant strains cannot be determined or anticipated based on currently available data. The spread of ML-resistant strains, with the increased relocation of dogs and the availability of mosquito vectors, is likely to be unavoidable [35]. More accessible in-clinic diagnostic tools for ML-resistant strains, such as the microfilariae suppression test [36], may help provide insights into future scenarios for this resistance issue.

The first ML-resistant *D. immitis* case in Europe was reported in 2024 [37]. In 2023, the infected dog traveled from the USA to Rome, Italy, and tested positive for heartworm within 6 months of arrival despite monthly preventive administration (milbemycin oxime plus isoxazoline). This case report proved that the relocation of infected animals can indeed spread heartworm disease, ML-resistant or not. A more thorough understanding of *D. immitis* is needed to cope with the threat of the spread of heartworm disease.

The endosymbiont *Wolbachia*

1) History and biological features of *Wolbachia*

Wolbachia was first discovered in 1924 in the gonads of *Culex pipientis*; in 1936, a complete description of *Wolbachia pipientis* was published [38]. With the development of electron microscopy techniques in the late 1960s and early 1970s, ultrastructural studies of *D. immitis* found *Wolbachia* inside the worms [38]. The general characteristics of *Wolbachia* showed similarity to the Rickettsiae. The Gram-negative, pleomorphic α-proteobacteria live in host-derived vacuoles in the intracellular niche and range in size from 0.2 to 4 μm [39, 40]. Unlike typical Gram-negative bacteria with a cell wall consisting of peptidoglycan and lipopolysaccharide (LPS), *Wolbachia* lacks the genes for LPS biosynthesis and may build a minimal peptidoglycan-like structure instead of LPS peptidoglycan [41]. These vertically transmitted bacteria replicate in the membrane-bound vacuoles inside the host cells, and it can remain intracellular during the cells' mitotic cycles [41].

Wolbachia is considered to be one of the most widespread intracellular bacteria in the animal kingdom, as it is estimated to be present in 40 % of all arthropod species, as well as the filariae of mammals [40]. The phylogeny of *Wolbachia* was analyzed using the sequences of 16S rRNA gene and the surface protein gene *wsp*. Seventeen possible phylogenetic supergroups (A – F, H – Q, and S) were classified in the genus *Wolbachia* [42]. Divisions at the genomic level were observed between *Wolbachia* supergroups found in arthropods and those found in nematodes, indicating differences in the host-*Wolbachia* interactions. For instance, the *Wolbachia* supergroups related to nematodes have smaller genomes (0.9 to 1.1 Mb) compared to the arthropod *Wolbachia* genomes (1.2 to 1.6 Mb), and nematode *Wolbachia* supergroups lack prophage-associated genes in the genome [43]. The C and D supergroups can be found in

nematode species of great importance in human and veterinary medicine. Table 1.3 lists the nematode species that harbor *Wolbachia* supergroups C or D.

Table 1.3

Nematode species that harbor *Wolbachia* supergroups C or D.

Supergroup C	Supergroup D
Dirofilaria immitis	*Brugia malayi*
Dirofilaria repens	*Brugia pahangi*
Loxodontofilaria caprini	*Brugia timori*
Onchocerca armillatta	*Wuchereria bancrofti*
Onchocerca eberhardi	*Litomosoides brasiliensis*
Onchocerca gutturosa	*Litomosoides solaria*
Onchocerca ochengi	*Litomosoides hameletti*
Onchocerca volvulus	*Litomosoides sigmodontis*
Onchocerca skrjabini	*Litomosoides gallzai*
Onchocerca dewittei japonica	

Note: adapted from [44].

2) *Wolbachia* in filarial nematodes – distribution and reproduction

The presence of *Wolbachia* in filarial nematodes seems to be limited to the family Onchocercidae (subfamilies Onchocercinae and Dirofilariinae), as not all filarial nematodes in the family carry *Wolbachica* (such as *Loa Loa* and *Acanthocheilonema viteae*) [45]. For species that harbor *Wolbachia*, this endosymbiont can be found in all life stages, but there appears to be a distinct variation in localization of the bacteria in tissues between different species and sexes of worms [46]. The major tissues that contain *Wolbachia* are hypodermal lateral cords and reproductive organs in most Onchocercidae nematodes, including *Brugia* spp. and *D. immitis* [46]. This discovery was first made in the mid-1970s, when *Wolbachia* distribution and mode of reproduction and transmission were observed in *O. volvulus, B. malayi, D. immitis, and L. sigmodontis* [38]. Researchers found that *Wolbachia* was present in the hypodermal tissues in all

life cycle stages of both sexes, as well as hypodermal precursor cells of embryos *in utero* and in the rachis and oocytes of the female worms [38].

Fischer et al. (2011) examined *Wolbachia* distribution in *B. malayi* in different tissues at each life cycle stage [47]. Most cells were endobacteria-free in the mf, L1, L2, and L3. In L3, *Wolbachia* was observed mainly in the lateral cord cells but not in the organs of *B. malayi*. Large numbers of *Wolbachia* were found in the developing lateral cords of the L4 *B. malayi* midbody regions [47]. The ratio of *Wolbachia*/filarial DNA indicated a substantial increase within seven days post infection of the mammalian host and further increased during L4 development [45]. The *Wolbachia* expansion was also observed in *D. immitis* L4, derived from L3 cultured in an immunodeficient mouse model and recovered 14 days post infection [10]. Fischer et al. (2011) observed no *Wolbachia* in the empty uterus branches in female *B. malayi* five weeks post infection after *B. malayi* finished the L4 to immature adult molt but had not yet become sexually mature. However, large numbers of *Wolbachia* clusters were observed in the basal borders of the hypodermal cords close to the ovaries [47]. *Wolbachia* spreads through the lateral cords by cell-to-cell transmission and then enters the posterior pseudocoelom [48]. As the *B. malayi* female becomes sexually mature and starts to produce mf, dense *Wolbachia* clusters are found in the ovaries. The morula stage embryos in the uterus showed numerous *Wolbachia*, while the lateral cords adjacent to the ovaries and uterus showed lower numbers of *Wolbachia* [47]. During embryogenesis, the *Wolbachia* in supergroups C and D go through asymmetric segregation to reach hypodermal cells before morphogenesis. This step may determine the distribution of *Wolbachia* in the nematodes [48]. In *D. immitis*, *Wolbachia* is found in the lateral cords of all life stages within the vectors and definite hosts.

Further investigation is required to fully understand the mechanism of *Wolbachia* reproduction in filarial nematodes. Kozek (2005) [39] described two potential methods, including the common bacteria method of binary fission, where a cell splits into two identical daughter cells, and the *Chlamydia*-like developmental cycle, where small, dense inclusions form and grow into bacterial form [39]. *Wolbachia* needs both asymmetric mitotic segregation and cell-to-cell invasion to ensure a successful vertical transfer in the filarial nematodes [43, 48].

3) *Wolbachia* in the filarial nematode – biological functions

Wolbachia is essential for some filarial nematodes to develop, reproduce, and survive. As described above, the dynamic of *Wolbachia* numbers and distribution in filarial nematodes is closely related to the changes in life cycle stages, especially during embryogenesis and L4 development, as *Wolbachia* enters the uterus and expands through the hypodermal lateral cords, respectively [10, 39, 45, 47-49]. Antibiotics that target *Wolbachia* (such as tetracycline class drugs) can disrupt embryogenesis in the female worms as a consequence of *Wolbachia* clearance [42, 46, 50-53].

Wolbachia may also be required in molting activities for *B. malayi* and *B. pahangi.* One study focused on the L1 to L3 molt in the mosquitoes with *Wolbachia*-depleted *B. malayi* mf, which were obtained by peritoneal lavage from *B. malayi* patent gerbils that were treated with tetracycline for 6 weeks. The mf were fed to mosquitos, and DNA was isolated in order to measure *Wolbachia* levels. The L3 recovered per mosquito showed a 100% reduction in *Wolbachia* ($p < 0.001$) when fed with tetracycline-treated mf, while the *Wolbachia* levels in mf were lowered by 89.0% [54]. The *Wolbachia*-depleted *B. malayi* mf showed decreased expression in chitinase (detailed below), which is considered essential for the sheathed mf to molt and migrate through the midgut of the mosquitoes [54].

A similar experiment was performed on *O. volvulus* mf, which were unsheathed, and the result showed that *Wolbachia* depletion impacted, but did not inhibit, the development of L3 from mf in the *Simulium damnosum* vectors [55]. They observed an increase in the ratio of L1 to L2 in mosquitoes in total larvae recovered compared to the ratio in untreated groups, which may indicate the delayed development of larvae in the *Wolbachia*-depleted group. However, the reduction was not significant until three months post treatment, which may be explained by the consumption of "leftover" bacterial breakdown products of the *Wolbachia* following doxycycline treatment [55].

Casiraghi et al. (2007) tested the impact of *Wolbachia* on molting *in vivo* using *B. pahangi* and tetracycline. They discovered that the L4 to immature adult molt was partially dependent on *Wolbachia*, as a decreased recovery of *B. pahangi* adult worms occurred in gerbils that had received tetracycline prior to the L4 to immature adult molt for both sexes. Also, the sex-ratio of adults was distorted when tetracycline was administered after the male L4 to immature adult molt but before the female L4 to immature adult molt [56].

Genomic and transcriptomic studies provide insight into the functions of *Wolbachia* in filarial nematodes. The complete genome DNA sequence for *Wolbachia* by Foster et al. (2005) demonstrated that *Wolbachia* may provide riboflavin, flavin adenine dinucleotide, heme, and nucleotides to *B. malayi*, as genes in *B. malayi* may be absent or incomplete to fulfill the production needs for the above molecules [57]. Luck et al. (2014) investigated the transcriptomic profile of *D. immitis* and *Wolbachia* at different life cycle stages. Their findings showed that *Wolbachia* genes are more actively transcribed in the mf stages than in other stages (L3, *in vitro*, developed L4, adult female, adult male) [18]. Similar to the genomic pattern in *B. malayi*, they found that *D. immitis* lacks specific genes coding for *de novo* synthesis of crucial metabolic

materials, including purines, pyrimidines, heme, and riboflavin, but the genome of *Wolbachia* in *D. immitis* (*w*Di) has complete pathways for these molecules [18]. Some common *Wolbachia*-related metabolic pathways/functions are detailed below.

- Heme synthesis

Heme belongs to the tetrapyrrole class compounds; it is a critical cofactor for numerous proteins, such as cytochromes, hemoglobins, peroxidase, and catalases, participating in various biological processes [58]. Genetic studies on *B. malayi*, *D. immitis*, and *O. ochengi* indicate incomplete heme *de novo* biosynthesis pathways in the nematode genome, while their *Wolbachia* analysis showed a complete and likely functional heme pathway [18, 58, 59]. At the transcriptomic level, the entire *w*Di heme biosynthesis pathway was overrepresented in the *D. immitis* mf stage, indicating the active tetrapyrrole/heme binding function [18]. However, *Wolbachia*-negative nematode species, such as *Loa Loa*, also have a genome that lacks heme synthesis pathways [60], indicating that heme acquisition may come from other exogenous sources than *Wolbachia* [61]. Potential sources include the host's erythrocytes, which can be a rich source of heme and could be ingested by *L. sigmodontis* and *O. volvulus* [62, 63]. Iron-containing storage granules were observed in the *O. volvulus* intestinal epithelium, indicating the uptake of iron-containing proteins such as hemoglobin [64].

- Chitinase transcription

Chitinase can be found in *Wolbachia*-containing and *Wolbachia*-deficient filarial nematodes. It catalyzes the degradation of nematode chitin during various nematode

development activities, including molting [54]. Chitinase expression seems to be stage-specific in different nematode species. Microfilaria-specific chitinase activity can be observed in sheathed mf (e.g., *B. malayi, W. bancrofti, L. Loa*). The L3-specific chitinase, which is homologous to the *B. malayi* mf-specific chitinase, can be found in filarial species that produce unsheathed mf (e.g., *D. immitis, A. viteae, O. volvulus*) [18]. In *B. malayi* mf treated with tetracycline, chitinase is down-regulated as evidenced in both transcriptomic and Western blot analyses, leading to the inability to molt in mosquito vectors [54]. This may indicate that *Wolbachia* participates in the transcriptional regulation of mf chitinase in nematode species with sheathed mf. In *D. immitis* mf, which is unsheathed, the cuticular endochitinase is highly expressed and may be worth further investigation [18].

4) *Wolbachia* and filarial nematode mitochondria interaction

Mitochondria are essential in metabolic energy generation in eukaryotic cells. Through DNA sequence analysis, the hypothesis that mitochondria evolved from endosymbionts, such as *Rickettsia*, has been validated [65]. *Wolbachia*, as an endosymbiont in filarial nematodes that participates in multiple metabolic pathways, may also influence mitochondrial functions. Strübing et al. (2010) [66] examined mitochondrial gene expression changes when *Wolbachia* was depleted with tetracycline treatment in *L. sigmodontis*, a murine filariid closely related to *Brugia* spp. They also included *A. viteae*, a filarial nematode naturally lacking *Wolbachia*, as a control [66]. In their findings, *Wolbachia* in *L. sigmodontis* were effectively depleted after 36 days of tetracycline treatment as determined by qPCR. Microarray results showed that 9 mitochondrial encoded subunits of the respiratory chain were significantly upregulated with the depletion of *Wolbachia* in *L. signodontis*, including cytochrome c oxidase subunits, cytochrome

b, ATPase subunit 6, and NADH-dehydrogenase subunits. At the same time, no changes were observed in tetracycline-treated *A. viteae* [66]. A 60 kDa heat shock protein (HSP60) antibody was proven to bind both *Wolbachia* and mitochondria in *O. volvulus*, but the *Wolbachia* and mitochondria can be differentiated by their distinct localization patterns. Pfarr et al. (2008) [67] found that HSP60 electron microscopy staining increased in mitochondria after *Wolbachia* depletion. They confirmed the upregulation of HSP60 genes by qPCR and found a 7.7-fold increase of HSP60 in nematode cells. With no change in HSP60 staining in *A. viteae*, they concluded that the upregulation and increase in HSP60 protein translation might respond to the loss of *Wolbachia* rather than a direct effect of the antibiotic treatment [67].

On the genetic level, *Wolbachia* infection seems to impact the mitochondrial diversity both in arthropods and nematodes [68, 69]. However, McNutly et al. (2012) compared the mitochondrial genomes of *Wolbachia*-dependent (*B. malayi, W. bancrofti, D. immitis,* and *O. volvulus*) and *Wolbachia*-independent (*A. viteae, L. loa, Onchocerca flexuosa, Setaria digitata,* and *Chandlerella quiscali*) filarial nematode species and found no distinct differences between the mitochondrial genome sequences based on the presence of *Wolbachia* [70]. Factors including the stages of parasites, and the sample collection and preservation, may introduce inconsistency in the findings. Further investigation into the genetic impacts of mitochondria-*Wolbachia* interactions in filarial nematodes deserves more attention.

5) *Wolbachia* and pathogenesis in vertebrate hosts of nematodes

Wolbachia can be chronically or acutely released into the vertebrate host during filarial nematode infection and induce different immune reactions [39, 46, 71, 72]. The female worms can shed the free *Wolbachia* or *Wolbachia* in egg fragments during mf production. Chronic

Wolbachia stimulation may dampen the host innate immune system, which facilitates the survival of filarial nematodes and opportunistic microorganism infections [39, 73, 74].

The acute release of *Wolbachia* from dead worms can induce pro-inflammatory responses systemically, which cause the majority of lesions in filarial infections [75-77]. In human filariasis and onchocerciasis, studies have proven both *in vitro* and *in vivo* that the presence of *Wolbachia* correlates with the increase in severity of the symptoms or pathology [78-84]. *Wolbachia* surface protein (WSP), in a manner similar to LPS, induces a pro-inflammatory innate immune reaction by activating host monocytes, macrophages, dendritic cells, and neutrophils in a Toll-like receptor (TLR)-dependent manner, then leads to the release of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and nitric oxide (NO) [80, 85-88]. Extracts from *Wolbachia*-deficient *A. viteae* failed to induce inflammatory reactions in macrophages, indicating the responses to be *Wolbachia* dependent [88]. Other pathogen-associated molecular patterns of *Wolbachia*, such as HSP60 and groEL, interact with the immune system via TLR2 and/or TLR6 [85]. Post-treatment reactions, including fever, tachycardia, headaches, and lymph node enlargement, can occur after treatment of *B. malayi* and onchocerciasis infections that induce the acute release of *Wolbachia* [75, 76]. The reduction of *Wolbachia* prior to adulticidal treatment, or treatment that inhibits abrupt *Wolbachia* release, should be considered to prevent potential inflammatory reactions.

6) *Wolbachia* and pathogenesis in heartworm disease

Heartworm disease can cause extensive pathology in vertebrate hosts. In addition to the lesions induced by the mechanical damage of adult worms in the pulmonary arteries (e.g., endothelial damage, inflammation, and proliferation) [7], the response of the host to pulmonary

thromboembolism, can also result in significant pathology [89]. The severity of cardiopulmonary pathology in the hosts is closely related to the worm burden, host immune response, infection duration, and host activity level.

As stated above, the release of *Wolbachia* from nematodes to the hosts can be acute, chronic, or a combination of both [39, 46, 72, 77]. The lungs, liver, and kidneys of dogs that have died naturally from heartworm disease showed the presence of WSP in tissues [90]. In the kidney, dense positive staining of WSP has been shown in renal tubules and the glomeruli, suggesting that the natural turnover of circulating mf may release *Wolbachia* into the hosts, causing potential renal pathology. In the liver, hepatic monocytes were also positive for WSP [7, 90]. In lung tissue, *Wolbachia* was found freely or in monocytes [90].

Wolbachia can induce inflammation. The whole microorganism or its products stimulate immune cells, including macrophages, monocytes, neutrophils, and dendritic cells, which then release pro-inflammatory cytokines and chemokines [91]. It is believed that WSP is the major immunogenic factor. Both Th1 and Th2 types of immune responses have been observed in filarial infections. In *D. immitis* infection, studies targeted to develop immunization strategies in dogs identified that the reaction against larval challenge was polarized toward a Th2-type immune response [92]. Marcos-Atxutegi et al. (2003) tested the soluble antigens of *D. immitis* L3 and adults in BALB/c mice and discovered both Th1 and Th2 responses based on the detection of IFN-γ and specific WSP-IgG2a, suggesting that *Wolbachia* and/or its molecules are also able to stimulate a Th1-type of immune response [92]. In dogs naturally infected with *D. immitis*, specific humoral responses to WSP predominately in the IgG2 subclass can be found, indicating a primarily Th1-type immune response [93]. Later, Morchón et al. (2007) administered rWSP to BALB/c mic via subcutaneous injection. They measured the production of

NO, mRNA levels of inducible nitric-oxide synthase (iNOS) and interferon (IFN)-γ, and Th1-type antibody responses. Their results showed an increase in all values after rWSP injection [94]. Macrophages that receive IFN-γ become classically activated, leading to the expression of iNOs and the production of NO, which can kill intracellular microorganisms [95]. An *in vitro* study examined how canine polymorphonuclear neutrophils (PMNs) respond to rWSP. They found a general increase in neutrophil chemokinesis and chemotaxis, as well as IL-8 production, which indicated an enhanced motility and recruitment of PMNs [96]. The inflammatory reactions induced by *Wolbachia* may lead to vasoconstriction and possibly bronchoconstriction, as well as parenchymal lung inflammation and edema [97].

7) *Wolbachia* as filaricidal drug target

The control and treatment of diseases caused by filarial nematodes can be difficult due to their complicated life cycles and the different biological and immunological characteristics in each life cycle stage of filarial worms. However, *Wolbachia* is present in all life stages in *Wolbachia*-dependent nematodes and is involved in multiple essential pathways related to embryogenesis and long-term survival [18 44, 52-55, 63, 67]. Thus, the elimination of *Wolbachia* is beneficial, as transmission can be blocked (sterilizing of adult females), there are deleterious effects on mf, and the potential pathology induced by *Wolbachia* may be reduced.

Antibiotics that have shown anti-*Wolbachia* activity include tetracycline-class antibiotics (doxycycline/minocycline), rapamycin, rifapentine, moxifloxacin, corallopyronin A, berberin, globomycin, and succinyl acetone [83, 87]. The only anti-*Wolbachia* drug currently in use against human filariasis and onchocerciasis, doxycycline, will be detailed in the section below.

Doxycycline in the treatment against filarial nematodes

1) Doxycycline - mechanism of action

Doxycycline, discovered in 1967, is a broad-spectrum antibiotic in the tetracycline class. It can kill and prevent the growth of many Gram-positive and Gram-negative bacteria, including *Rickettsia* [98, 99]. Doxycycline is highly lipophilic, which allows the molecule to travel through multiple membranes of Gram-negative bacteria, using OmpF and OmpC porin channels to reach the target binding sites, the 70S ribosomal RNA, and primarily the 30S subunit [100]. Doxycycline functions as a bacteriostatic medication by inhibiting the contact of aminoacyl-tRNA with the bacterial ribosome, disrupting protein synthesis and leading to a deficiency in essential protein synthesis in the bacteria, thus killing the bacteria [98]. Doxycycline has also shown immunomodulating features that can relieve pulmonary inflammation in a mouse model by inhibiting inflammatory pathways. An *in vitro* study indicated potentially anti-fibrosis functions, which were tested in mammalian cells [25].

2) Doxycycline in filarial nematode disease

The FDA has approved multiple formulations of doxycycline for the prevention or treatment of infections within the categories: rickettsial infections, Lyme disease, anthrax, acute intestinal amebiasis, and malaria prophylaxis [101]. Tetracycline and doxycycline have been proven to effectively reduce *Wolbachia* in many filarial nematodes, sterilize female worms, inhibit embryogenesis, and, over time, clear mf [52, 53, 79, 84, 102].

Though the history of incorporating doxycycline into filarial disease treatment is short [103], the benefits of adding doxycycline have been demonstrated. The off-label use of

doxycycline in filarial nematode disease was recorded via the U.S. Centers for Disease Control and Prevention (CDC) in the clinical treatment of onchocerciasis [104]. The treatment with a 6-week course of doxycycline (200 mg orally daily) was able to kill more than 60% of the adult female worms and to sterilize 80 – 90% of the females 20 months post-treatment [104]. Doxycycline also helps to relieve the mf burden and symptoms in patients with lymphatic filariasis. Patients with Bancroftian filariasis who received doxycycline for 6 weeks at 200 mg/day showed a 96% reduction in *Wolbachia* and a 99% reduction in mf levels 1 year post-treatment [102]. The same regimen of doxycycline can improve mild to moderate lymphedema of ongoing infections [83, 105]; a shorter treatment with doxycycline (200 mg/day, 21 days) also showed a reduction in lymphatic pathology in Bancroftian filariasis [81].

Doxycycline in canine heartworm treatment

1) Canine heartworm treatment protocol by the American Heartworm Society

The AHS classified heartworm disease into four classes based on the severity of symptoms. A summary of clinical signs corresponding to each class is listed in Table 1.4. from AHS Canine Guidelines for the Prevention, Diagnosis, and Management of Heartworm (*Dirofilaria immitis*) Infection in Dogs [25].

Table 1.4

Summary of Clinical Signs of Canine Heartworm Disease

Mild (Class 1)	Asymptomatic or cough
Moderate (Class 2)	Cough, activity intolerance, abnormal lung sounds
Severe (Class 3)	Cough, activity intolerance, dyspnea, abnormal heart and lung sounds, enlarged liver (hepatomegaly), syncope (temporary loss of consciousness from reduced blood flow to the brain), ascites (fluid accumulation in the abdominal cavity), death

Caval Syndrome (Class 4)	Sudden onset of severe lethargy and weakness accompanied by hemoglobinemia and hemoglobinuria

Note: Content from [25].

The AHS recommends a three-dose, melarsomine dihydrochloride injection protocol for canine heartworm treatment (Fig 1.2). The key points of heartworm treatment are listed in Table 1.5.

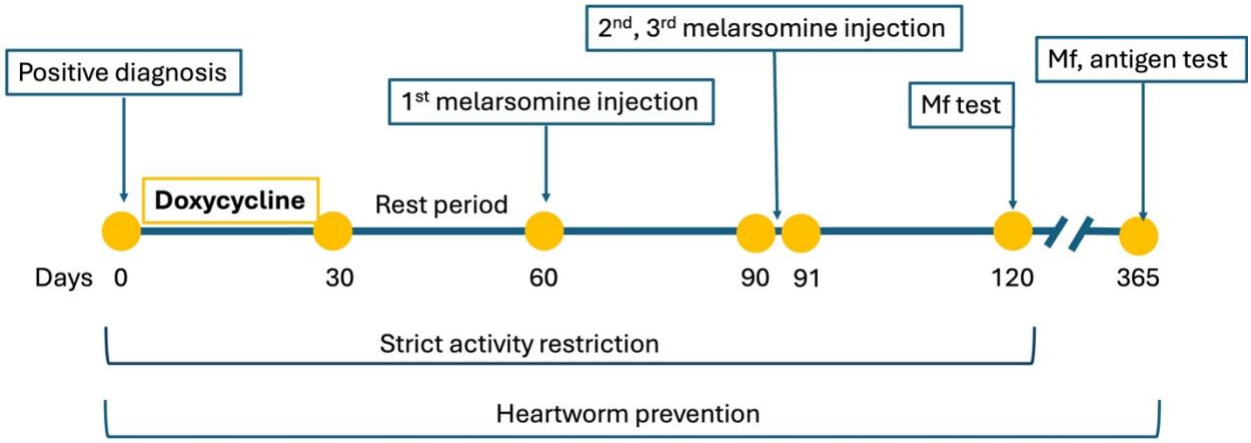

Figure 1.2

Canine heartworm treatment timeline

Table 1.5

Key points in heartworm treatment

Treatment stage	Event	Justification
Overall goal		Heartworm elimination in all life stages with minimal post-treatment complications while improving the clinical condition of the animal.
Throughout treatment	ML preventive administration	To reduce new infections and eliminate existing susceptible larvae.
	Activity restriction	Essential for minimizing cardiopulmonary complications resulting from both infection and the treatment regimen.

Pre-adulticidal treatment	Patient evaluation	Thoracic radiographs can help in evaluating the potential for post-adulticide treatment complications.
	Stabilize patient	Glucocorticosteroids, diuretics, vasodilators, positive inotropic agents, and fluid therapy may be given to dogs that exhibit significant clinical signs.
	Doxycycline therapy	Four weeks of doxycycline treatment and one-month rest period after completion of treatment to ensure the reduction of *Wolbachia* within *D. immitis*, reducing the potential pathology and disrupting heartworm transmission. Doxycycline and ML have a synergistic effect, eliminating all developing larvae pre-adulticidal treatment.
	Steroids	Anti-inflammatory doses of glucocorticoids help reduce clinical signs of thrombus formation post-melarsomine injection.
Adulticidal treatment	1st melarsomine injection	The only adulticidal drug approved by the FDA. Given by deep intramuscular injection into the belly of the epaxial lumbar muscles between the 3rd and 5th lumbar vertebrae.
	2nd & 3rd melarsomine injection	The 2nd & 3rd doses given one-month after the 1st injection (24 hours apart). Two-dose melarsomine protocol (two injections 24 hours apart only) eliminates fewer adult worms than 3 doses.
Recovery	mf and antigen testing	Evaluate the treatment and determine if additional treatment is needed.

Note: Adapted from [25]

Doxycycline is recommended as an adjunct therapy prior to the administration of adulticidal melarsomine dihydrochloride to reduce *Wolbachia* harbored within *D. immitis*, thus reducing the pathology associated with dead worms and also blocking heartworm transmission [25]. The current recommendation for doxycycline usage is 10 mg/kg, twice daily (BID) for 4 weeks, with a one-month rest period between the finish of doxycycline and the beginning of melarsomine treatment, to reduce *Wolbachia* within the parasite further [25].

2) Benefits of introducing doxycycline in heartworm disease treatment

- Elimination of *Wolbachia*

Doxycycline can effectively reduce the amount of *Wolbachia* in *D. immitis*. Studies in clinical and experimental settings have yielded repeatable results, which indicate that there is a decrease and potential elimination of *Wolbachia* in different stages of *D. immitis* [106-111]. *In vitro* treatment, however, may not result in *Wolbachia* DNA reduction at the end point of the experiments. Doxycycline is a bacteriostatic drug that targets the 70S ribosome in *Wolbachia*, disrupting the translation of essential proteins, which leads to the death of bacteria. Thus, the decrease of *Wolbachia* DNA may need more time to be observed [112, 113]. Table 1.6 summarizes the doxycycline usage in the studies mentioned above. All studies included untreated control groups.

Table 1.6

Doxycycline reduces *Wolbachia in vivo*

Stage	Doxycycline regimen	Detection method	Reference
Adult, mf	10 mg/kg SID, interval treatment for 20 weeks	Immunohistochemistry, qPCR, electron microscopy	[106, 107]
mf	10 mg/kg SID, intermediated treatment for 20 weeks	ELISA for anti-WSP antibodies	[110]
mf	10 mg/kg or 5 mg/kg, BID, for consecutive 28 days	Quantitative PCR	[108]
mf	10 mg/kg or 5 mg/kg BID, or 10 mg/kg SID, for consecutive 30 days	ELISA for anti-WSP antibodies	[111]
mf	10 mg/kg BID for consecutive 30 days with monthly Advocate®	Quantitative PCR	[109]

- Disruption of subsequent heartworm disease after transmission

Doxycycline blocks the embryogenesis of filarial nematodes via *Wolbachia* depletion. Though mf in the blood takes weeks to months to clear, the ability of those doxycycline-treated mf to develop into mature adults was impacted [51, 106, 109, 114, 115]. McCall et al. (2008, 2014, 2023) investigated the ability of *D. immitis* L3 to complete a subsequent lifecycle when the

L3 were produced from the blood of doxycycline-treated dogs [51, 114, 116]. All dogs were experimentally infected and mf positive. In the 2014 study, the dogs received doxycycline at 10 mg/kg, BID, for 30 days. Blood was collected on days 73 to 77 after the start of doxycycline treatment and artificially fed to mosquitoes. The L3 were collected 15 days after feeding and used to infect subsequent heartworm-naïve dogs. The researchers checked for mf and antigen from 5 months to 302 days post infection. No positive antigen or mf in blood was observed, and no live or dead adult *D. immitis* were recovered during necropsy [51]. In the 2023 study, doxycycline (10 mg/kg, SID, 30 days) combined with oral ivermectin (6 µg/kg, given on days 0 and 30) was used in the treatment group. The blood was collected on days 22, 29, and 42 after the beginning of the treatment, and L3 produced were injected into heartworm-naïve dogs; no adult worms were recovered from dogs that received treated L3 [114].

Doxycycline inhibits the development of *D. immitis* L3 in the early transmission stage. In a *B. pahangi* and *D. immitis* co-infection model, dogs were inoculated with 200 *B. pahangi* and 50 *D. immitis* L3 and then received 10 mg/kg BID doxycycline treatment for varied duration [117]. The dogs received doxycycline on days 0-29 post infection, during which time the L3 of both parasites typically molt to L4. At necropsy, neither *D. immitis* nor *B. pahangi* were recovered [117].

- Reduction of post-adulticidal treatment pathology, complications, and clinical signs

As mentioned in previous sections, the dying heartworms cause the most severe damage to the hosts. The administration of doxycycline before the melarsomine injection can significantly reduce pathology as compared to dogs that received melarsomine injection without doxycycline treatment [107]. Ivermectin was used in some studies as a single treatment and

combined with doxycycline to evaluate the effect of different treatment regimes, as ivermectin exhibits microfilaricidal activity.

Kramer et al. (2011) assessed lung pathology in *D. immitis*-experimentally infected dogs treated with three injections of melarsomine with or without prior doxycycline treatment [118]. Doxycycline was given 20 mg/kg SID for 4 weeks. Melarsomine injection started 8 weeks after the completion of doxycycline treatment. All groups were euthanized at week 24 post infection. Dogs treated with melarsomine alone formed lung lesions associated with thromboembolism, such as severe alveolar wall thickening and lung tissue hepatization. In contrast, dogs that received doxycycline pre-treatment showed decreased severity of the lesions, especially perivascular inflammation [118].

A study examined pathogenesis when long-term, doxycycline (10 mg/kg SID) was used on heartworm-positive dogs from weeks 0-6, 10-12, 16-18, 22-26, and 28-34, with or without IVM (6 µg/kg, orally, once per week for 34 weeks). Four groups of dogs were included in the experiment; group 1 received doxycycline as described above, group 2 served as control and received no treatment. Group 3 received doxycycline and ivermectin followed by melarsomine injection, and group 4 received melarsomine injection only (first injection on week 24, followed by two additional injections 24h apart 1 month later) [107]. All dogs were euthanized at week 36. The number of *D. immitis* adults recovered at necropsy was significantly reduced in all treatment groups compared to the no-treatment control group, indicating the adulticidal activity of doxycycline. Dogs that received melarsomine injection without pre-treatment (in this study, doxycycline combined with ivermectin) had significantly more inflammation and/or thickening of the alveolar walls, and periarterial inflammation in the lungs compared to the pre-treatment group [107].

Post-adulticidal conditions were evaluated in a clinical study in dogs naturally infected with heartworm that received doxycycline 10 mg/kg BID versus no doxycycline before adulticidal treatment (2.5 mg/kg 3-dose melarsomine injection). Fewer respiratory complications and heartworm disease-related deaths occurred in the doxycycline-treated group compared to the no doxycycline group (6.52% versus 19.14%; 0% versus 4.25%, respectively) [119].

3) Controversy about the doxycycline treatment regimen

The current recommended regimen for doxycycline is 10 mg/kg, BID, for 28 days. A one-month rest period is recommended before the administration of adulticidal treatment [25]. Several aspects of the recommendation can be further optimized, including the dosage and duration of doxycycline treatment and the necessity of the one-month rest period.

- Dosage

In a retrospective study, 386 dogs received doxycycline therapy (doses varied from 5 to 30 mg/kg per day, mean dose 15.4 mg/kg/day, median dose 16.0 mg/kg/day) for various infectious diseases, except for heartworm, including: *Ehrlichia canis* (60.1%), respiratory tract infections (11.7 %), skin infections (7.8 %), and other infectious diseases (*Anaplasma phagocytophilum*, leptospirosis, borreliosis, hemotrophic mycoplasmas). Each disease composed at or less than 5% of the study population [120]. Gastrointestinal (GI) signs and elevated liver enzyme activities were noted. Side effects that were most commonly observed were vomiting, diarrhea, and anorexia in 18.3 %, 7.0 %, and 2.5% of dogs, respectively. Increased alanine aminotransferase and alkaline phosphatase activity were shown in 39.4 % and 36.4% of dogs, respectively [120]. Savadelis et al. (2018) tested doxycycline dosages of 5 mg/kg versus 10

mg/kg twice daily in heartworm-positive dogs to evaluate the severity of the side effects within the 28-day treatment period. Though not statistically significant, dogs in the 10 mg/kg doxycycline group had more animals that presented with GI signs, and the signs were more severe than those in the 5 mg/kg group [108]. Though not significant, a study in a shelter setting evaluated doxycycline at 10 mg/kg SID versus 10 mg/kg BID and found a lower rate of GI signs among dogs receiving the lower dose (36.7% and 52.1%, respectively) [121]. A lower doxycycline dose will likely reduce potential side effects associated with medication administration.

The efficacy in *Wolbachia* clearance and decrease in pathology should be evaluated to determine whether the dose of doxycycline can be changed in heartworm treatment. Savadelis et al. (2018) indicated that *Wolbachia* elimination was not as effective when using 5 mg/kg of doxycycline versus 10 mg/kg, given that 2 out of the 8 dogs from the 5 mg/kg group remained *Wolbachia* DNA positive at the end of the 28-day treatment period (0 out of 8 dogs were positive in the 10 mg/kg group). Moorhead et al. (2023) examined the severity of pathology in dogs experimentally infected with adult *D. immitis* via surgical transplantation that received doxycycline at different doses (5 mg/kg, 7.5 mg/kg, 10 mg/kg, BID) for 28 days. The researchers evaluated lesions in the heart, lung, liver, and kidney, and found no significant differences between dosages [122]. An ELISA for detecting *Wolbachia* using anti-recombinant WSP antibody was used in another study examining levels of *Wolbachia* in blood via optical densities (OD). All dosage groups (5 mg/kg/12 h, 10 mg/kg/12 h, 10 mg/kg/24 h) showed a significant reduction in OD, which corresponded to the *Wolbachia* amount in the blood at the endpoint of doxycycline treatment (day 30) [111]. However, direct measurement of *Wolbachia* in adult *D. immitis* after doxycycline treatment at different dosages has not been performed. The evaluation

of *Wolbachia* in adult *D. immitis* may increase understanding of the efficacy of *Wolbachia* depletion using a lower doxycycline dosage.

- Duration

According to the AHS protocol, doxycycline treatment requires 28 consecutive days of twice-daily oral drug administration. This frequency and duration of treatment with doxycycline have been proven effective in *Wolbachia* elimination in terms of reduction in complications and respiratory signs caused by adulticidal treatment [25]. Research indicates that the presence of *Wolbachi*a in the blood starts to decrease one week after doxycycline treatment and continues to decrease throughout the treatment [108]. McCall et al. (2023) tested the ability of *D. immitis* L3 to complete a normal life cycle in naïve dogs, when the L3 were raised from mosquitoes fed on blood from animals that had received doxycycline and prophylactic doses of ivermectin [114]. They treated the microfilaremic dogs with monthly ivermectin (6 µg/kg) and 30 days of doxycycline at 10 mg/kg, once per day. No adult *D. immitis* were recovered from naïve dogs infected with L3 raised from blood (mf) collected 22 days after the treatment started, while L3 raised from untreated mf completed the life cycle [114]. The duration and dosage of doxycycline treatment were lower than recommended by the AHS [25]. Although *Wolbachia* levels were neither measured in mf nor L3 in the study mentioned above [114], one can infer that the depletion of *Wolbachia* was enough to prevent *D. immitis* from completing its life cycle [123].

A modified, three-dose, melarsomine treatment protocol was evaluated in shelter settings. This protocol used doxycycline 10 mg/kg SID instead of BID, and administered the first melarsomine injection 14 days after the start of doxycycline treatment versus 30 days [121]. This

protocol was utilized on dogs with asymptomatic or mild heartworm disease. The results found no significant differences in new respiratory signs (11.4% and 7.4%) or respiratory complications (2.5% and 3.7%) between the shortened and AHS protocols, respectively [121].

No *Wolbachia* levels in mf, L3, or adult *D. immitis* were measured with quantitative methods in the above studies. Quantifying *Wolbachia* weekly after the start of doxycycline treatment could fill the gap between the phenotype (*i.e*., the inability of L3 to complete the normal life cycle) and potential mechanisms.

- One-month rest period

The one-month wait period is hypothetically needed so that the worms may eliminate *Wolbachia* and its metabolites. Doxycycline is bacteriostatic versus bactericidal; therefore, there is no direct killing of *Wolbachia* but most likely an inhibition of necessary functions that aid *Wolbachia* in maintaining its symbiosis with the worm. Also, the worms may weaken as they are deprived of *Wolbachia* [25, 98].

The retrospective study described above [121] showed that clinical signs were not significantly affected when the one-month rest period was eliminated. The Toronto Humane Society (THS) recommends this shortened protocol, especially in shelter settings. However, we should note that this data was collected only on asymptomatic animals or animals with mild heartworm disease [121]. Animals with class 2 or 3 heartworm disease [25], especially in endemic areas, have a higher risk of severe complications and death during heartworm treatment with the AHS protocol, which is expected due to the advanced clinical disease in these animals

[124]. No data was obtained as to whether the THS protocol could obtain the same results in animals with class 2 and above heartworm infections.

Moorhead et al. (2023) measured worm weights after doxycycline treatment and the subsequent one-month rest period [122]. They observed a significant decrease in mean worm weight between days 30 and 60 (days after the start of doxycycline treatment), which aligned with the hypothesis that the one-month rest period can reduce the mass of the worms [25]. However, they found no significant difference in pathology between days 30 and 60. This may be due to the strict activity restriction for the experimental animals and the absence of melarsomine injection, which causes adult worm death and resultant pathology [7, 26, 122].

The impact of doxycycline on *Wolbachia* and *D. immitis* at different life cycle stages

A thorough understanding of the interaction between *D. immitis* and its endosymbiont, *Wolbachia,* is beneficial in clinical practice and requires basic science research. In the following sections, we investigated the impact of *in vivo* doxycycline administration on *Wolbachia* and, consequently, *D. immitis* at different filarial nematode life cycle stages. The studies performed and described in this dissertation may help to fill in the knowledge gaps.

Chapter 2 focused on the L3 to L4 stages of *D. immitis*. In this chapter, we examined if the presence of *Wolbachia* was required for L3 to molt into L4 *in vitro*. Previous studies showed the inability to complete a full life cycle with mf obtained from doxycycline-treated dogs [51, 114]. This study revealed possible inhibition mechanisms on *D. immitis* larvae development after *Wolbachia* were depleted with doxycycline.

As summarized in the previous sections, *Wolbachia* contributes to the pathogenesis of heartworm disease in canine hosts. Chapter 3 examined the *Wolbachia* levels in adult *D. immitis* after completing different doxycycline treatment regimens. Doxycycline administration is correlated with the severity of clinical signs and lesions after post-adulticidal treatment [107, 118, 119]. The *Wolbachia* levels in adult *D. immitis* may predict the severity of potential lesions after worm death. The data presented in this dissertation may help in the decision-making process for veterinary practitioners regarding the necessity of the one-month rest period and the dosage of doxycycline.

In Chapter 4, we explored the influence of doxycycline on the mf of *D. immitis* via changes in gene expression levels and mitochondrial functions. Genomic and transcriptomic studies of nematodes and their *Wolbachia* revealed potential interactions between the two organisms [18, 57, 59, 61, 125]. We focused on heme-related bioactivities, chitinase activities, and mitochondrial functions. The results may further explain the mechanisms that lead to the importance of *Wolbachia* in the fitness of and disease caused by the nematode and potential drug target discovery.

Overall, the data and results provided in this dissertation could contribute to a more comprehensive understanding of interactions between heartworm, *Wolbachia*, and doxycycline. In conjunction with these current studies, additional studies may further benefit the drug discovery field and our basic understanding of heartworm disease control and treatment.

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CHAPTER 2

IN VITRO MOLTING OF *DIROFILARIA IMMITIS* THIRD-STAGE LARVAE DERIVED FROM MICROFILARIAE COLLECTED FROM DOXYCYCLINE-TREATED DOGS

Chu, Y. To be submitted to a peer-reviewed journal (Parasites & Vectors)

Abstract

Background: *Dirofilaria immitis*, canine heartworm, contains an endosymbiont, *Wolbachia*, in all life stages. Doxycycline, an antibiotic, has been incorporated into heartworm treatment protocols to eliminate *Wolbachia*. Previous studies indicate that subsequent infection cannot be established using viable third-stage larvae (L3) developed from doxycycline-treated microfilariae (mf). The stages in which the development of larvae is impacted by doxycycline remains unknown. We examined the impact of doxycycline at the third-stage to fourth-stage larval molt, as it is the first event for *D. immitis* after they invade the vertebrate host.

Methods: Microfilaremic blood was collected weekly from an untreated dog and dogs that received 28-day doxycycline treatment at 10 mg/kg as recommended by the American Heartworm Society. Blood was collected weekly until the end of doxycycline treatment. The blood was used for L3 production and mf isolation. *Wolbachia* levels in microfilariae (mf) and L3 were measured using real-time quantitative PCR. L3 were cultured *in vitro* for nine days to assess whether molting occurred. The Fisher's exact test and Bonferroni correction were used for statistical analysis.

Results: The molting of L3 from the doxycycline-treated groups did not show a significant difference compared to the L3s from the control group at weeks 0, 1, 2, 3, and 4. The *Wolbachia* levels in mf and L3 decreased starting from seven days post-treatment and remained less than five percent of controls throughout the treatment.

Conclusions: Doxycycline treatment can eliminate *Wolbachia* in both mf and subsequently developed L3. The molts of the mf to L3 in the mosquito and the L3 to L4 molt *in vitro* appear not to be impacted by the reduction or elimination of *Wolbachia*.

Keywords: *Dirofilaria immitis, Wolbachia*, doxycycline, molting, heartworm

Background

Canine heartworm disease (HWD), caused by *Dirofilaria immitis*, is an endemic disease across the world. In the United States, heartworm disease has been diagnosed in all continental 48 states [1]. Infected dogs carrying circulating microfilariae (mf) can be reservoirs for subsequent infection. Besides canids, more than 30 species of animals, including humans, can be infected by this parasite [2]. A study focused on the incident rates of HWD in the USA between 2013 – 2016 indicated an overall increase of 21.7 %, with relatively no change in the proportion of dogs receiving heartworm prevention [3]. The spread of heartworm will require additional efforts to control and treat the disease.

Dirofilaria immitis is a filarial parasite that is related to the human pathogens, *Onchocerca volvulus*, *Brugia malayi,* and *Wuchereria bancrofti*. *Dirofilaria immitis* needs a vertebrate host and an invertebrate host to complete its life cycle. The life cycle begins with a mosquito taking a blood meal from an infected host containing the circulating mf; the ingested mf then develops into third-stage infective larvae (L3) in the mosquito. When the mosquito takes another blood meal from a susceptible host, the L3 migrate to the head part of the mosquito and enter the host through the bite wound caused by the feeding activity [4, 5]. In the subsequent host, the L3 take three to 12 days to molt into fourth-stage larvae (L4), before they eventually molt into immature adults and are present in the pulmonary artery by 120 days post-infection. Adult male and female worms will mate and produce mf [4-6]. When another mosquito takes a blood meal from the infected dog, the life cycle continues.

To treat HWD, the American Heartworm Society (AHS) recommends the use of three doses of melarsomine dihydrochloride via intra-muscular injection as the adulticidal treatment. Prior to the first dose of melarsomine, AHS recommends a 28-day course of doxycycline at 10 mg/kg twice daily (BID). Doxycycline is a bacteriostatic agent that belongs to the tetracycline family. It can effectively reduce *Wolbachia* in the parasite, and subsequently reduce pathology and complications that may occur in the host after adulticidal treatment [7-9]. Doxycycline has been shown to be effective against early infection of *D. immitis* in dogs by McCall et al. (2011) in a *Brugia pahangi* and *D. immitis* co-infection model. The dogs were infected with 200 *B. pahangi* L3 and 50 *D. immitis* L3 via subcutaneous injection. The dogs that received doxycycline on days 0-29 post-infection, which span over the L3 to L4 molt, had no heartworms develop, nor was any *B. pahangi* recovered on necropsy days (days 218-222 post-infection) [10].

Wolbachia is a *Rickettsia*-like, intracellular, bacterial endosymbiont that is found in a variety of filarial nematodes that cause filariasis and dirofilariasis [11]. Though the roles of this endosymbiont need more investigation, some studies provide insights into the functions of this bacterium, indicating that five metabolic biosynthetic pathways are only found in *Wolbachia* but not in its nematode hosts, including heme, riboflavin, FAD, glutathione, and nucleotide synthesis pathways [7, 12]. *Brugia malayi* does not harbor six out of seven genes required for heme *de novo* synthesis, along with the lack of all five genes required for riboflavin biosynthesis. The *de novo* biosynthesis pathways of purines and pyrimidines are found in *Wolbachia* from *B. malayi*, which suggests that *Wolbachia* may support nucleotide synthesis for the host, especially in oogenesis and embryogenesis, where DNA synthesis is in high demand [7]. At the same time, the genome of *Wolbachia* from *B. malayi* indicated the expression of most enzymes for both pathways [7]. The depletion of *Wolbachia* has been proven to sterilize the parasites in several

filarial nematode species, including *B. pahangi, B. malayi, and D. immitis*, which will impact the production of mf [13, 14].

Wolbachia may also be required in molting activities for *B. malayi* and *B. pahangi* [15, 16]. Casiraghi et al. (2002) treated *B. pahangi*-infected gerbils (*Meriones unguiculatus*) with tetracycline. They assessed the impact by looking at the worm recovery when 1) tetracycline was given before the molt of both sexes, 2) after the molt of males but before the molt of females, and 3) after both sexes molted. The gerbils were euthanized 54 days post infection, and worms were collected for qPCR to measure the *Wolbachia* levels. Their results indicated that tetracycline treatment impacted the *B. pahangi* L4 to L5 molting *in vivo* (all compared to control groups – 1) reduced worm recovery; 2) sex-ratio distortion; 3) no significant difference in worm recovery and sex-ratio distortion), and the *Wolbachia* levels in the recovered worms decreased significantly [15]. Another study focused on the L1 to L3 molt of *B. malayi* in the mosquito. They obtained *Wolbachia*-depleted mf by treating *B. malayi*-infected gerbils with tetracycline for six weeks and collecting mf via peritoneal lavage. The mf were used for mosquito feeding and DNA isolation for *Wolbachia* level measurement. The L3 recovered per mosquito were reduced significantly ($p < 0.001$) when fed with tetracycline-treated mf, while the *Wolbachia* levels in mf were lowered by 89.0% [16].

McCall et al. (2014, 2023) investigated the ability of *D. immitis* L3 to complete normal development in heartworm-naïve dogs, when the L3 were produced from the blood of doxycycline-treated microfilaremic dogs [17, 18]. In the study published in 2014, the microfilaremic dogs received doxycycline at 10 mg/kg, BID, for 30 days. The blood was collected on days 73 to 77 post-doxycycline treatment and fed to mosquitoes. They infected heartworm naïve dogs with L3 produced from the doxycycline-treated dogs and examined the

blood for mf and antigen beginning five months after infection until 302 days post infection, when they were necropsied and examined for adult worms. No mf in blood or detectable antigen were observed, and no live or dead adult *D. immitis* were recovered. The dogs infected with L3 developed from the blood of untreated microfilaremic dogs had ten live male and 17 live female heartworms recovered [17]. In another study published in 2023, the authors examined the ability of *D. immitis* L3 developed from the blood of dogs treated with doxycycline (10 mg/kg, once daily for 30 days) and ivermectin (6 μg/kg, given on days 0 and 30) at different time points throughout the treatment regimen. The blood was collected on days 22, 29, and 42 after the beginning of the treatment, and L3 produced by mosquitoes were injected into heartworm-naïve dogs. The subsequently infected dogs were necropsied on days 163 – 183 post infection, and no adult worms were recovered from dogs that received L3 developed from doxycycline/ivermectin-treated mf, while dogs that received L3 developed from untreated mf had a total recovery of 26 – 43 heartworms [18]. However, no measurement of *Wolbachia* levels in either mf or L3 was performed in the above two studies, leaving a gap in knowledge in the correlation between *Wolbachia* levels and the ability of L3 to develop into adult worms in subsequent hosts. Meanwhile, we do not know at which point the worms stopped developing in the host (L3 to L4 molt, L4 to immature adult molt), or whether the depletion of *Wolbachia* impacted the immunomodulatory effect that the worms have against the host.

Our study explores the impact of doxycycline on mf and subsequently produced L3. We measured the *Wolbachia* levels in mf and L3 at different time points during doxycycline treatment. Previous studies have shown inhibition of molting in *Brugia spp*. with tetracycline treatment [14-16, 19]; we were interested in the first molt after the L3 enter the host. Due to

limitations in the ability to track *D. immitis* L3 to L4 molting *in vivo*, we established a *D. immitis in vitro* culture/molting system to observe the impact of *Wolbachia* on molting.

Method

Study animals

All experiments with purpose-bred dogs were performed according to the University of Georgia Institutional Animal Care and Use Committee guidelines using approved Animal Use Protocol A2022 04-006.

The blood from three dogs were used for production of L3s in this study. All dogs were experimentally infected with *D. immitis* Missouri (MO) strain and housed in a specific pathogen-free environment. One dog served as the negative control, and two dogs received doxycycline Hyclate (Epic Pharma, LLC, Laurelton, NY) at 10 mg/kg twice daily for 28 consecutive days. Blood was collected from the jugular vein of each dog before the start of doxycycline treatment and weekly after the treatment began. Blood was used in mosquito feeding for L3 production as described in the protocol by the Filariasis Research Reagent Resource Center (FR3) [20], and mf were isolated for DNA analysis.

Microfilaria isolation from whole blood

The blood was diluted with saponin solution, consisting of 0.2 % saponin (Tokyo Chemical industry, Tokyo, JPN) and 0.85 % sodium chloride (Sigma-Aldrich, St. Louis, MO) in distilled water at a 1:11 ratio (i.e., 3 mL blood with 33 mL solution). The mixture was incubated at 37^{o}C for 15 minutes for hemolysis. The hemolyzed sample was centrifuged at 850 x *g* for 10 minutes at room temperature, and the supernatant was removed. The mf were washed twice by

filling the tubes with phosphate-buffered saline (PBS) and repeating the centrifugation step above. After the second wash, mf were resuspended with 10 mL PBS and counted by taking two, 10 µL aliquots and visualizing at 10X magnification via light microscopy. Microfilariae were stored at -80ºC until DNA isolation was performed.

Mosquito feeding and L3 collection

Laboratory-raised, *Aedes aegypti*, black-eyed, Liverpool strain was cultured and fed with blood collected from control and treatment dogs. Third-stage larvae were collected 15 days post feeding per the FR3 protocol [20]. Briefly, mosquitoes were stunned at low temperatures, gently crushed in a mortar with a pestle, transferred to a 150-mm mesh sieve, quickly washed, and then soaked with 2% ciprofloxacin (Sigma-Aldrich, St. Louis, MO)-supplied HBSS solution. The L3 were manually counted under light microscopy.

DNA isolation and quantitative real-time PCR

DNA samples were isolated from mf and L3 of the control and treatment groups. DNeasy Blood & Tissue kit (QIAGEN, Valencia, CA) was used for DNA isolation. All samples were processed according to manufacturer's instructions. Isolated DNA was stored at -20ºC for qPCR analysis.

Quantitative PCR was performed to determine the ratio of *Wolbachia ftsZ* DNA to *D. immitis* 18s ribosome DNA using the primer set listed below (Table 2.1). SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) with Bio-Rad CXF96 Touch Real-Time PCR Detection System were used. The reactions were designed based on the protocol provided with the SYBR Green Supermix mentioned above. Briefly, the reaction was started at

98°C for two minutes, followed by 40 cycles of 95°C for 15 s, 54°C for 30 s, 60°C for 30 s, signal detection, and melting curve generation (from 60 to 90°C, at 0.5°C interval). Raw data were collected with CFX Maestro Software (Bio-Rad).

Table 2.1
Primers used for *Wolbachia* level measurement.

Gene name		Primer sequence (5' – 3')	Reference
D. immitis 18s rRNA	Forward	TGAGAAACGGCTACCACATC	GenBank: AF036638 [21]
	Reverse	GATAACCGGCCTCATAGAGAAC	
*D. immitis Wolbachia Fts*Z	Forward	GCTGGTGCCTTACCTGATATT	GenBank: AJ495000 [21]
	Reverse	CCACCCATTCCTGCTGTTAT	

In vitro molting assessment

Third-stage larvae were washed according to the protocol from the Zamanian lab [22]. Briefly, the L3 were transferred to a 1.5-mL microcentrifuge tube with 500 µL of wash media RPMI-1640 with L-glutamine (Lonza Bioscience, Walkersville, MD) supplied with 100 U/mL penicillin and 100 µg/mL streptomycin (Penicillin-Streptomycin solution, Thermo Fisher Scientific, Waltham, MA), 10 µg/mL ciprofloxacin, and 100 µg/mL gentamycin (Sigma-Aldrich, St. Louis, MO] and centrifuged at 1,000 x *g* for 10 minutes under room temperature. The L3 condensed at the bottom were transferred to new microcentrifuge tubes pre-filled with 500 µL washing media. The centrifugation and transfer steps were repeated twice. The L3 were transferred to a petri dish containing culture media consisting of washing media plus 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA). The L3 were examined under a dissecting microscope, and one L3 was aliquoted to one well of the 96-well plate. An additional 200 µL of culture media was added to each well containing a single L3. The L3 were cultured in an incubator at 37 and five percent CO_2.

Observations of L3 were made under an optical microscope at 40X magnification. The presence of a fully detached cuticle was defined as a successful molt. The observations were performed for nine consecutive days.

Data analysis

The qPCR results were collected as C_t values. All C_t values were processed by CFX Manager software (Bio-Rad, Hercules, CA, USA), and $2^{-\Delta\Delta Ct}$ (fold change) was calculated using the Livak method [23]. The readouts for the *Wolbachia ftsZ* gene for each sample were normalized to the internal control (*D. immitis* 18S rRNA gene) and then to the control at each time point.

Fisher's exact test was applied to determine the dependency of doxycycline treatment versus *in vitro* L3 to L4 molting rate. To calculate whether the time-to-molt event significantly differed between the control and treated groups, the Log-rank (Mantel-Cox) test was compared to the survival curves. Bonferroni correction was applied to both analyses.

Results

Quantitative real-time PCR

The *Wolbachia* levels in mf and L3 showed a similar trend (Fig 2.1). Following seven days of treatment, the *Wolbachia* levels in treatment groups decreased to less than five percent of the control group in both mf and L3s. The *Wolbachia* levels were further reduced compared to day seven of the treatment as the treatment progressed.

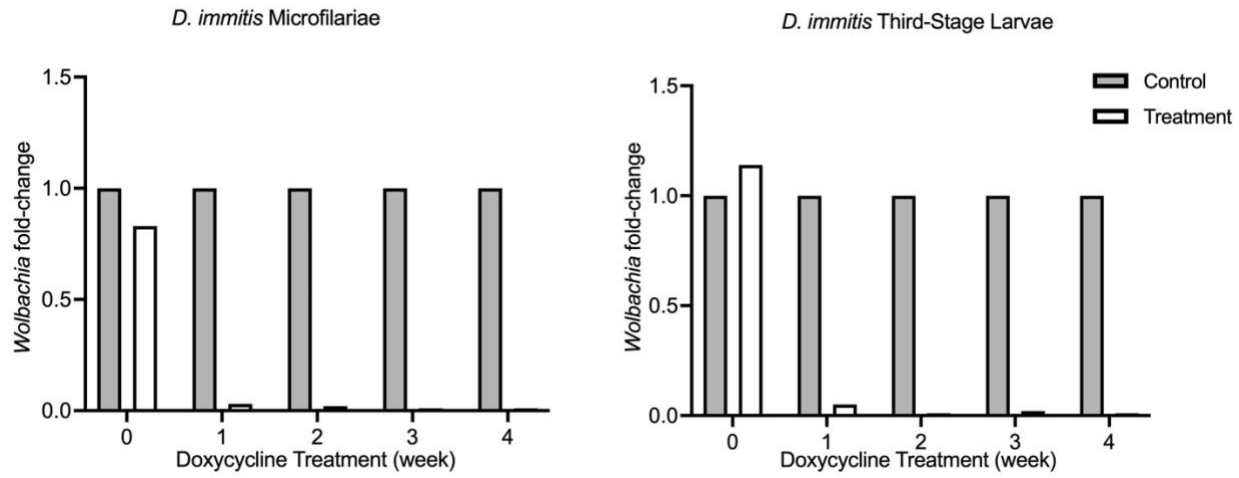

Figure 2.1.

Fold-change of *Wolbachia* levels in *D. immitis* mf and L3. The *Wolbachia* levels were measured using qPCR with *ftsZ/Di* 18s rRNA DNA ratio in both mf and L3. The results indicated a substantial decrease after one week into the treatment (less than 5 % of the control group) and remained at low levels throughout the treatment.

In vitro L3 to L4 molting

We defined molting completion by the identification of a fully detached cuticle in the culture plate. Using Fisher's exact test, we analyzed whether the molting rate was correlated with doxycycline by examining the number of L3 that had molted by the end of the observation period (day nine). No significant difference was found before the start of treatment (week 0, $p = 0.6954$) or at the end of the treatment (week 4, $p = 0.1515$), or during the treatment process (week 1, $p = 0.1994$; week 3, $p = 0.2517$) except for week 2 ($p = 0.0076$) (Table 2.2). The L3 molted on week 2 from the control group reached a molting rate of 83.33%, while the molting rate of other weeks were 31.25%, 72.92%, 51.22%, and 51.02 % for weeks 0, 1, 3, and 4, respectively. At the same time, the molting rate for the treatment group on week 2 reached 60.82%, while the molting rate of other weeks was 26.80%, 82.29%, 63.04%, and 64.58% for weeks 0, 1, 3, and 4, respectively.

Table 2.2
Molting number of control and doxycycline-treated L3

		Week 0		Week 1		Week 2		Week 3		Week 4	
Doxycycline		-	+	-	+	-	+	-	+	-	+
Molt	Yes	15	26	35	79	40	59	21	58	25	62
	No	33	71	13	17	8	38	20	34	24	34
p		0.6954		0.1994		0.0076**		0.2517		0.1515	

The time-to-molt event was analyzed by recording the date that the L3 molted and excluded any L3 that died before the observation's end point (day nine). We did not observe any significant impact of doxycycline on the date-to-molt events. We used the Log-rank (Mantel-Cox) test in the Kaplan-Meier survival curves to measure the correlation between the molting pattern and doxycycline treatment. The molting pattern was not significantly correlated to the length of doxycycline treatment at all weeks (week 0, $p = 0.6222$; week 1, $p = 0.0222$; week 2, $p = 0.0267$; week 3, $p = 0.1147$; week 4, $p = 0.0854$) (Figure 2.1).

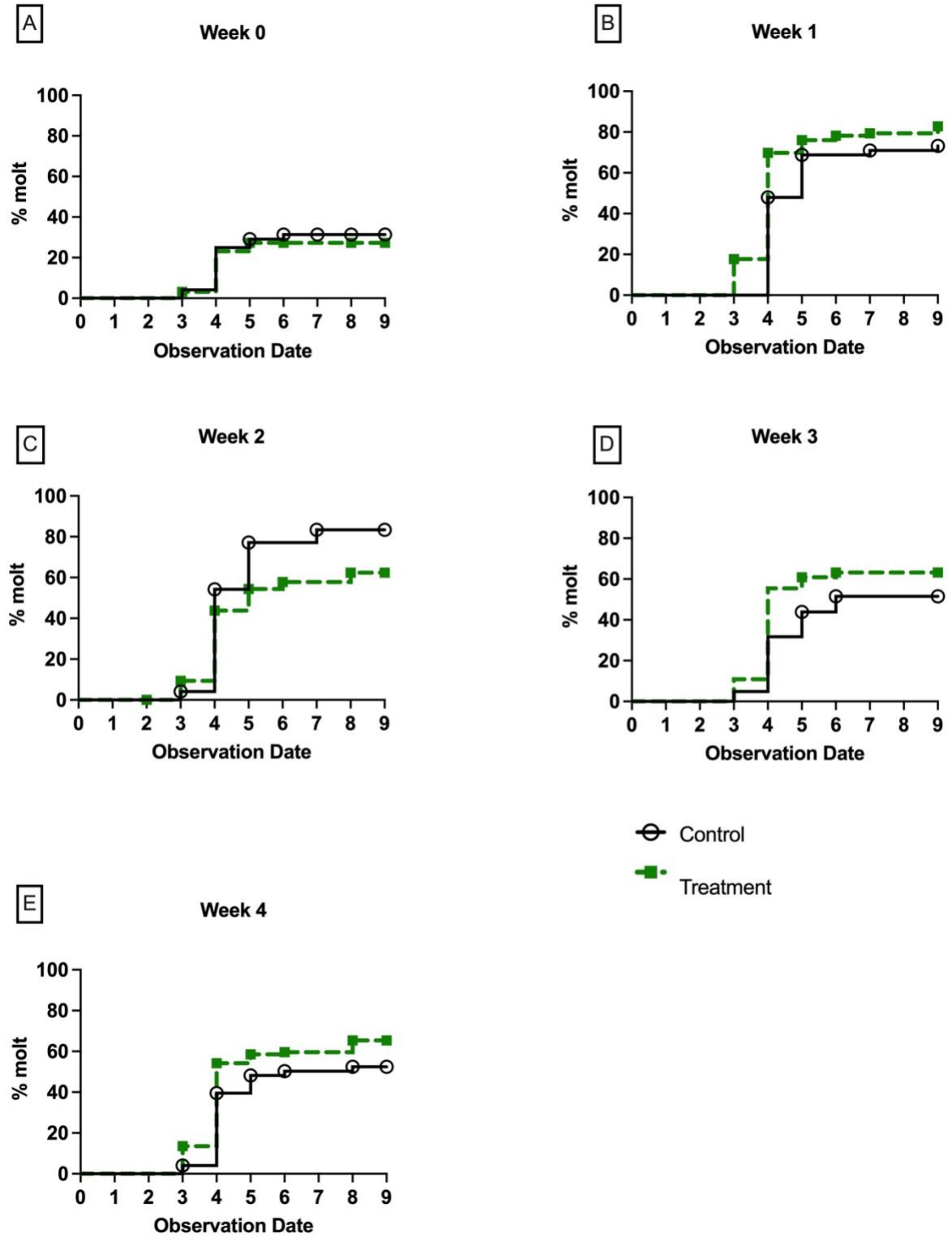

Figure 2.2
Time-to-molt event of *D. immitis* L3 through doxycycline treatment. The observation of a fully detached cuticle indicates the success of the molt event. The death occurred before molt was excluded from the group. The molt percent represents the ratio of successfully molted L3 on

given date to the total number of L3 included in the experiment. The doxycycline treatment in all weeks does not significantly impact the time-to-molt event.

Discussion

Heartworm disease affects multiple hosts (such as canids, felids, bears, ferrets, seals, sea lions, and humans) and is distributed in all continents except Antarctica [2]. Doxycycline is an important component of heartworm treatment due to its effect on the bacterial endosymbiont *Wolbachia* [11, 24]. Previous studies by McCall et al. (2014, 2023) [17, 18] indicated that *D. immitis* L3 developed from doxycycline-treated mf can grow into viable L3 in mosquitoes but lose the ability to mature into adults in subsequent vertebrate hosts. Based on the previous studies, we inferred that the removal of *Wolbachia* leads to the inability of parasites to complete lifecycles. However, the mechanism behind this remains to be determined. Several hypotheses include the inhibition of L3 to L4 molt, the inhibition of L4 to immature adult molt, and if *Wolbachia* depletion leads to altered host immune reactions against the worms.

We focused on the L3 to L4 molt, which occurs by day 3 - 12 post infection [6]. Due to the lack of an *in viv*o tracking method of L3, we had to use an *in vitro* culture method and observed the L3 for nine consecutive days. We hypothesized that L3 raised from doxycycline-treated mf would be unable to molt *in vitro.* Our results disproved our hypothesis. The results showed that the *Wolbachia* levels in mf and L3 were reduced sufficiently seven days after the beginning of doxycycline treatment and remained less than five percent of the control group throughout the treatment. However, the molting rate and time-to-molt were not impacted (Table 2.2, Figure 2.1) except the molting rate for week 2 ($p = 0.0076$, $p < 0.01$). The significance of the molting rate at week 2 may be due to biological variation from different batches of L3.

Our study investigated the impact of doxycycline without ivermectin at the preventive dose, which is microfilaricidal and may impact the general health of the mf, to reduce potential variations due to other factors besides *Wolbachia* elimination [25]. Our data showed that *Wolbachia* levels decreased to almost non-detectable in mf and L3 after doxycycline treatment. While *Wolbachia* levels had been examined in mf, they had not been examined in L3 derived from mosquitoes fed blood from doxycycline-treated dogs. These results suggest that the inability of mf from doxycycline-treated dogs to complete their life cycle in subsequent hosts is due to disruption in the life cycle at a step beyond the L3 molt.

Multiple studies have been performed examining the effect of tetracycline treatment versus the molting of filarial nematodes, including *B. malayi, B. pahangi, Onchocerca volvulus,* and *D. immitis* [16, 19, 26, 27]. Our data indicates that the presence of *Wolbachia* may not be necessary for *D. immitis* L3 to molt to L4 *in vitro.* Smith et al. (2000) examined the *in vitro* impact of tetracycline against the development of several filarial nematodes, and their results indicated that inhibition of molting occurred. They cultured *B. malayi* and *B. pahangi* with *Rhodotorula minuta* supplemented by arachidonic acid, while *D. immitis* was cultured without cellular coculture. Tetracycline (10 µg/ml) was added at the beginning of the culture (ten days for *Brugia* spp., three days for *D. immitis*). Tetracycline treatment inhibited *B. malayi* L3 to L4 molting by 93.9% of the controls on day ten, and the *D. immitis* L3 to L4 molting reached inhibition at 57.5% of the controls on day three. They performed PCR to determine *Wolbachia* DNA levels, and samples collected from tetracycline-treated larvae still showed amplified bands on gel electrophoresis [19]. The discrepancy between their study and ours has several potential explanations. The duration of treatment may explain the decrease of *Wolbachia* in our L3 samples. The first timepoint we tested for *Wolbachia* was seven days after the beginning of the

treatment, while Smith et al. (2000) tested for *Wolbachia* three days post-treatment. The treatment conditions (*in vitro* vs. *in vivo*) could contribute to the difference in molting results. The actual medication used (tetracycline vs. doxycycline, with doxycycline being more lipophilic), and the endpoint of molting observation (three days vs. nine days) may also impact the outcomes.

L4 obtained from *in vitro* or *in vivo* molting has been shown to have some differences. Though not in naïve definitive hosts, Marriott et al. (2023) developed an immunodeficient mouse model and obtained *in vivo* grown L4 from L3 [28]. They cultured L3 *in vitro* with EMEM media supplemented with 10 % fetal bovine serum or with the addition of Madin-Darby Canine Kidney cells or rhesus monkey kidney epithelial cells on top of the fetal bovine serum. They compared the morphology as well as the *Wolbachia* levels in L4 obtained from mice 14 days post infection and from *in vitro* culture 14 days post culture. The L4 cultured *in vitro* were significantly smaller compared to the L4 cultured *in vivo. In vitro*-cultured L4 also presented with microscopic degenerative phenotypes, including malformed cuticle, hypodermis, buccal cavity, esophagus, and intestine [28]. *Wolbachia* numbers in the *in vivo*-cultured L4 expanded 66-fold on average, while the *in vitro*-cultured L4 did not show a notable change in *Wolbachia*. This could suggest that the host immune system, the nutrition those L3 could receive, as well as the host-parasite interaction contribute to the fitness of the worms and their endosymbiont, *Wolbachia*, and explain the differences in levels of *Wolbachia* between *in vivo* and *in vitro*-derived larvae

Wolbachia levels in mf were measured weekly throughout the 28-day doxycycline treatment in a clinical study [21]. Savadelis et al. (2018) studied eight dogs with doxycycline treatment at 10 mg/kg twice daily for 28 days and a monthly correct dosage of ivermectin/pyrantel (Heartgard® Plus, Boehringer Ingelheim, Duluth, GA). The dogs were

brought to the clinic weekly for blood collection, and qPCR was performed to detect the *Wolbachia* level in mf at weeks 0, 1, 2, 3, and 4 post treatment start point. Unlike our study, which showed a substantial decrease of *Wolbachia* in mf one week post treatment starts, six out of eight dogs remained positive for *Wolbachia* DNA in mf on week 1. All dogs were cleared of *Wolbachia* DNA in mf at the end point of the study [21]. This difference may be due to several factors. They defined *Wolbachia* DNA positive as the qPCR result that could amplify *Wolbachia* DNA within 38 cycles of the reaction. In contrast, our qPCR looked at the relative changes in *Wolbachia* DNA compared to *D. immitis* DNA, which focused on the difference in C_t values between the *Wolbachia* gene and the *D. immitis* gene. Also, clinical studies have limitations in terms of animal conditions, the original burden of infections, and the compliance of pet owners. Those aspects may impact the efficacy of doxycycline treatment, thus leading to the delay in *Wolbachia* DNA reduction.

Answering the questions of whether the *D. immitis* L3 can also molt *in vivo* and exactly how doxycycline treatment inhibits *D. immitis* development *in vivo* requires further investigation. Tracking the L3s once they enter the hosts is time-consuming and cost-intensive [6]. For our study, we developed the *in vitro D. immitis* L3 to L4 molting system with only RPMI-1640 and heat-inactivated FBS. This system allows us to determine whether each cultured L3 can molt with minimal other variables (i.e. cell viability if cell culture is involved). We proved that doxycycline-treated *D. immitis* L3 can molt *in vitro* into L4. This could suggest that the inhibition of worm development in the host may occur after the L3 to L4 molt.

Conclusion

Our research demonstrated that doxycycline can effectively reduce *Wolbachia* levels in mf and subsequently derived L3 at one week after the start of treatment. Our research also suggests that under *in vitro* conditions, the depletion of *Wolbachia* via *in vivo* doxycycline treatment does not impact the molting rate or the time-to-molt of *D. immitis* L3 to L4.

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CHAPTER 3

REAL-TIME PCR AND IMMUNOHISTOCHEMISTRY DETECTION OF *WOLBACHIA* IN ADULT *DIROFILARIA IMMITIS* FROM DOGS TREATED WITH DOXYCYCLINE AND IVERMECTIN

Chu, Y. Submitted to Parasites & Vectors, September 20, 2024.

Abstract

Background

Wolbachia is present in all life stages of *Dirofilaria immitis*. *Wolbachia* surface protein (WSP) can be highly immunogenic and induce acute inflammatory reactions in the host upon worm death. To avoid the abrupt release of *Wolbachia* and its antigens from deceased parasites, the American Heartworm Society (AHS) has suggested using doxycycline and having a one-month wait period between the doxycycline treatment and the adulticidal process for *Wolbachia* elimination. Studies have shown that the 28-day, 10 mg/kg BID administration of doxycycline can effectively clear *Wolbachia* in the bloodstream of the host. The one-month wait period is hypothesized to allow for further reduction of *Wolbachia*. However, the levels of *Wolbachia* in adult parasites after doxycycline treatment remain unknown.

Methods

Forty-five purposely bred dogs were intravenously transplanted with 20 *Dirofilaria immitis* adults, consisting of 12 females and eight males. The dogs were divided into nine groups of five dogs each. Two groups each received 5, 7.5, or 10 mg/kg doxycycline BID orally for 28 days and ivermectin (IVM) monthly (6 µg/kg.) Three groups remained untreated as controls. Study animals were necropsied on days 0, 30, and 60 following the start of treatment. Adult worms were collected at necropsy and preserved for later analysis. Quantitative PCR and immunohistochemistry for WSP were performed on worms collected at each time point. The data were analyzed using a Linear Mixed Model (LMM). Multiple comparisons were adjusted using Tukey's test.

Results

The qPCR results showed that all treatment doses significantly reduced *Wolbachia* levels compared to the control groups at 30 and 60 days. The intradose comparison indicated a significant decrease on day 60 compared to day 30. No significant differences were found between different doses on the two examination dates. Immunohistochemistry indicated the markedly reduced presence of *Wolbachia* in treatment groups.

Conclusion

All dosages of doxycycline effectively decreased *Wolbachia* levels by both timepoints (30 and 60 days). The *Wolbachia* levels in *D. immitis* adults decreased further after the one-month wait period but were still present. Immunohistochemistry for WSP correlated with the real-time PCR findings.

Background

Heartworm disease, caused by *Dirofilaria immitis*, is a long-existing and continuing threat to the well-being of canine species worldwide. *Dirofilaria immitis* requires an intermediate host (mosquito) and a definitive host (i.e., canids) to complete its life cycle. *Dirofilaria immitis* adults can live in the pulmonary artery of the definitive host for up to seven years and produce microfilariae (mf) if both adult males and females are present. To eliminate the parasites, the American Heartworm Society (AHS) recommends three injections of melarsomine dihydrochloride (MEL), with one month between the first and second doses, and twenty-four hours between the second and third doses, to kill the adult parasites. Prior to the adulticidal treatment, the AHS advises the use of a macrocyclic lactone along with doxycycline as a supplementary treatment [1].

Doxycycline is a tetracycline-class antibiotic that can treat a wide range of infections caused by bacteria, including *Mycoplasma* and *Rickettsia* species [2]. Doxycycline is also used as an adjunct therapy in the treatment of elephantiasis, dirofilariasis, and onchocerciasis by targeting the endosymbiont, *Wolbachia* [3]. *Wolbachia* is hypothesized to perform biosynthetic activities that were found to be absent in the nematode genome, including heme utilization, lipid synthesis, and enzyme metabolism [4-7]. The *Wolbachia* genus belongs to the Alphaproteobacteria class in the order *Rickettsiales*. This bacterium is found in many filarial nematodes in the family *Onchocercidae,* including *Brugia malayi, B. pahangi*, *Onchocerca volvulus*, and *D. immitis*, in all life stages. In human filariasis and onchocerciasis, *Wolbachia* surface protein (WSP), in a manner similar to lipopolysaccharide, induces a pro-inflammatory innate immune reaction by activating host monocytes, macrophages, dendritic cells, and neutrophils [8, 9]. Post-treatment reactions, including fever, tachycardia, headaches, and lymph node enlargement, can happen after treatment of *B. malayi* and onchocerciasis infection [10, 11]. The reduction of *Wolbachia* prior to adulticidal treatment or using treatments that prevent sudden *Wolbachia* release should be considered to prevent potential inflammatory reactions. Though the roles of *Wolbachia* in filarial nematodes are still unclear, multiple studies have shown that the lack of *Wolbachia* impacts the fertility of the parasite and viability in different developmental stages [6, 12-14]. The elimination of *Wolbachia* can reduce the inflammatory immune reactions against bacteria released from the parasite, as well as prevent the potential pathological changes that the inflammation may induce.

Doxycycline has been tested in multiple clinical conditions for human filariasis. Compared to other antihelminth treatments alone, the addition of doxycycline showed increased efficiency in mf elimination and parasite-killing [15, 16]. The decrease in *Wolbachia* levels in

response to doxycycline treatment has been demonstrated in multiple studies; however, the optimal dosage of doxycycline in heartworm treatment remains unclear. Members of our group have previously investigated the elimination of *D. immitis* mf and adults, as well as lung pathology of the hosts, using different dosages of IVM and doxycycline either alone or together [17]. The infection in that reported study was induced by adult *D. immitis* transplantation. Doxycycline was given at 10 mg/kg once per day orally starting six weeks after transplantation from weeks 0-6, 10-12, 16-18, 22-26, and 28-34, with or without IVM (6 μg/kg, orally, once per week for 34 weeks). The groups that received MEL treatment started at week 24 and followed the AHS protocol. All dogs were euthanized at week 36. The concentration of mf was tracked throughout the experiment, and qPCR was performed on the adult worms. The number of *D. immitis* adults recovered at necropsy was significantly reduced in all treatment groups compared to the control group (efficacy: doxycycline 8.7%, IVM 20.3%, doxycycline + IVM 78.3%). Both doxycycline and IVM showed significant efficacy in mf reduction, including the group that received both treatments. Meanwhile, the copy number of the *Wolbachia* ftsZ gene in adult worms was significantly decreased in the doxycycline and doxycycline + IVM treatment groups. *Wolbachia* surface protein (WSP) immunohistochemistry indicated a reduction in *Wolbachia* in doxycycline and doxycycline + IVM groups, but not in the IVM-only group [18]. Histopathology suggested a reduction in lung lesions in animals that received doxycycline + IVM compared to MEL or doxycycline + IVM + MEL groups [19]. A clinical study [20] showed total clearance of circulating *Wolbachia* DNA in antigen- and mf-positive dogs (n = 17) after 10 mg/kg, twice per day, 30-day doxycycline administration with 2.5% moxidectin.

Dirofilaria immitis infection can induce cardiovascular and pulmonary lesions in multiple ways. Kramer et al. (2011) assessed lung pathology in *D. immitis*-experimentally infected dogs

treated with three injections of MEL with or without prior doxycycline treatment [21]. Doxycycline was given 20 mg/kg orally, once per day, for 4 weeks. Melarsomine injection started 8 weeks after the completion of doxycycline treatment per AHS recommendations. All groups were euthanized at week 24 post infection. Dogs treated with MEL alone formed typical lung lesions associated with thromboembolism and severe alveolar wall thickening and hepatization, while dogs that received doxycycline pre-treatment showed decreased severity of the lesions, especially with regards to perivascular inflammation.

A clinical study by Nelson et al. (2017) [22] looked at the rates of respiratory complications and heartworm disease-related deaths in dogs naturally infected with heartworm that received doxycycline 10 mg/kg BID prior to MEL injection (n = 47) versus no doxycycline before MEL (n = 47). All dogs received MEL 30 days after the completion of doxycycline. They found fewer respiratory complications and heartworm disease-related deaths in the doxycycline-treated group compared to the no doxycycline group (6.52% versus 19.14% and 0% versus 4.25%, respectively).

The present study aimed to investigate *Wolbachia* levels in adult *D. immitis* after doxycycline treatment at different dosages, as well as before and after the one-month wait period (also referred to as the "rest period") at the end of the doxycycline regimen. Our objective was to evaluate whether a 28-day, 5, 7.5, or 10 mg/kg, BID, doxycycline treatment, combined with monthly ML preventives (IVM at 6 µg/kg), can significantly reduce the *Wolbachia* levels in adult females and males at 30 and 60 days post treatment. We measured *Wolbachia* levels using qPCR and examined the ratio of *Wolbachia ftsZ* DNA to *D. immitis* 18S DNA. By performing immunohistochemistry using anti-WSP antibody, we were able to examine the presence of *Wolbachia* in *D. immitis* cross-sections in the different treatment groups. We evaluated the

necessity of the one-month wait period based on changes in *Wolbachia* levels in adult *D. immitis* at the beginning and end of the one-month "rest period".

Methods

Study animals

All animals used in this study were housed in a specific-pathogen-free facility for the duration of the study. Forty-five, young adult dogs were intravenously transplanted with 20 adult *D. immitis* (GA-3 isolate, 12 females and 8 males) each and were randomly divided into nine groups of five dogs each. The dogs and transplanted worms were allowed to rest for 75 days before doxycycline and IVM treatment was started. Groups 1, 2, and 3 served as non-treated controls. Groups 4-9 received different doxycycline regimens (Figure 3.1, Table 3.1). Groups 4 and 7, groups 5 and 8, and groups 6 and 9 received BID doxycycline at 5 mg/kg, 7.5 mg/kg, or 10 mg/kg, respectively. All treatment groups received monthly ivermectin at the preventive dose of 6-12 µg/kg. On day 0 post treatment, control group 1 was euthanized and necropsied. On day 30 post-treatment, control group 2 and treatment groups 4, 5, and 6 were euthanized and necropsied. On day 60, control group 3 and treatment groups 7, 8, and 9 were euthanized and necropsied. Adult worms were collected at necropsy, sexed, labeled, and stored in 70% ethanol for DNA analysis or 4% paraformaldehyde (PFA) for anti-WSP immunohistochemistry.

Table 3.1
Experimental treatment design for all groups with different doxycycline regimens.

Necropsy (days post-treatment)	Control	Doxycycline 5 mg/kg	Doxycycline 7.5 mg/kg	Doxycycline 10 mg/kg
	IVM 6 µg/kg monthly			
0	Group 1			
30	Group 2	Group 4	Group 5	Group 6
60	Group 3	Group 7	Group 8	Group 9

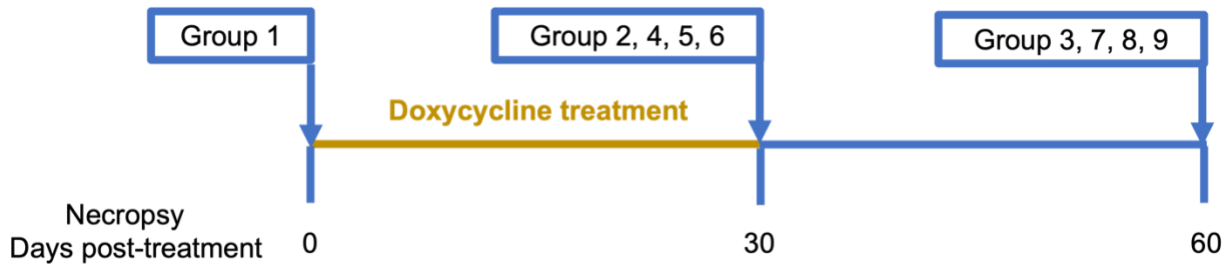

Figure 3.1
Experimental timeline of necropsy dates of each group.

DNA isolation and quantitative real-time PCR

Three adult female and male *D. immitis* from each dog were used for DNA isolation. One adult female and one adult male were randomly picked from the extra worms used for intravenous transplantation and served as standard controls for all female and male samples, respectively. Each single worm was stored in 70 % ethanol and soaked overnight in 50 mL phosphate-buffered saline (PBS) before liquid nitrogen homogenization. The worms were broken into powder, aliquoted per the suggestion of the DNeasy Blood & Tissue kit (QIAGEN, Valencia, CA), and processed according to the kit protocol. Isolated DNA was stored at –20ºC until further analysis.

Relative quantitative PCR (qPCR) was performed to determine the ratio of *Wolbachia* ftsZ DNA to *D. immitis* 18S DNA to assess the change in *Wolbachia* levels in adult worms. Primers were chosen based on previous research [23]. The *Wolbachia* amplicon (GenBank: AJ495000) included forward (5'-GCT GGT GCC TTA CCT GAT ATT-3') and reverse (5'-CCA CCC ATT CCT GCT GTT AT-3') primers to amplify a 110-bp fragment. The *D. immitis* amplicon (GenBank: AF036638) included forward (5'-TGA GAA ACG GCT ACC ACA TC-3') and reverse (5'-GAT AAC CGG CCT CAT AGA GAA C-3') primers that amplified a 112-bp

fragment. The assay used SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) with Bio-Rad CXF96 Touch Real-Time PCR Detection System. Raw data were collected with CFX Maestro Software (Bio-Rad, Hercules, CA).

Immunohistochemistry staining

Two female and two male, adult *D. immitis* from each group were randomly selected for immunohistochemistry. The adult worms were fixed in 4% paraformaldehyde and embedded in paraffin for sectioning. The worms were held in position using 10% agarose and trimmed to expose the cross-section before embedding. Briefly, the slides were deparaffinized, rehydrated, and blocked with peroxidase suppressor and blocking buffer. Sections were incubated with anti-WSP monoclonal antibody (BEI Resources, NIAID, NIH. NR-51684) with the dilution of 1:250; mouse IgG2a kappa isotype control (Invitrogen. Catalog # 14-4724) served as negative control. A goat anti-mouse IgG (H+L) antibody, HRP (Invitrogen. REF 32430) was used at a dilution of 1:50 as the secondary antibody. The sections were stained using the horseradish peroxidase (HRP) staining technique with AEC (3-amino-9-ethylcarbazole) substrate according to the recommendations of the manufacturers (BD PharmingenTM AEC Substrate Kit; BD Biosciences, catalog # 551015) and as described in the supplementary material protocol, and counterstained with hematoxylin. ProlongTM Gold anti-fade Mountant (Invitrogen, REF P10144) was used to coverslip the sections, and all stained slides were photographed within 48 hours to avoid the fading of color.

Data analysis

The qPCR results were collected as C_t values. All C_t values were processed by CFX Manager software (Bio-Rad, Hercules, CA, USA) and $2^{-\Delta\Delta Ct}$ (fold change) was calculated using the Livak method [24]. The readouts for the *Wolbachia* fst Z gene for each sample were normalized, first to the internal control (*D. immitis* 18S rRNA gene), then to the standard control (one adult male or female *D. immitis* from the same batch of transplantation). The fold change of Wolbachia is presented in the descriptive data. The fold change data were log-transformed to match the assumptions of normality and homoscedasticity of model residuals to apply to a linear mixed model. All analyses were performed using SAS 9.4 (Cary, NC, USA). Multiple comparisons were adjusted by using Tukey's test. The graphs were made with GraphPad Prism version 10.0.0. (Boston, MA, USA).

Results

Real-time PCR

Within *D. immitis* female adults recovered at necropsy, the *Wolbachia* levels in all treatment groups were significantly decreased from the control groups (control vs. 5 mg/kg; control vs. 7.5 mg/kg; control vs. 10 mg/kg) on day 30 (all $p < 0.0001$) and day 60 (all $p < 0.0001$.) based on statistical analysis performed on log e transformed fold-change data. No significant differences were found between different dosage groups (5 mg/kg vs. 7.5 mg/kg; 5 mg/kg vs. 10 mg/kg; 7.5 mg/kg vs. 10 mg/kg) on day 30 ($p = 0.40$, $p = 0.31$, $p = 1.00$, respectively) or day 60 ($p = 0.13$, $p = 0.25$, $p = 0.98$, respectively). Between days 30 and 60, all dosage groups (5 mg/kg, 7.5 mg/kg, 10 mg/kg) showed a significant decrease in *Wolbachia* levels within each dosage (all $p < 0.0001$.) (Figure. 3.2 A).

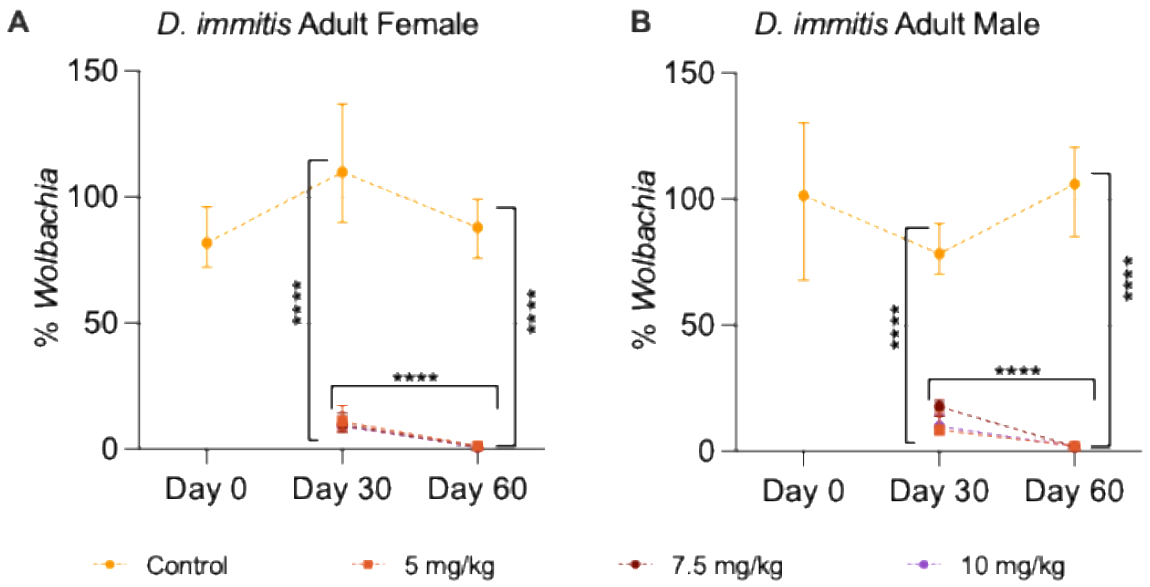

Figure 3.2
Relative *Wolbachia* levels (*ftsZ/Di* 18s rRNA ratio) in *D. immitis* female (A) and male (B) adults for each group at each time point showed in percentage-fold change. Horizontal asterisks indicate significant differences between time points within each dosage of treatment groups (intra-dose comparison between days, $p < 0.0001$). Vertical asterisks indicate significant differences between treatment (5 mg/kg, 7.5 mg/kg, and 10 mg/kg) and control groups at a single time point (all $p<0.0001$).

The *Wolbachia* levels in *D. immitis* male adults presented a similar decline over time. All treatment groups displayed a significant decrease in *Wolbachia* levels compared to control groups (control vs. 5 mg/kg; control vs. 7.5 mg/kg; control vs. 10 mg/kg) on day 30 (all $p < 0.0001$) and day 60 (all $p < 0.0001$). Among all dosage groups (5 mg/kg vs. 7.5 mg/kg; 5 mg/kg vs. 10 mg/kg; 7.5 mg/kg vs. 10 mg/kg), no significant differences were found on day 30 ($p = 0.11$, $p = 0.84$, $p = 0.44$, respectively) or day 60 ($p = 0.98$, $p = 0.69$, $p = 0.89$, respectively) post treatment. The intradose comparisons between days revealed a significant decrease in *Wolbachia* levels on day 60 compared to day 30 within all treatment groups (5 mg/kg, 7.5 mg/kg, 10 mg/kg; all $p < 0.0001$.) (Figure. 3.2B).

Tables 3.2 and 3.3 summarize the *Wolbachia* levels in female and male adults in percentages of control standard. To quantify the changes in *Wolbachia* levels, we used the Livak method to calculate the relative *Wolbachia* levels. We defined the *Wolbachia* levels in control standards as 100%, and all data are shown in percentages of the control standards. The interquartile range (IQR) indicates the range of *Wolbachia* levels that include 50% of the population given certain conditions (doxycycline dosages, examination date). The amount of *Wolbachia* in adult *D. immitis* significantly decreased, both before (all $p < 0.0001$) and after the one-month wait period (all $p < 0.0001$). On day 30 post treatment, the median *Wolbachia* levels in adult females were reduced to 11.1% (5 mg/kg doxycycline), 9.6% (7.5 mg/kg doxycycline), and 9.2% (10 mg/kg doxycycline) of the female control standard. On day 60 post treatment, the median *Wolbachia* levels in adult females further decreased to 1.3% (5 mg/kg doxycycline), 0.9% (7.5 mg/kg doxycycline), and 0.8% (10 mg/kg doxycycline) of the control standard. The median *Wolbachia* levels in adult males showed a similar trend. On days 30 and 60 post infection, the median *Wolbachia* levels were at 8.4% and 2.1% (5 mg/kg doxycycline), 17.7% and 1.9% (7.5 mg/kg doxycycline), and 10.1% and 1.9% (10 mg/kg doxycycline) of the male control standard, respectively.

Table 3.2

Percentage median and interquartile range of *Wolbachia* in Adult Female *D. immitis*

Days post-treatment	Group	Doxycycline	% Median	% Interquartile range (IQR)
0	1	Control	81.8	(72.1, 96.3)
30	2	Control	109.9	(89.9, 136.8)
	4	5 mg/kg	11.1	(7.4, 17.3)
	5	7.5 mg/kg	9.6	(6.8, 14.4)
	6	10 mg/kg	9.2	(7.1, 11.3)
60	3	Control	88	(75.8, 99.1)
	7	5 mg/kg	1.3	(1.0, 2.1)

	8	7.5 mg/kg	0.9	(0.9, 1.0)
	9	10 mg/kg	0.8	(0.5, 1.6)

Table 3.3
Percentage of *Wolbachia* in Adult Male *D. immitis*

Days post-treatment	Group	Doxycycline	% Median	% Interquartile range (IQR)
0	1	Control	101.4	(67.9, 130.4)
30	2	Control	90.4	(70.1, 78.4)
	4	5 mg/kg	8.4	(6.2, 15.2)
	5	7.5 mg/kg	17.7	(13.8, 20.4)
	6	10 mg/kg	10.1	(7.1, 14.6)
60	3	Control	106.0	(85, 120.7)
	7	5 mg/kg	2.1	(1.4, 2.8)
	8	7.5 mg/kg	1.9	(1.3, 2.8)
	9	10 mg/kg	1.9	(1.0, 2.1)

Immunohistochemistry

Dirofilaria immitis female and male adults were sectioned and stained with anti-WSP antibody. *Wolbachia* clusters were present in the lateral cords of female and male adults from the control groups across the dates examined (Figures 3.2 and 3.3, respectively). All samples were immunoreactive for WSP despite treatment with doxycycline and the time at which the sample was collected. Dense, robust clusters of *Wolbachia* were present in the control female and male adults (Figure 3.3, A-C; Figure 3.4, A-C). Fewer *Wolbachia* were observed in groups 4, 5, and 6 (all dosages at day 30) in both female and male adults (Figure 3.3, D-F; Figure 3.4, D-F). Single, scattered, and deformed *Wolbachia* were observed in groups 7, 8, and 9 (Day 60, all dosages; Figure 3.3, G-I; Figure 3.4, G-I). The reduction of positive staining of WSP aligned with the real-time PCR results.

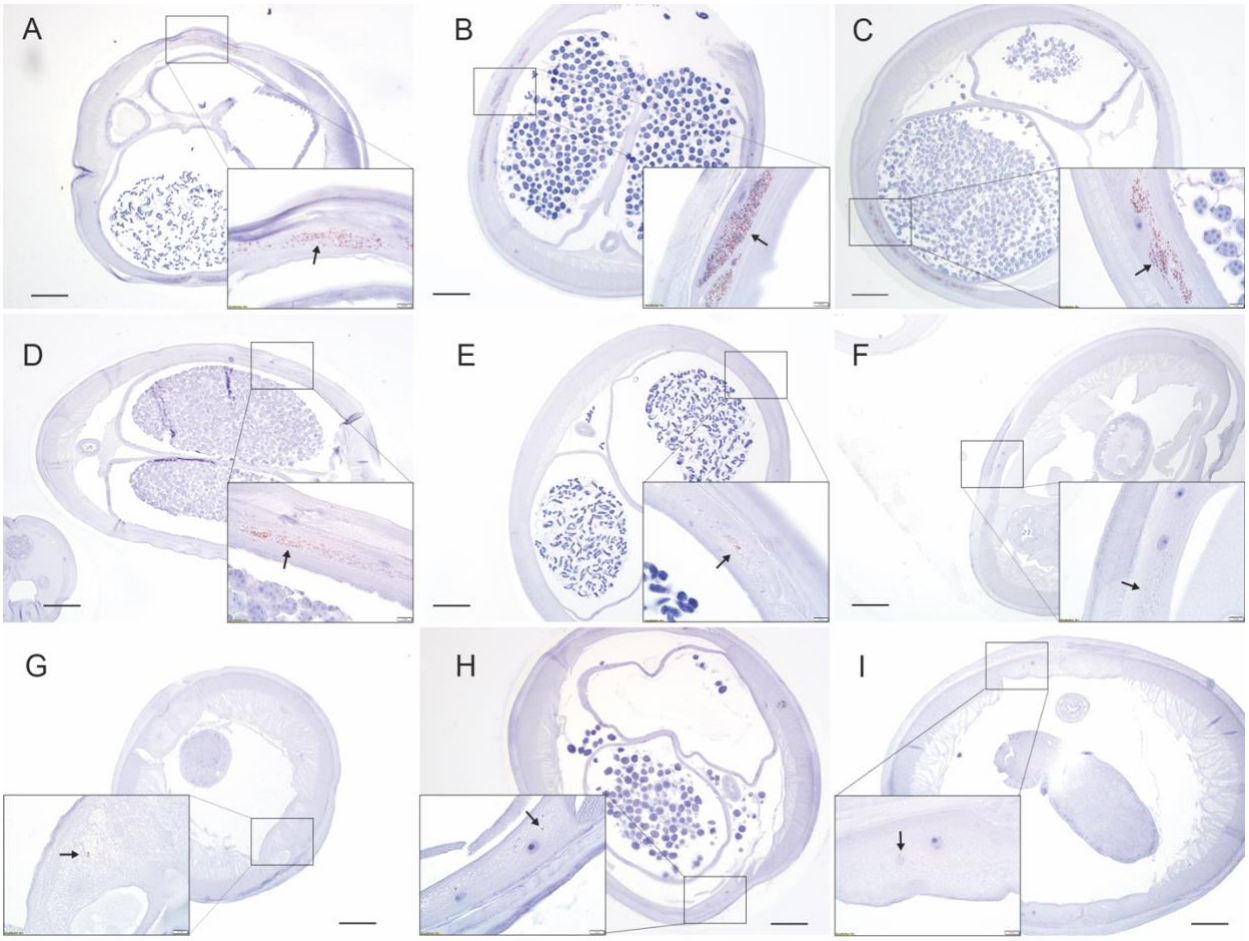

Figure 3.3
Detection of *Wolbachia* in adult female *D. immitis* by immunohistochemistry with anti-WSP antibody. The rectangular box indicates 10X zoom-in. Scale bar = 100 µM.
A – C. Cross-sections from groups 1, 2, and 3 (control groups necropsied on 0, 30, 60 days post-treatment), respectively, showing dense *Wolbachia* clusters (arrows) in the lateral cord. D – F. Cross-sections from groups 4, 5, and 6 (doxycycline 5 mg/kg, 7.5 mg/kg, 10 mg/kg necropsied 30 days post-treatment), respectively. Scattered *Wolbachia* clusters (arrows) in the lateral cord. G – I. Cross-sections from groups 7, 8, and 9 (doxycycline 5 mg/kg, 7.5 mg/kg, 10 mg/kg necropsied 60 days post-treatment), respectively. Single *Wolbachia* (arrows in G, H) and deformed *Wolbachia* (arrows in I) in the lateral cord.

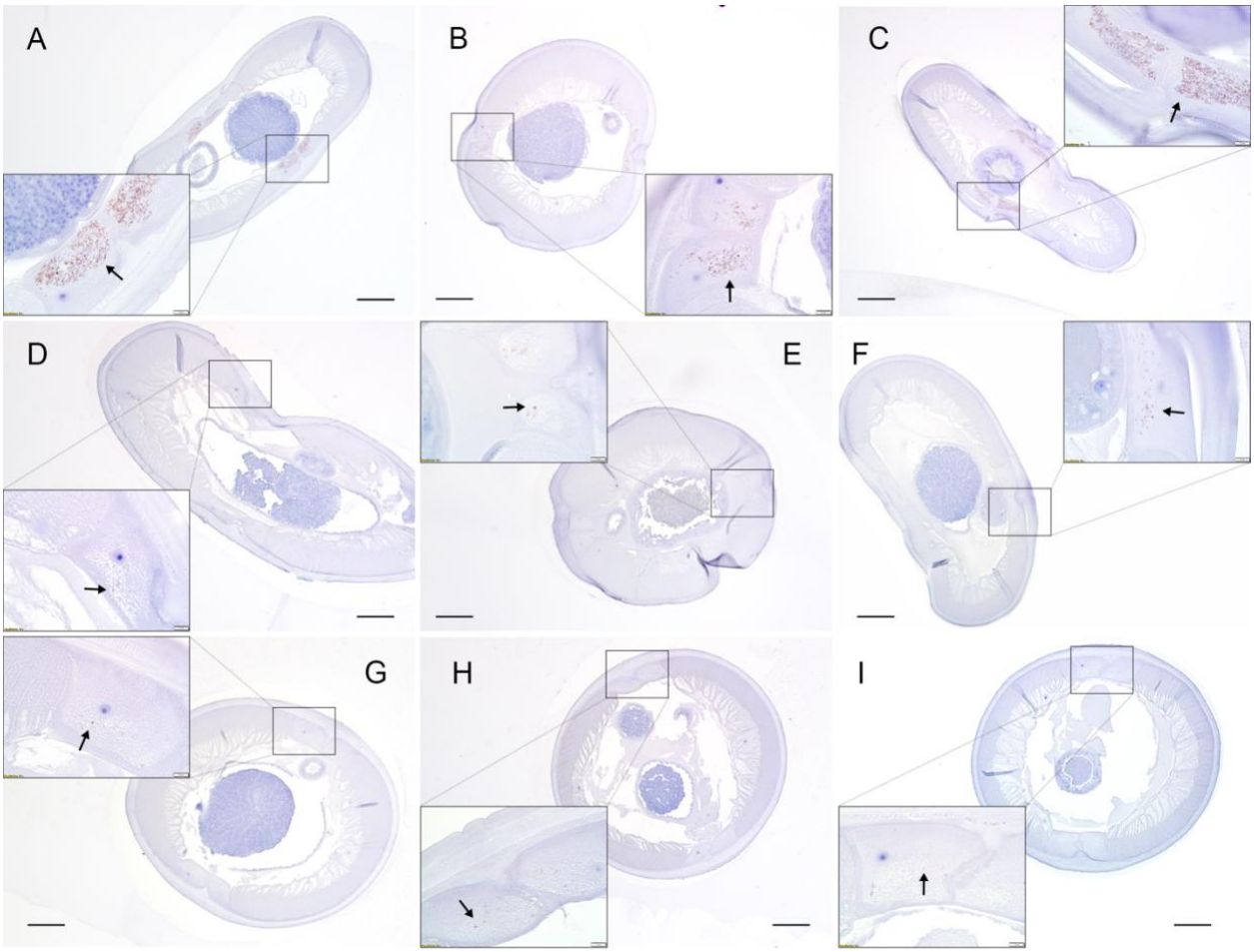

Figure 3.4
Detection of *Wolbachia* in adult male *D. immitis* by immunohistochemistry with anti-WSP antibody. The rectangular box indicates 10X zoom-in. Scale bar = 100 µM.
A – C. Cross-sections from groups 1, 2, and 3 (control groups necropsied on 0, 30, 60 days post-treatment), respectively. Dense *Wolbachia* clusters (arrows) in the lateral cord. D – F. Cross-sections from groups 4, 5, and 6 (doxycycline 5 mg/kg, 7.5 mg/kg, 10 mg/kg, necropsied 30 days post-treatment), respectively. Scattered *Wolbachia* (arrows) in lateral cord. G – I. Cross-sections from groups 7, 8, and 9 (doxycycline 5 mg/kg, 7.5 mg/kg, 10 mg/kg necropsied 60 days post-treatment), respectively. Single *Wolbachia* (arrows) in the lateral cord.

Discussion

The recently updated AHS heartworm treatment protocol indicated the use of 10 mg/kg doxycycline for 28 days before adulticidal treatment to both reduce the pathology resulting from dead and dying worms and prevent the development of L3s in subsequent hosts [1]. Our study focused on the *Wolbachia* levels in adult worms after treatment with IVM and different dosages of doxycycline. We also examined the efficacy of multiple doxycycline dosages in reducing *Wolbachia* immediately after completion of the course of doxycycline and one month later. Our results suggest that the relative *Wolbachia* numbers in adult *D. immitis* females and males decrease significantly after doxycycline and IVM treatment among all dosage groups (Figure 3.2, Table 3.2, 3.3).

The one-month wait period after doxycycline treatment and before the first melarsomine injection was hypothesized to clear the *Wolbachia* and WSP that may persist in the worms [1]. Our data show that *Wolbachia* levels were significantly decreased after the one-month wait period for each dosage group (all $p < 0.0001$). This finding aligns with the hypothesis and suggests that conserving the one-month wait period would be beneficial in reducing complications caused by *Wolbachia*.

Although a significant decrease in *Wolbachia* was observed among all treatment groups, none showed complete clearance of *Wolbachia*, as indicated by the qPCR and immunohistochemistry results. *Wolbachia* surface protein persisted in the highest dosage group even after the one-month wait period (10 mg/kg doxycycline, Figure 3.3 I). No statistical analysis can be performed on the staining results; however, a visible decrease in WSP can be observed in all treatment groups on days 30 and 60 post treatment. It should be noted, however, that due to the size and thickness of the cuticles of adult *D. immitis*, the worms were stiff post

fixation and difficult to bend or place at a certain angle or position for histology. To obtain cross-sections of the worms, we stabilized the samples in 10% agarose before trimming and embedding in paraffin. We could not control the way worms were held in the 10% agarose. Thus, the cross-section obtained from each worm was random. The *Wolbachia* in *D. immitis* adults are not evenly distributed, which may cause bias when interpreting the IHC data. We observed a general decrease in positive staining (data not shown) and captured representative sections from each group (Figures 3.3 and 3.4). If cross-sections at the exact same location for each worm could have been obtained, we may have been able to perform statistical analysis according to the surface area of positive staining.

However, we did not find any positive staining in any of the study animal tissue sections, including those containing dead worms, from the control and treatment groups (data not shown). This may be because only very few worm deaths occurred, and the dead worm cuticles remained intact, thus preventing *Wolbachia* from entering the host's tissue.

In Moorhead et al. (2023) [25], we compared the mean worm weight change between groups. For all dose groups, this was significantly increased from the start of the doxycycline treatment (day 0) to the completion of the doxycycline treatment (day 30), and then significantly decreased after the rest period (day 60). Our current study showed that the *Wolbachia* levels in adult worms were significantly decreased in treatment groups 30 days post treatment, which could have impacted the reproductive system of *D. immitis*. The decrease in mean worm weight 60 days post treatment compared to 30 days may be due to a negative impact of *Wolbachia* elimination on mf production and the general health of adult *D. immitis*. However, no significant difference in the worm weight change was observed between the 60 days and the 0 day post-treatment groups. The increase in mean weight changes shown in the 30 day post-treatment

groups may be due to the natural growth of the adult *D. immitis*. Although the *Wolbachia* levels decreased significantly in the 30-day post-treatment groups, studies have shown that the elimination of circulating mf takes longer than 30 days [17, 18, 23], which may suggest that the embryos or other reproduction-related structures in *D. immitis* remain functional at 30 days post treatment.

This is the first study that has been able to link the amount of *Wolbachia* in adult *D. immitis* to different doxycycline dosages. The data we presented could aid in decision-making for practitioners. Our results suggest that the one-month wait period may be beneficial, as the *Wolbachia* levels further reduced compared to 30 days post treatment with statistical significance. As shown in tables 3.2 and 3.3, the *Wolbachia* levels decreased to 8.4%-17.7% of standard control in all treatment groups at the completion of treatment, then further decreased to 0.8% - 2.1% after the one-month rest period. However, we did not perform the AHS-recommended MEL injections to kill the adult worms at the end of the study, as the intact adult worms were needed to quantify the *Wolbachia*. Thus, we could not correlate the *Wolbachia* levels in adult worms to potential complications or lesions induced by WSP or other *Wolbachia* metabolites following MEL treatment. A study by Kramer et al. (2011) [21] evaluated the severity of lesions in experimentally infected *D. immitis* dogs treated with doxycycline alone (20 mg/kg SID for 4 weeks) or doxycycline and IVM (20 mg/kg doxycycline SID for 4 weeks, 6 µg/kg IVM monthly) before administration of MEL (8 weeks after the completion of doxycycline) versus MEL alone. Their study showed that treatment with doxycycline, which could lead to the depletion of *Wolbachia*, resulted in reduced lesion scores following MEL injection. Due to the limitations stated above, they did not perform assays to quantify the *Wolbachia* levels in the parasite at the time of MEL injection. They inferred that the reduction in

populations of *Wolbachia* could reduce the potential lesions. However, whether the further decrease of *Wolbachia* after the rest period, as we found in our current study (for example, 17.7% to 2.1%; Table 3.3, group 4, 7), will significantly impact the complications and lesion formation after MEL injection requires additional study.

We found no significant differences in *Wolbachia* levels between the dosages of doxycycline on days 30 and 60, and between the dates. Though this study was not designed to test for equality of the efficacy of doxycycline against *Wolbachia*, our findings may support the use of reduced doxycycline dosages in situations where the ideal dose of 10 mg/kg cannot be used. Doxycycline given at less than 10 mg/kg may lead to an increased time for mf clearance from the peripheral blood of the host [23]. We should note that while doxycycline is targeted at *Wolbachia* in heartworm and other filarial nematode treatments, this antibiotic also shows efficacy in anti-inflammatory and immunomodulatory activities, which may aid in the reduction of lesion severity [2]. Another limitation of this study is that we transplanted the adult *D. immitis* instead of natural infection in order to assess treatments for the same worm burden (20 worms total). In clinical practice, other factors, such as the age and health condition of the patients, worm burden, and the compliance of the pet owners may also impact the efficacy of the treatment. Our study can potentially inform the decision-making process in the choice of doxycycline dosages as well as the necessity of the one-month wait period.

Conclusion

Our study showed that all dosages of doxycycline effectively decreased *Wolbachia* levels at both time points (days 30 and 60 following the start of treatment). The *Wolbachia* levels in *D. immitis*

adults decreased further after the one-month wait period but were still present. Immunohistochemistry for WSP correlates with the real-time PCR findings.

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CHAPTER 4

THE IMPACT OF DOXYCYCLINE ON *DIROFILARIA IMMITIS* MICROFILARIAE GENE EXPRESSION

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Abstract

Background: *Wolbachia* is an endosymbiont present in many filarial nematode species, including *Dirofilaria immitis*. Genetic and transcriptomic studies have proven the necessity of *Wolbachia* in multiple biosynthesis pathways in the nematode including: heme, riboflavin, flavin adenine dinucleotide (FAD), glutathione, and nucleotide synthesis. Doxycycline is widely used in filariasis disease treatment worldwide and is recommended by the American Heartworm Society as a supplemental treatment for heartworm disease to reduce *Wolbachia* in the parasite. For this study, we picked genes related mitochondria, heme-binding protein, and chitinase to investigate if the 28-day doxycycline treatment *in vivo* can change the expression levels of those genes in *Dirofilaria immitis*.

Method: Five purposely bred dogs with *D. immitis* MO strain patent infection were used in this study. Three dogs received doxycycline treatment at 10 mg/kg, twice daily, for 28 consecutive days. Two dogs served as untreated control. Blood was taken on days 0 and 28 of treatment in all groups, and on day 60 in three treatment groups. Microfilariae (mf) were isolated for DNA and RNA extraction. Primers were designed based on the previous literature and transcriptome data available on WormBase ParaSite (https://parasite.wormbase.org/index.html). PCR was performed to test the amplification product of the primers. Quantitative PCR was performed to evaluate the fold change in the gene of interest.

Results: Among eight genes of interest, one set of primers detecting COX1 was able to both amplify desired fragments using PCR and generate results using qPCR. The doxycycline regimen reduced *Wolbachia* levels in mf. We did not find a significant difference in the fold change of the COX1 gene in *Wolbachia*-depleted mf compared to non-treated mf.

Conclusion: Doxycycline can efficiently reduce the *Wolbachia* levels in *D. immitis* mf after a 28-day, 10 mg/kg, twice-daily treatment regimen. The decrease in *Wolbachia* levels appeared not to impact the overall biogenesis of mitochondria in *D. immitis* mf after 28 days of treatment or after 60 days of treatment.

Background

Wolbachia is a gram-negative *Alphaproteobacteria* endosymbiont that belongs to the order *Rickettsiales* [1]. It is considered to be the most widespread intracellular bacterium in the animal kingdom, infecting about 40% of all arthropod species [2, 3]. Besides insects, *Wolbachia* in superfamily C, D, F, and J can be found in onchocercid nematode species, including *Wuchereria bancrofti, Brugia malayi, B. pahangi., Onchocera* spp., and *Dirofilaria immitis* [4]. The bacteria reside in host-derived vacuoles in the intracellular niche in the hosts' cells and replicate in the membrane-bound vacuoles [3, 5].

The interaction between *Wolbachia* and filarial nematodes is believed to be mutualistic. *Wolbachia* was proven essential in nematode embryogenesis, molting, growth, and survival [6]. The analysis of the *Wolbachia* genome indicated that several biosynthetic pathways required the participation of *Wolbachia*, as the hosts of *Wolbachia* lack part of or whole genes mandatory for these activities [7, 8]. The pathways include: heme, riboflavin, flavin adenine dinucleotide (FAD), glutathione, and nucleotide synthesis. Heme belongs to the tetrapyrrole class compounds; it is a critical cofactor for numerous proteins, such as cytochromes, hemoglobins, peroxidase, and catalases, participating in various biological processes [9]. Genetic studies on *B. malayi, D. immitis,* and *O. ochengi* indicated incomplete heme *de novo* biosynthetic pathways in the

nematode genome, while their *Wolbachia* showed a complete and likely functional heme pathway [8-10]. At the transcriptomic level, the entire *Wolbachia* of *D. immitis* (*w*Di) heme biosynthesis pathway was overrepresented in the *D. immitis* mf stage, suggesting an increased need for heme during this stage [10]. Cytochromes, the heme-containing enzymes that are often found in oxygen-binding and metabolism activities, would also be expected to be overrepresented in the mf stage of *D. immitis* [10]. However, *Wolbachia*-negative nematode species, such as *Loa Loa*, also have a genome that lacks heme synthesis pathways, indicating that heme acquisition may come from other sources than *Wolbachia* [11].

In *Wolbachia*-dependent nematodes, the molting process may require components provided by this endosymbiont. Quek et al. (2022) examined the impact of *Wolbachia* on the molt of mf in mosquitoes [12]. They treated gerbils (*Meriones unguiculatus*) that had a patent infection of *B. malayi* with tetracycline for 2, 4, and 6 weeks. The mf were collected via peritoneal lavage, and DNA isolation and quantitative PCR were performed to determine the reduction of *Wolbachia.* The mf were fed to mosquitoes and allowed to develop for 12 days before L3 recovery [12]. The reduction in *Wolbachia* levels in mf and the recovery rate in L3 were observed. The decrease of *Wolbachia* in mf for the 2 and 4-week treatment groups were 6% and 39.4%, respectively. However, the reduction of L3 recovery in the 2 and 4-week groups was similar at 31.4% and 36.4%, respectively. The 6 weeks of tetracycline can reduce the *Wolbachia* burden of mf to 11%, with a full inhibition of L3 development in mosquitoes [12].

The transcriptomic and western blotting analysis of the 6-week treatment group proved a substantial decrease in chitinase, an enzyme that catalyzes the degradation of nematode chitin, suggesting a correlation between this enzyme and the presence of *Wolbachia* [12]. Chitinase can be found in *Wolbachia*-containing and *Wolbachia*-independent filarial nematodes. Chitinase

expression seems to be stage-specific among different nematode species. Microfilarial-specific chitinase activity can be found in sheathed mf (e.g., *B. malayi, W. bancrofti, Loa Loa*), and L3-specific chitinase, which is homologous to the *B. malayi* mf-specific chitinase, can be found in filarial species that produce unsheathed mf (e.g., *D. immitis, Acanthocheilonema viteae, Onchocerca volvulus*) [10]. Although no chitinase activities were observed in *D. immitis* mf, the transcriptomic study revealed several genes that may be worth attention. Chitinase precursor protein (nDi.2.2.g01593) was present throughout all stages in the study, and cuticular endochitinase (nDi.2.2.g09584) showed a significant upregulation in the mf stage compared to other stages [10]. Whether the depletion of *Wolbachia* can impact the expression of chitinase in *D. immitis* mf is not known.

Mitochondria are organelles critical to energy production, such as ATP synthesis, in eukaryotic cells. DNA sequence analysis indicated a remarkable resemblance between the genomes of *Rickettsia prowazekii*, a bacterium that can only reproduce within eukaryotic cells, and the mitochondrial genome. Mitochondria are highly dynamic, undergoing division and fusion, and morphology changes constantly [13]. Though multiple copies of DNA may exist in one mitochondrion due to the constant dividing and merging of the organelle, the mitochondria DNA (mtDNA) copy number is a valid biomarker of mitochondrial function and quantity [14, 15]. Mitochondrial biogenesis regulates mitochondrial turnover, content, and number vital to sustaining the homeostatic demands [16]. Mitochondria DNA copy number, or mtDNA levels, is often utilized to estimate mitochondrial biogenesis, as mtDNA depletion or over-replication can be an indication of a mitochondrial disorder [17]. Multiple methods were able to measure the changes in mtDNA copy number, including qPCR [18].

In nematodes that harbor *Wolbachia*, the depletion of this endosymbiont may influence the function of mitochondria [8, 19, 20]. Strübing et al. (2010) treated *Litomosoides sigmodontis*, a *Wolbachia*-dependent murine nematode, with tetracycline and showed an upregulation in mitochondria-encoded subunits [20]. The study included *A. viteae* as the control, as this nematode is naturally *Wolbachia* free. The microarray assay indicated that the mitochondrial-encoded subunits, including cytochrome c oxidase (COX) subunits, cytochrome b, ATPase subunit 6, and NADH-dehydrogenase subunits were upregulated in *L. sigmodontis* but not in *A. viteae* [20]. The mitochondria in *O. volvulus* showed an upregulation of HSP60 when the *Wolbachia* was depleted, while no expression changes were found in *A. viteae* that received tetracycline treatment. The researchers inferred that the increase in HSP60 expression may correlate to *Wolbachia* reduction rather than tetracycline treatment [19]. A genetic study on *O. volvulus* and its *Wolbachia* suggested that in the soma, transcription of membrane transport and respiration-related genes in *Wolbachia* were upregulated. They concluded that *Wolbachia* may have functions that resemble mitochondria, generating ATP for the nematode hosts [8]. However, McNulty et al. (2012) found no significant difference between the mitochondrial genome sequences in *Wolbachia*-dependent versus *Wolbachia*-independent nematodes [21]. The relationship between *Wolbachia* and nematode mitochondria requires further investigation.

In this study, we picked several genes of interest, including heme-related, chitinase-related, and mitochondria-related genes. We measured their expression level changes in *Wolbachia*-containing and *Wolbachia*-depleted *D. immitis* mf. Investigating doxycycline-induced gene expression changes in *D. immitis* mf could identify new targets for blocking disease transmission and developing drugs for prevention and treatment.

Methods

Genes of interest selection

The National Center for Biotechnology Information (NCBI) [22] and the database for nematodes, WormBase ParaSite [23], were the sources of all sequences used in this study. The genes were selected based on previous publications indicating the potential interactions between *Wolbachia* and its nematode hosts (Table 4.1).

Table 4.1

Genes of interest selection based on previous publications.

Gene ID	Justification	Reference
nDi.2.2.2.g00287	Cytochrome P450 superfamily proteins	[8-10]
nDi.2.2.2.g03793		
nDi.2.2.2.g02876		
nDi.2.2.2.g09584	Chitinase, chitinase precursor protein, and endocuticular chitinase	[10, 12]
nDi.2.2.2.g09661		
nDi.2.2.2.g01593		
nDi.2.2.2.g08626	Chitin synthase	[10, 12]
NC005305	*Dirofilaria immitis* mitochondrial genome. cytochrome c oxidase subunit 1 from different isolates	[19, 20]
OQ316630		
PQ045537		

Primer design

Primers were designed using the Geneious Prime (Geneious Prime 2024.0.7, https://www.geneious.com) primer design tool. The parameters for primers included a product size between 70 and 200 bp, a Tm of 58 to 60 ºC, and a G+C content between 30 and 80 percent.

The primers tested in this study are shown in Table 4.2.

Table 4.2

Primer sequence for genes of interest

Primer name	Sequence 5' – 3'	Product size	Transcript template

1	CYP450	F	ACCCACTCCAAAATCACGGA	150 bp	nDi.2.2.2.t03793
		R	ACCCACTCCAAAATCACGGA		
2	CYP4V2	F	GGACAACGATTCGCACTTATGG	96 bp	nDi.2.2.2.t02876
		R	GCGCATCTGATCTGTACGAA		
3	CYP23A1	F	TGGGTGGTGCAAATGGTGTA	186 bp	nDi.2.2.2.t00287
		R	GATTTCCCCCGCAGAACACT		
4	CYP23A1-2	F	AACGACAGTGCTTGGGTGAA	148 bp	
		R	TGGTGAGCTTGTCAAACCGA		
5	Chitin synthase	F	ACACGGCTACAACTGGAACC	122 bp	nDi.2.2.2.t08626
		R	CCTTCACATGCGCCTTCAAG		
6	Chitinase precursor protein	F	CGACCACCATAAGTTGTGCG	119 bp	nDi.2.2.2.t01593
		R	GAGTGAATTTTCGAGCGGCC		
7	Chitinase	F	CCAAACGGTGTAGCTGAGGA	162 bp	nDi.2.2.2.t09661
		R	TCCCAAAGACCGAACATCGT		
8	Chitinase – 2	F	CTGGTGGTACTGCGGCTTAT	116 bp	
		R	ACCGTACCACTGGTTTCCCT		
9	Cuticular Endochitinase	F	AGGTTATGGTGGTGCGTTCA	164 bp	nDi.2.2.2.t09584
		R	TTTCTGATGGTGGTGGCGTT		
10	Cuticular Endochitinase - 2	F	GTGCAGTTGAGGAACATGCC	103 bp	
		R	ACAGCAGCGGTGAGAAGTAG		
11	Cuticular Endochitinase - 3	F	AGCGAGTATTGGTGCATCAG	104 bp	
		R	TCGTTTCCTTGCCACCTTTT		
12	Cuticular Endochitinase - 4	F	CCAATATCCCAGTGACTGCC	133 bp	
		R	TGTTCCTGCTGCATTCATCA		
13	COX 1	F	GCAGGATTGGGAGGTTCTGT	198 bp	NC_005305
		R	GGCACCCAATATCAAAGGCA		
14	COX 1 – 2	F	TTACCGGTGTTTGGGATTGT	114 bp	OQ316630
		R	AGTCCCCAATACAGCAATCCA		
15	COX 1 – 3	F	TTGGTATTGTCAGTGCCTGTT	183 bp	PQ045537
		R	ACTAACAATCCCAAACACCGG		

Note: F: Forward; R: Reverse.

Animals

The handling of all animals included in this experiment strictly adhered to the Animal Use Protocol A2022 04-006, approved by the University of Georgia Institutional Animal Care and Use Committee. Five purpose-bred dogs participated in this experiment. All dogs were infected with the *D. immitis* MO strain via subcutaneous injection of 50 *D. immitis* L3 and had

circulating mf in the blood prior to the start of the treatment. Animals 1 – 3 were male, infected 1 year before the beginning of the treatment, and animals 4 – 6 were female. The experimental animal chart is shown in Table 4.3. Dogs 1 to 3 received doxycycline hyclate (Epic Pharma, LLC, Laurelton, NY) at 10 mg/kg twice daily for 28 consecutive days. Dogs 5 and 6 served as control. Blood from all dogs was collected on days 0 and 28 of the treatment. Additional blood was collected from dogs 1 to 3 on day 60 of the treatment (approximately one month after the finish of doxycycline administration) (Table 4.3). The mf in the blood were isolated for further analysis.

Table 4.3

Experimental animal chart. Six animals were included in the study with different treatments of doxycycline. Animals 1 – 3 received 10 mg/kg doxycycline for 28 days. All animals had blood collection on day 0 and day 28 of the treatment. Animals 1 – 3 had additional blood collection on day 60 of the treatment.

Animal	Doxycycline treatment (10 mg/kg, 28 days, BID)	Blood draw (mf isolation)		
		Day - 0	Day - 28	Day - 60
1	√	√	√	√
2	√	√	√	√
3	√	√	√	√
4		√	√	
5		√	√	

Microfilaria isolation from whole blood

The mf were isolated from whole blood as follows. Briefly, the blood was diluted at a 1 to 11 ratio in a saponin hemolysis solution containing 0.2 % saponin (Tokyo Chemical Industry, Tokyo, JPN) and 0.85% sodium chloride (Sigma-Aldrich, St. Louis, MO) in distilled water. The mixture was incubated in a 37ºC water bath for 15 minutes before centrifuging at 850 x *g* for 10 minutes at room temperature. The supernatant was removed, and the mf were washed with PBS

twice. Microfilariae were resuspended with 10 mL PBS and counted by examining two, 10 µL aliquots. The mf in PBS were stored at –80ºC.

DNA isolation

The mf samples in PBS were thawed on ice and centrifuged at 4,000 x *g* for three minutes at 4ºC. After removing the PBS, the mf were processed for DNA isolation per the instructions provided with the DNeasy Blood & Tissue kit (QIAGEN, Valencia, CA). Isolated DNA was stored at -20ºC for downstream experiments.

RNA extraction and complementary DNA synthesis

The mf samples in PBS were thawed on ice and centrifuged at 4,000 x *g* for three minutes at 4ºC. After removing the PBS, 300 µL TRI Reagent® (Zymo Research Corporation, Irvine, CA) was added to the mf pellet and mixed thoroughly. The mixture was transferred to a 2 mL, round-bottom, microcentrifuge tube containing one, stainless steel, 3-mm bead (autoclaved; uxcell, Hong Kong). To homogenize the samples, the 2-mL tubes were vortexed at the highest speed for 20 minutes, pausing every 5 minutes to incubate samples on ice for 3 minutes before vortexing. Homogenized samples were processed for RNA isolation per the manufacturer's instructions with the Direct-zol RNA MiniPrep Kit (Zymo Research Corporation, Irvine, CA). RNA samples were aliquoted and stored at –80ºC.

The complementary DNA (cDNA) was synthesized from each mf RNA sample using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The cDNA was aliquoted and stored at –20°C.

Primer validation

All primers designed were tested using NCBI primer blast [24] with Refseq mRNA database and Nucleotide database. All primers were also mapped to the reference sequences *dirofilaria_immitis*.PRJEB1797.WBPS19.mRNA_transcripts and *dirofilaria_immitis*.PRJEB1797.WBPS19.genomic from WormBase ParaSite [23, 25] in Geneious Prime to check off-target bindings.

The primers were then tested using PCR. The templates for primers related to CYPs and chitinase were cDNA synthesized from the RNA of an untreated mf sample. The templates for mitochondrial primers were DNA isolated from an untreated mf sample. The primers for *D. immitis* GAPDH and *D. immitis* 18S rRNA were used as positive controls for cDNA and DNA templates in the PCR, respectively. The REDExtract-N-Amp™ Tissue PCR Kit (MilliporeSigma, Burlington, MA) was used, and the reactions were set up according to the kit protocol. The amplification cycle started at 94°C for three minutes, followed by 39 cycles of (94°C for 1 min, 54°C for 1 min, 72°C for 1 min), then a final extension at 72°C for 10 minutes. The PCR products were used for gel electrophoresis using a 2% agarose gel and examined using a UV imager.

Quantitative PCR

Quantitative PCR utilized SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) with Bio-Rad CXF96 Touch Real-Time PCR Detection System. Briefly, the reaction started at 98°C for two minutes, followed by 40 cycles of (95 for 15 s, 54°C for 30 s, 60°C for 30 s, signal detection) and melting curve generation (from 60 to 90°C, at 0.5°C interval). Raw data were collected with CFX Maestro Software (Bio-Rad, Hercules, CA). DNA templates were used in the reaction for mitochondrial COX1 and *Wolbachia ftsZ* gene (primers included forward 5'-GCT GGT GCC TTA CCT GAT ATT-3' and reverse 5'-CCA CCC ATT CCT GCT GTT AT-3', GenBank: AJ495000) quantification, and *D. immitis* 18S rRNA (primers included forward 5'-TGA GAA ACG GCT ACC ACA TC-3' and reverse 5'-GAT AAC CGG CCT CAT AGA GAA C-3', GenBank: AF036638) was used as the housekeeping gene. Complementary DNA templates were used in reactions for all other gene quantification, and *D. immitis* GAPDH (primers included forward 5'-TCA TTC CAG CAA GCA CTG GT-3' and reverse 5'-GTT GGC ACA CGA AAA GCC AT-3', [26]) was used as the housekeeping gene.

Data analysis

The samples were categorized into three groups, first, based on doxycycline treatment (untreated vs. treated) and then on mf collection dates in the *Wolbachia*-depleted samples (day 28 vs. day 60), as indicated in Figure 4.1. The qPCR results were collected as C_t values. All C_t values were processed by CFX Manager software (Bio-Rad, Hercules, CA, USA), and $2^{-\Delta\Delta Ct}$ (fold change) was calculated using the Livak method [27]. The data collected were first normalized to the corresponding housekeeping genes and then to one mf sample in the untreated group. Unpaired two-tailed *t*-tests were performed on the expression level changes between the control (*Wolbachia*$^{+}$) and the day 28 *Wolbachia*-depleted group (Day 28 *Wolbachia*-), the

Wolbachia^{+} group and the day 60 *Wolbachia*-depleted group (Day 60 *Wolbachia*-), and between the days 28 and 60 *Wolbachia*- groups.

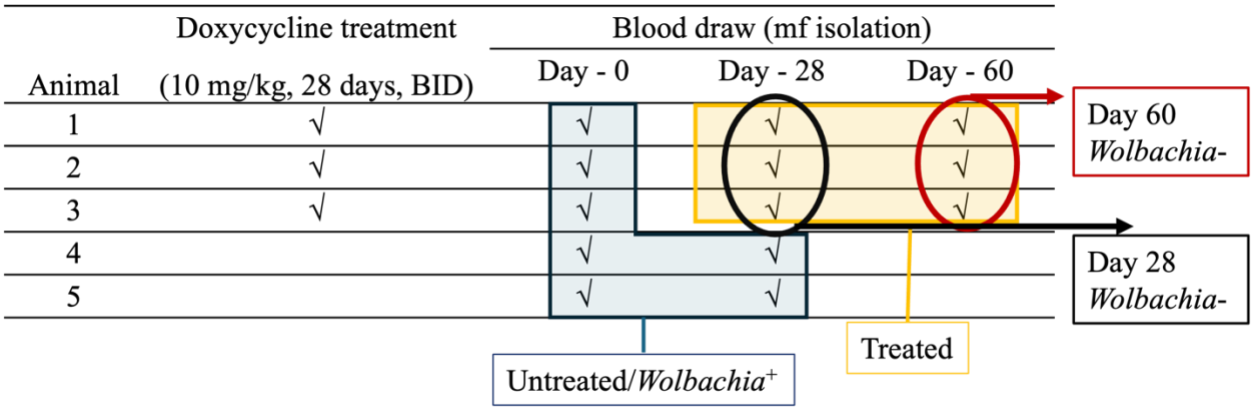

Figure 4.1

Categories of samples. The samples were categorized into treated and untreated groups (*Wolbachia*+ and *Wolbachia*-). The samples were categorized based on days post the beginning of treatment (day 28 and day 60) to evaluate if time is an impacting factor for potential gene expression/mitochondrial biogenesis changes.

Results

Primer design and validation

The primer sets that showed bright bands at the correct size in PCR validation were Chitinase precursor protein (primer set 6) and COX 1 – 2 (primer set 14). CYP 450 (primer set 1) and cuticular endochitinase – 4 (primer set 12) were primer sets that showed very faint bands. Other primer sets did not generate any amplification products through conventional PCR (Figure 4.2).

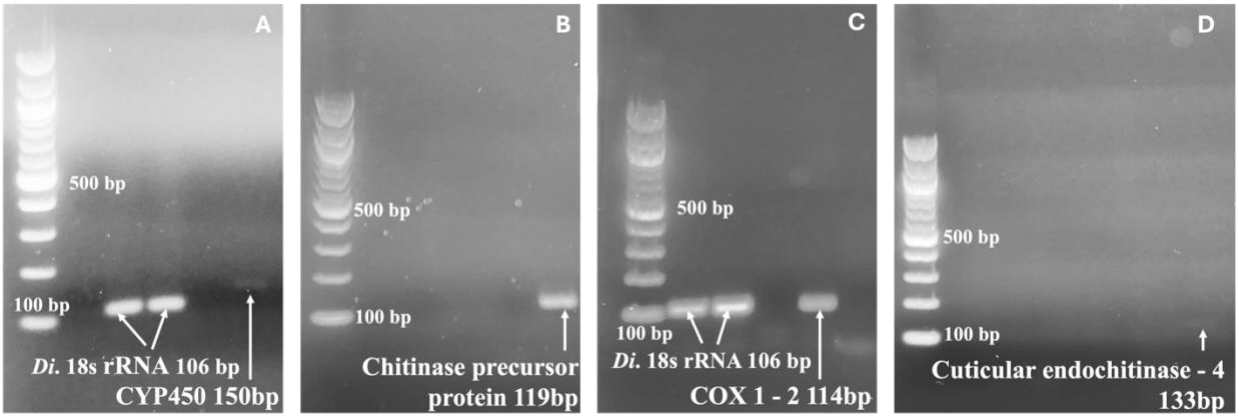

Figure 4.2

Conventional PCR gel electrophoresis results. Fig 4.2A, B, and D used cDNA as an amplification template, and Fig 4.2C used DNA as a template. Fig 4.2A shows the amplification product of primer set Di. 18s rRNA and CYP450. The Di. 18s rRNA bands were located at 106 bp. The faint band for CYP450 was located at 150 bp. Fig 4.2 B, C, and D show the bands for amplification products of Chitinase precursor protein (119 bp), COX1 – 2 (114 bp), and Cuticular endochitinase – 4 (113 bp), respectively.

Quantitative PCR

The primer set quantifying the mitochondrial COX1 gene was the only primer set that yielded consistent qPCR results. The samples were classified into three groups, first, based on doxycycline treatment (untreated vs. treated) and then on mf collection dates in the treated *Wolbachia*-depleted samples (day 28 vs. day 60). The result of the *Wolbachia ftsZ* gene quantification is shown in Figure 4.3. The result of the *Wolbachia ftsZ* and COX1 gene quantification for all groups is shown in Figure 4.4.

A significant decrease can be found in *Wolbachia* levels between doxycycline-treated and untreated groups ($p < 0.0001$). No significant difference in *Wolbachia* levels was found between Day 28 *Wolbachia*- and Day 60 *Wolbachia*- groups ($p = 0.1477$). No significant differences were observed in COX1 levels between *Wolbachia*+ and Day 28 *Wolbachia*- groups ($p = 0.6488$),

Wolbachia+ and Day 60 *Wolbachia*- groups ($p = 0.1636$), and Day 28 and Day 60 *Wolbachia*- groups ($p = 0.1710$).

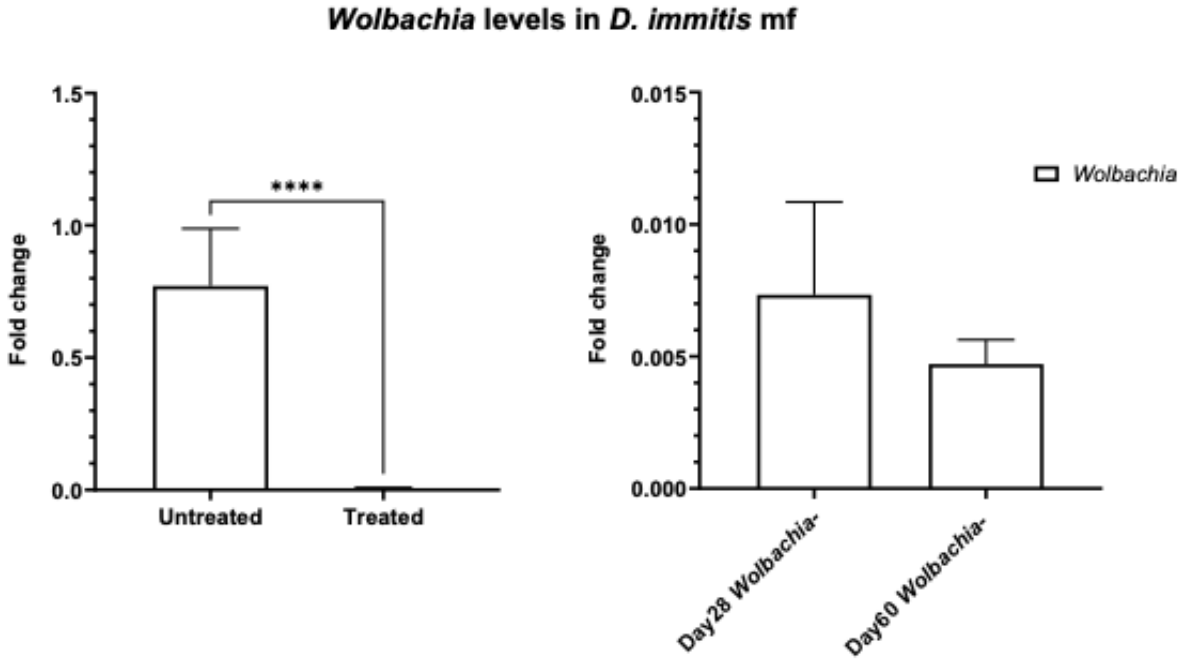

Figure 4.3

Wolbachia levels in *D. immitis* mf. *Wolbachia* levels were shown in fold change (*ftsZ/Di* 18s rRNA ratio) to the control sample (one untreated mf DNA). Asterisks indicate significant differences between the *Wolbachia* levels in untreated and treated groups ($p<0.01$). No significant differences were found between the *Wolbachia* levels on day 28 and day 60 in the treated groups ($p = 0.2787$).

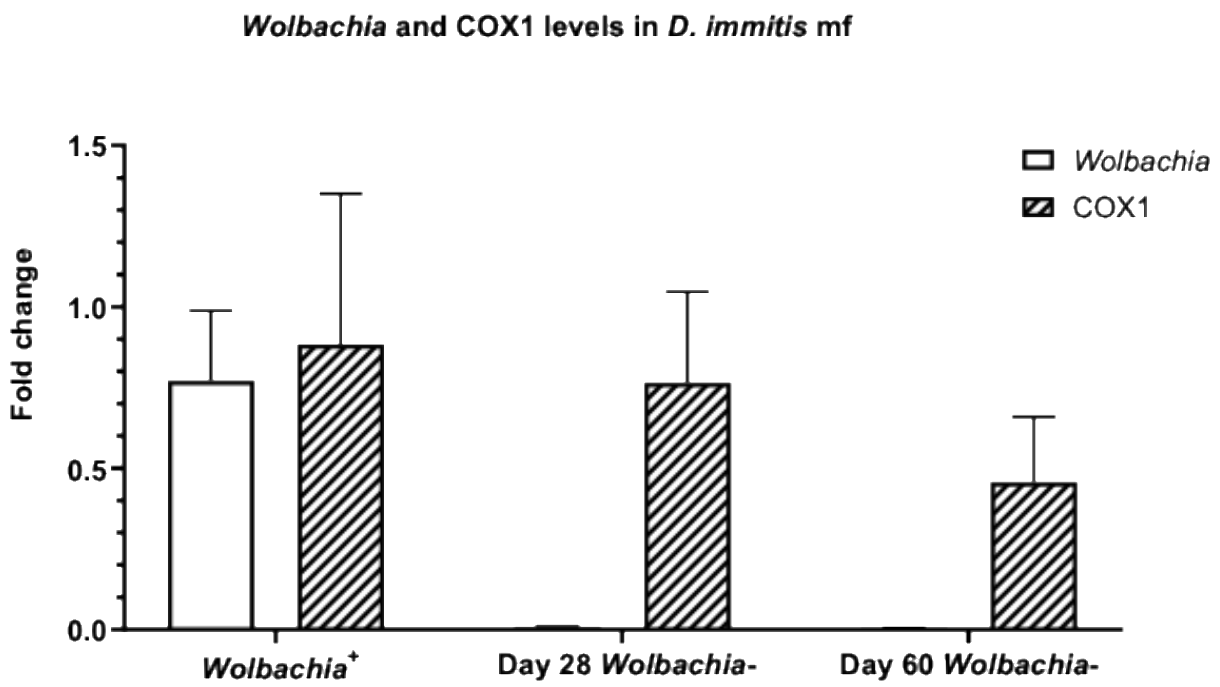

Figure 4.4

Wolbachia levels and COX1 quantity in *D. immitis* mf. Wolbachia and COX1 levels were shown in fold change (*fstZ/Di* 18s rRNA ratio and COX1/*Di* 18s rRNA ratio) to the control sample (one untreated mf DNA). No significant differences between COX1 levels in *Wolbachia*+ and Day 28 *Wolbachia*- groups ($p = 0.7523$), *Wolbachia*+ and Day 60 *Wolbachia*- groups ($p = 0.0860$), and Day 28 *Wolbachia*- and Day 60 *Wolbachia*- groups ($p = 0.0637$).

Discussion

In this study, we chose eight genes to test for changes in expression levels. The genes can be classified into three functional categories: heme-related, chitinase-related, and mitochondria-related. As stated above, the categories of genes we selected were shown to be influenced by *Wolbachia* depletion in nematodes other than *D. immitis* [7-9, 11, 12, 19, 20]. For heme-related genes, we picked the cytochrome P450 (CYPs) superfamily. These are heme-containing enzymes widely present in all kingdoms [28]. Among the superfamily, we selected genes nDi.2.2.2.g03793, nDi.2.2.2.g00287, and nDi.2.2.2.g02876; all are orthologues to *O. volvulus, B.*

malayi, and *B. pahangi* Cytochrome P450. We used the COX1 gene in mitochondria to indicate an overall quantity of mitochondria. We expected the heme-related genes and mitochondrial biogenesis to be upregulated due to the depletion of *Wolbachia*, as the nematode host would need to compensate for the decline in metabolic activities. For chitinase-related genes, we selected nDi.2.2.2.g01593 (chitinase precursor protein), nDi.2.2.2.g09584 (cuticular endochitinase), and nDi.2.2.2.g09661 (chitinase) based on the study published by Luck et al. (2014) [10]. We included the gene encoding for chitin synthase (nDi.2.2.2.g08626) to test whether the production of chitin was impacted. The chitinase-related genes may be downregulated, as in *B. malayi* *Wolbachia*-depleted mf [12]. It may also not change since *D. immitis* mf are unsheathed and may not require chitinase expression after mf enter the mosquitoes.

The primer set that showed a consistent signal in our study targeted the mitochondrial COX1 gene. Mitochondria are organelles that go through constant changes in the cytoplasm. Besides energy production, mitochondria are also important in regulating cellular homeostasis and survival [29]. Mitochondrial biogenesis regulates mitochondrial turnover, content, and number vital to sustaining homeostatic demands [16]. Mitochondria DNA copy number, or mtDNA levels, is often used to estimate mitochondrial biogenesis, as deviations in mtDNA quantity can indicate mitochondrial dysfunction [17]. The qPCR analysis of mtDNA to nucleotide DNA ratio with the Livak calculation method allowed us to estimate mitochondrial biogenesis in the nematode cells. Our results indicated that mitochondrial biogenesis did not change significantly, while the *Wolbachia* levels in the mf changed dramatically with doxycycline treatment (Figure 4.2, Figure 4.3). We examined doxycycline-treated mf collected at different dates (day 28 and day 60) to evaluate if the changes in mitochondrial biogenesis required additional time. The results disproved our hypothesis.

In *Wolbachia*-dependent nematodes, the depletion of *Wolbachia* can induce the upregulation of mitochondrial gene expression [19, 20]. Luck et al. (2014) indicated that at the transcriptional level, the mf stage of *D. immitis* has a very high expression of *Wolbachia* metabolic pathways among other stages (third-stage larvae, fourth-stage larvae, adult male, and adult female) [10]. It would be reasonable to hypothesize that the depletion of *Wolbachia* in mf stages of *D. immitis* could greatly impact the overall metabolic pattern of the parasite. Our results indicated that *Wolbachia* depletion in *D. immitis* mf did not influence the amount of mtDNA in the nematode, which may indicate that *Wolbachia* depletion may not affect mitochondrial biogenesis. This may be due to a relatively low density of *Wolbachia* in the mf, as shown by Fischer et al. (2011) [30]. Thus, the impact could be less obvious. Studies demonstrating the change in mitochondrial gene expression used adult worms instead of mf [19, 20]. Also, the measurement of mtDNA amount may not provide a full picture of the reaction of mitochondria to the depletion of *Wolbachia*. The transcription and/or translation of certain genes and proteins may be impacted, which would not be detected via qPCR of mtDNA.

Unfortunately, the other primer sets could not amplify or produce reliable qPCR results. Thus, we could not investigate the gene expression changes in the remaining genes of interest. In our study, we picked four sets of primers at different locations on transcript nDi.2.2.2.t09584, as the expression level of this cuticular endochitinase gene was highest in the mf stages [10]. However, none of the primer sets functioned as expected. A database with full annotation of the *D. immitis* genome and transcriptome through all life cycle stages would be incredibly beneficial for future studies.

Further optimization of reaction conditions may help improve the performance of the primers. A transcriptome of *Wolbachia*-depleted *D. immitis* mf could be a more comprehensive

approach to studying the impact of doxycycline on *Wolbachia* and, consequently, *D. immitis* mf. This study and future studies could help provide insights into the mechanism of *Wolbachia* functioning in the nematode, which may offer new perspectives in filariasis drug discovery.

Conclusion

Our study showed that doxycycline can effectively eliminate *Wolbachia* in mf when given at 10 mg/kg twice daily for 28 days. The mtDNA amount in *D. immitis* appeared not to be impacted by the depletion of *Wolbachia* in the mf.

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CHAPTER 5

CONCLUSION

Significance

The endosymbiont *Wolbachia* is a promising target for filariasis, onchocerciasis, and dirofilariasis control and treatment [1-7]. Doxycycline is the bacteriostatic antibiotic that targets *Wolbachia*. It is currently used in onchocerciasis and dirofilariasis treatment [8, 9]. A thorough investigation of the interaction between doxycycline and *Wolbachia* and, consequentially, *Dirofilaria immitis* can benefit clinical practice and mechanism research.

Dirofilaria immitis has a complicated life cycle that needs two hosts to complete. The sensitivity to treatment varies depending on the stages. The AHS recommends ML, including ivermectin, milbemycin oxime, moxidectin, and selamectin, as monthly heartworm preventives. This class of drugs works efficiently against L3 and L4 stages but has a limited impact on mature adult worms *in vivo* when used at preventive doses [9-12]. Missing doses may increase the chance of getting heartworm if the treatment-sensitive window passes [9]. Diethylcarbamazine was a preventive medication against heartworm due to its efficacy against developing heartworm larvae and circulating mf, but not against older worms [13]. Doxycycline also shows a preventive effect on early infection of *D. immitis* in dogs when dosing daily for 30-day periods [14]. Doxycycline showed 100% efficacy in heartworm disease prevention when administrated between days 0 – 29 post infection, which aligned with the development of L3 to L4 *in vivo*. The efficacy of doxycycline was reduced (69.6%) when the drug was administrated between days 65

– 94 post infection, which indicated that doxycycline is less efficient when targeting later stages in the *D. immitis* lifecycle (L4 to immature adult). Regardless, *Wolbachia* presents persistently throughout the life cycle stages of *D. immitis*, making it a notable target for disease control and treatment.

In this dissertation, we investigated the impact of doxycycline on *Wolbachia* and *D. immitis* at different life cycle stages (mf, L3, adult), providing insights into the molecular mechanisms of microbial-nematode interactions in heartworm development and clinical treatment of heartworm. In chapter 2, we presented the first study testing the molting of *D. immitis* L3 to L4 *in vitro* when *Wolbachia* was depleted. In chapter 3, we presented the first study examining *Wolbachia* levels in adult *D. immitis* after different dosages of doxycycline treatment as well as the change in *Wolbachia* levels after the subsequent one-month rest period. In chapter 4, we investigated the impact of *Wolbachia* depletion at a molecular level using qPCR in *D. immitis* mf, which may provide insights into new targets of prevention medications. This chapter summarizes the major research findings in chapters 2, 3, and 4, along with the objectives and hypotheses. The significance, limitations, and future direction will be reviewed.

In vitro **molting of** ***Dirofilaria immitis*** **third-stage larvae derived from microfilariae collected from doxycycline-treated dogs**

Previous studies suggest that *D. immitis* L3 developed from blood (mf) collected in doxycycline-treated dogs cannot complete a normal life cycle [15-17]. Several possible explanations for this phenotype include obstruction of the L3 to L4 molt, the L4 to immature adult molt, and the suppression of maturation to sexually mature adults. We focused on the L3 to

L4 molt, the first molt in the vertebrate host. Because of the lack of an *in vivo* tracking method, we had to use an *in vitro* culture system to observe this molt. We hypothesized that the inability of doxycycline-treated mf to develop into adulthood is due to the loss of *Wolbachia*, thus impacting the L3 to L4 molt *in vitro*.

The objectives of this study included validating whether the *Wolbachia*-depleted *D. immitis* L3 can molt into L4 *in vitro* and determining the change in relative *Wolbachia* levels in mf and L3 during doxycycline treatment. This study filled in the gap in knowledge regarding *Wolbachia* levels in L3 developed from *Wolbachia*-depleted mf and presented the change in *Wolbachia* levels throughout the 28-day doxycycline treatment, which may provide insight into the required duration of doxycycline treatment for heartworm disease. Three dogs were included in this study, one served as control and two were treated with doxycycline at 10 mg/kg, BID, for 28 days. Blood was collected before the start of treatment and weekly during the treatment. Third-stage larvae were produced by artificially feeding mosquitos with blood containing mf. Both mf and L3 were processed for DNA isolation and qPCR analysis, and L3 were placed in culture *in vitro* for 9 days to observe molting.

We proved that the sample culture conditions (RPMI-1640 with FBS as nutrition resource, no cell co-culture needed) can produce consistent molting results. As expected, the *Wolbachia* levels in mf and L3 were reduced following doxycycline treatment. *Wolbachia*-depleted mf can develop into L3 in mosquitoes, and the L3 remained *Wolbachia*-depleted. The decrease of relative *Wolbachia* levels in mf and L3 occurred sooner than anticipated during treatment. The *Wolbachia* levels in mf and L3 were reduced to less than 5% of the control group and further decreased as the treatment continued. Also, no significant difference was observed in the molting rate or time to molt between control and treatment groups in the L3 to L4 *in vitro*

molting experiment. The results suggest that the depletion of *Wolbachia* does not impact the *D. immitis* L3 to L4 molt *in vitro*. This could indicate that the inhibition of worm development in the host may occur after the L3 to L4 molt. This is the first study that looked at the molting of *Wolbachia*-depleted *D. immitis* L3 *in vitro*.

Like other *in vitro* studies, the main limitation of this study is the discrepancy between *in vitro* and *in vivo* environments. A reliable and cost-effective *in vivo* animal model that can represent the immune system of the definitive hosts (canine in this experiment), which would allow us to obtain L4 molted from L3, is unavailable. The *D. immitis in vitro* L3 to L4 molting system has been utilized in many studies, especially in stage-specific studies, such as secretion profiles, genetic analysis, and drug-larvae interactions [18-26]. *In vitro* culture provides a cost-effective and simple technique for parasite research. However, mf in dogs decreases after doxycycline treatment; thus, this experiment is is time-restricted. The production of L3 also depends on multiple factors, such as the infection rate and survival of mosquitoes. Assuming that a stable supply of *Wolbachia*-depleted mf and L3 can be obtained (i.e., treat mf *in vitro* with doxycycline and feed to mosquitoes), additional studies, such as transcriptomic and proteomic analyses, may be able to be performed.

Real-time PCR and immunohistochemistry detection of *Wolbachia* in adult *Dirofilaria immitis* from dogs treated with doxycycline and ivermectin

The heartworm treatment protocol by AHS recommends the use of doxycycline at 10 mg/kg, BID, for 28 days, followed by a one-month rest period before the adulticidal melarsomine injection [9]. The purpose of the above pre-adulticidal treatment is to reduce

inflammatory reactions, such as post-adulticidal respiratory signs and pathology in pulmonary arteries, induced by *Wolbachia* and/or its products [27-30]. The optimal dosage of doxycycline and the necessity of the one-month rest period remains controversial. This study is the first to measured *Wolbachia* levels in adult *D. immitis* after doxycycline treatment at different dosages (5 mg/kg, 7.5 mg/kg, 10 mg/kg) and at different time points (before and after the one-month rest period), which can be a valid indicator for the potential pathology and post-adulticidal treatment signs.

We hypothesized that the *Wolbachia* in *D. immitis* can be effectively reduced by all dosages of doxycycline and will be further reduced after the one-month rest period. To test this hypothesis, we evaluated the *Wolbachia* amounts in adult worms at different doxycycline dosages and at different time points via quantitative real-time PCR and immunohistochemistry. Purpose-bred dogs (n = 45) were randomly divided into nine groups (five per group) and assigned to different treatments. Groups 1, 2, and 3 served as untreated controls; groups 4 and 7, groups 5 and 8, and groups 6 and 9 received doxycycline of 5 mg/kg, 7.5 mg/kg, and 10 mg/kg, respectively. Group 1 was euthanized on day 0 of the treatment; groups 2, 4, 5, and 6, and groups 3, 7, 8, and 9 were euthanized on days 30 and 60 of the treatment, respectively. The adult worms were collected during necropsy for DNA isolation/qPCR and immunohistochemistry.

All dosages of doxycycline appeared to effectively reduce the amount of *Wolbachia* in *D. immitis* adult worms, independent of the sex of the worms, on days 30 and 60 of the treatment. Within each dosage group, the *Wolbachia* levels in adult worms decreased significantly on days 60 compared to days 30 of the treatment in all treatment groups. The immunohistochemistry results (anti-*Wolbachia* surface protein antibody staining), though non-quantifiable, aligned well with the qPCR results. The staining results indicated the reduction of *Wolbachia* in the lateral

cords of adult worms after doxycycline treatment, and a further reduction of *Wolbachia* after the one-month rest period. However, both the qPCR and immunohistochemistry results demonstrated that the *Wolbachia* persist in adult worms in all groups.

This study allowed us to correlate the dosage of doxycycline used in treatment, as well as the one-month rest period, to the levels of *Wolbachia* in the adult worms. *Wolbachia* induces an inflammatory reaction in the hosts and leads to the development of lesions [6, 31, 32]. The goal of heartworm treatment includes the reduction of post-adulticidal complications. The results of this research may help in the clinical decision-making process in the choice of doxycycline dosages as well as the necessity of the one-month rest period.

One limitation of the study was that no melarsomine injection was performed in this study; thus, the observation of clinical signs and pathology post adulticidal treatment was not feasible. Another limitation of this study is that the statistical tests for equality require a pre-defined range of expected outcomes. No previous data exist for *Wolbachia* levels in adult worms after doxycycline treatment; we, therefore, could not perform a statistical analysis of the efficacy between different dosages. However, the qPCR and immunohistochemistry results suggest a possible equal efficacy in *Wolbachia*-reduction between the different doxycycline dosages.

In future studies, we would like to develop methods that may correlate *Wolbachia* levels in blood to *Wolbachia* levels in adults. This may provide more information for decision-making in clinical settings where blood samples are relatively easy to obtain.

The impact of doxycycline on *Dirofilaria immitis* microfilariae metabolism-related gene expression

Previous studies on filarial nematodes revealed essential information regarding the interaction between nematodes and *Wolbachia*. Multiple metabolic pathways, including heme, riboflavin, FAD, glutathione, and nucleotide synthesis, require components from *Wolbachia* [4, 33, 34]. Molting, especially chitinase production, was impacted by the depletion of *Wolbachia* in *B. malayi* [35]. Mitochondria showed changes in gene expression when *Wolbachia* was depleted in nematodes that naturally harbor *Wolbachia* [36, 37]. Here, we used doxycycline-treated mf and examined the expression of genes involved in heme-, chitinase-, and mitochondrial-related functions with qPCR. We hypothesized that heme- and mitochondrial-related genes would be upregulated to compensate for the loss of *Wolbachia.* In contrast*,* the chitinase-related genes may be down-regulated due to the loss of *Wolbachia*.

We searched for genes of interest in the WormBase ParaSite and NCBI databases [38, 39]. Cytochrome P450 superfamily proteins, chitinase, chitinase precursor proteins, endocuticular chitinase, chitin synthase, and mitochondrial cytochrome c oxidase subunit 1 (COX1) were selected and primers were designed using the Geneious Prime (Geneious Prime 2024.0.7https://www.geneious.com) primer design tool. The primer sets were tested using conventional PCR and gel electrophoresis. Primer sets that showed bands at the correct size were used for qPCR analysis. The *Wolbachia ftsZ* primer set was included to validate the *Wolbachia* levels in mf. The mf samples collected included doxycycline-treated samples on days 28 and 60 of the treated and untreated mf samples.

We found no significant change in COX1 levels, while a significant decrease was observed in *Wolbachia* levels in *D. immitis* mf. The primer set for COX1 (including forward 5'-

TTACCGGTGTTTGGGATTGT-3' and reverse 5'- AGTCCCCAATACAGCAATCCA-3') yielded consistent qPCR results. Other primer sets, however, did not function as expected.

This exploratory study intended to investigate the impact of doxycycline on metabolism-related gene expression in *D. immitis* mf. As presented in a transcriptome study by Luck et al. (2014), the expression levels of *Wolbachia*-related metabolic pathways were highest in mf stages compared to other stages [26]. This would suggest that the depletion of *Wolbachia* should have a more distinct influence on the nematode host, including mitochondrial biogenesis. However, the distribution of *Wolbachia* in mf is not as dense as it is in adult stages [40], which may counteract such changes. The measurement of mitochondria DNA (mtDNA) is a valid biomarker for mitochondrial biogenesis; however, other aspects of mitochondria function, such as the activities of enzymes, could not be assessed by qPCR assay. Further tests are required for a more comprehensive mitochondrial function analysis.

Though the performance of many primer sets fell short of our expectations, this study provided insight into the interaction between *Wolbachia* and the mitochondria in the nematode host. The enhancement of the *D. immitis* transcriptome database with full annotation would greatly benefit this study and future studies investigating gene expression changes. Future directions could include RNAseq on *Wolbachia*-containing and *Wolbachia*-depleted *D. immitis* samples. RNAseq analysis can provide a more inclusive understanding of the mechanism and signaling pathways that *Wolbachia* influences on its nematode hosts, as well as potential disease control and treatment drug targets.

Future directions

In the future, we would like to build an *in vitro* system that can produce *Wolbachia*-depleted mf and L3 as needed. This system will eliminate the natural variations of an *in vivo* treatment (i.e., drug metabolism, experimental animal health conditions, and insectary productivity). This could also benefit genetic and transcriptomic studies, as both require substantial genetic materials to perform corresponding assays.

We plan to correlate the *Wolbachia* levels in blood with the *Wolbachia* levels in adult worms collected from the experiments in Chapter 3. If the correlation exists and can be measured, this could significantly benefit heartworm disease treatment studies. Clinical data, including post-adulticidal signs, could be linked to the *Wolbachia* levels in the adult worms without having to recover any worms This could potentially be a biomarker to determine the necessity of the one-month rest period and the optimal doxycycline dosage for each dog.

Lastly, a transcriptome study on *Wolbachia*-depleted *D. immitis* would allow a better understanding of the functions of *Wolbachia* in the nematode. Identifying stage-specific gene expression changes, especially in the infective stage, mf, and adults, may provide insights into new drug targets for disease control and treatment.

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