Developmental regulation of *Dirofilaria immitis* microfilariae and evaluation of ecdysone signaling pathway transcript level using droplet digital polymerase chain reaction

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April 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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Acknowledgments

This thesis would not have been possible without the support and guidance from a number of people. First and foremost, I would like to thank my supervisor, Dr. Roger Prichard, for giving me the opportunity to work under your supervision. Thank you for giving me the freedom and space to work on the project but also being there to guide me through my doubts and questions. I would also like to thank my advisory committee member, Dr. Jianguo Xia for your insightful feedback during our meetings and your confidence in me. I also appreciate all the support and help from all the present and past members of the Prichard lab: to Kathy Keller and Hua Che, thank you for always ready to lend a helping hand and for willing to discuss any matter related to my work. I am also very grateful to you for helping me with solving my RNA extraction mystery and for showing me how to use the droplet digital PCR machine. You two have been my greatest source of support and encouragement during my time here. To Dr. Cristina Ballesteros, thank you for taking the time to guide me through primer design and qPCR setup so my experiment could go smoothly. To Emily Curry, thank you for taking the time to show me how to extract microfilariae from dog blood and for going through your RNA extraction protocol with me, and to Dr. Nour Rashwan, thank you for being patient with me when I needed to use the cell culture hood. I would also like to thank Dr. Thavy Long for answering my questions and for sharing valuable experiences with me. I also appreciate all the help and support for the SEM analysis that I received from Dr. Youssef Chebli. I thank the NIH/NIAID Filariasis Research Reagent Resource Center (FR3) for providing the microfilariae of *Dirofilaria immitis* (Missouri strain) and Zoetis for providing the female and male adults *D. immitis* (Kentucky strain) used in my research. I thank Dr. George K. Christophides for providing the *Anopheles gambiae* 4a-3B cell line. I acknowledge all the funding sources which facilitated my research: The Robert P. Harpur Fellowship in Parasitology, the grants provided to Dr. Roger Prichard, and the Graduate Excellence Fellowship. I would also like to give special thanks to all the friends that I've made at the Institute of Parasitology, thank you all for making me feel welcomed and for cheering me on. I would also like to thank Elizabeth Siciliani for helping me with the translation of the abstract. Last but not the least, I would like to thank my family and my partner for all their love and support throughout this journey.

Contribution of authors

All parts of this manuscript were written by Tsai-Chi Shang Kuan. Revisions and corrections were provided by supervisor Dr. Roger Prichard.

All experiments and analysis presented in this manuscript were performed by Tsai-Chi Shang Kuan, with help from Kathy Keller during the extraction of *D. immitis* microfilariae.

Abbreviations

20E	20-Hydroxyecdysone
cDNA	Complementary DNA
DBD	DNA-binding domain
ddPCR	Droplet digital polymerase chain reaction
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleoside triphosphates
EcR	Ecdysone receptor
EcRE	Ecdysone-response element
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FBS	Fetal bovine serum
FR3	Filariasis Research Reagent Resource Center
GluCls	Glutamate-gated chloride channels
HMDS	Hexamethyldisilazane
HRE	Hormone response element
L1	First stage larvae
L3	Third stage larvae
L4	Fourth stage larvae
LBD	Ligand-binding domain
LC_{95}	95% lethal concentration
LOE	Loss of efficacy
MO	Missouri
MLs	Macrocyclic lactones
MurA	Muristerone A
NRs	Nuclear receptors
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pgp	P-glycoprotein

PonA	Ponasterone A
qPCR	Real-time polymerase chain reaction
RPMI	Roswell Park Memorial Institute
RXR	Retinoid X receptor
SD	Standard deviation
SEM	Scanning electron microscopy
SNP	Single-nucleotide polymorphism
USP	Ultraspiracle

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Abstract

Dirofilaria immitis, or the dog heartworm, causes cardiac congestion in dogs and is a major problem in many parts of the world. Current prevention involves the use of macrocyclic lactones, which mainly target the third- and fourth-stage larvae, but drug resistance has been reported. Therefore, understanding the development of the microfilarial stage may provide an alternative target for disease intervention. As microfilariae are known to undergo developmental arrest in their mammalian host and resuming a rapid molt once inside the arthropod host, this study aims to look at the developmental regulation of *D. immitis* microfilariae in their arthropod host using an in vitro approach and to understand the role of ecdysteroid signaling system in their development with the help of droplet digital PCR. A mixture of DMEM and Ham's F-12 media at 1:1 was found to provide a favorable environment for the early maturity of *D. immitis* microfilariae, supporting both the motility and the development of the larvae. The addition of fetal bovine serum to the culture had inhibitory effects on the growth and motility of the microfilariae, while the presence of *Anopheles gambiae* cells in the culture was able to induce the early appearance of the "sausage" stage. On further testing, media conditioned with *An. gambiae* cells also had similar effects, suggesting that factors released from the mosquito cells offer conditions favorable to microfilarial growth. On the other hand, ecdysteroids, or the insect molting hormones, which have been found to exert biological effects on the molting and embryogenesis of the third-stage larvae and adults, were found to have no effects or negative effects on the development of the microfilariae. Interestingly, developing microfilariae still displayed upregulation in the transcript levels of the ecdysteroid signaling system components. Altogether, a better understanding of the physiological interaction between *D. immitis* microfilaria and its mosquito vector, as well as how the parasite crosses its developmental checkpoints within the intermediate host would provide better insight into potential new targets that might be exploitable in the development of novel approaches to heartworm control.

Résumé

Dirofilaria immitis, le ver du cœur du chien, provoque une congestion cardiaque chez les chiens et devient un problème majeur au niveau mondiale. La prévention actuelle implique l'utilisation de lactones macrocycliques, qui ciblent principalement les larves de troisième et quatrième stades de leur cycle de la vie, mais une résistance aux médicaments a été rapportée. Par conséquent, la compréhension du développement du stade microfilarien peut fournir une cible alternative pour l'intervention contre la maladie. Les microfilaires sont connues pour subir un arrêt du développement chez son hôte mammifère et reprendre une mue rapide une fois à l'intérieur de son hôte arthropode. Cette étude vise à examiner la régulation du développement des microfilaires *D. immitis* dans son hôte arthropode en utilisant une approche *in vitro* et à comprendre le rôle du système de signalisation des ecdystéroïdes dans son développement à l'aide de la droplet digital PCR. Un mélange de DMEM et de Ham's F-12 à 1:1 s'est révélé fournir un environnement favorable à la maturité précoce des microfilaires de *D. immitis*, soutenant à la fois la motilité et le développement des larves. L'ajout de sérum bovin fœtal à la culture a eu des effets inhibiteurs sur la croissance et la motilité des microfilaires, tandis que la présence de cellules d'*Anopheles gambiae* dans la culture a pu induire l'apparition précoce du stade "sausage". Lors de tests supplémentaires, les médias conditionnés avec cellules d'*An. gambiae* ont également eu des effets similaires, suggérant que les facteurs sécrétés par les cellules de moustiques offrent des conditions favorables à la croissance microfilarienne. Cependent, les ecdystéroïdes, ou les hormones de mue des insectes, qui se sont avérés exercer des effets biologiques sur la mue et l'embryogenèse des larves de troisième stade et des adultes, se sont révélés n'avoir aucun effet ou effet négatif sur le développement des microfilaires. De façon intéressant, les microfilaires qui développent affichaient toujours une régulation positive dans les niveaux de transcription des composants du système de signalisation des ecdystéroïdes. Dans l'ensemble, une meilleure compréhension de l'interaction physiologique entre la microfilaire *D. immitis* et son moustique vecteur, ainsi que la façon dont le parasite franchit ses points de contrôle du développement au sein de l'hôte intermédiaire fournirait une meilleure compréhension des nouvelles cibles potentielles qui pourraient être exploitables dans le développement de nouvelles approches de contrôle du ver du cœur.

1. Introduction

1.1. *Dirofilaria immitis*: a widespread threat

Dirofilaria immitis is a parasitic roundworm in the *Nematoda* phylum. Also known as the dog heartworm, it is the causative agent of cardiovascular dirofilariasis, a serious and potentially fatal disease in pets that is spread by mosquitoes. As the name implies, dogs and wild canids are the main hosts for *D. immitis*; however, cats and ferrets are occasionally infected, and therefore serve as a key reservoir population harboring the parasite for mosquitoes. Due to factors such as relocation of infected dogs, environmental changes, and the presence of one or more species of vector-competent mosquitoes, studies have documented the spread of dirofilariasis, causing it to become a major concern in several areas of the world, including the United States and many parts of Canada (Figure 1) [1-3].

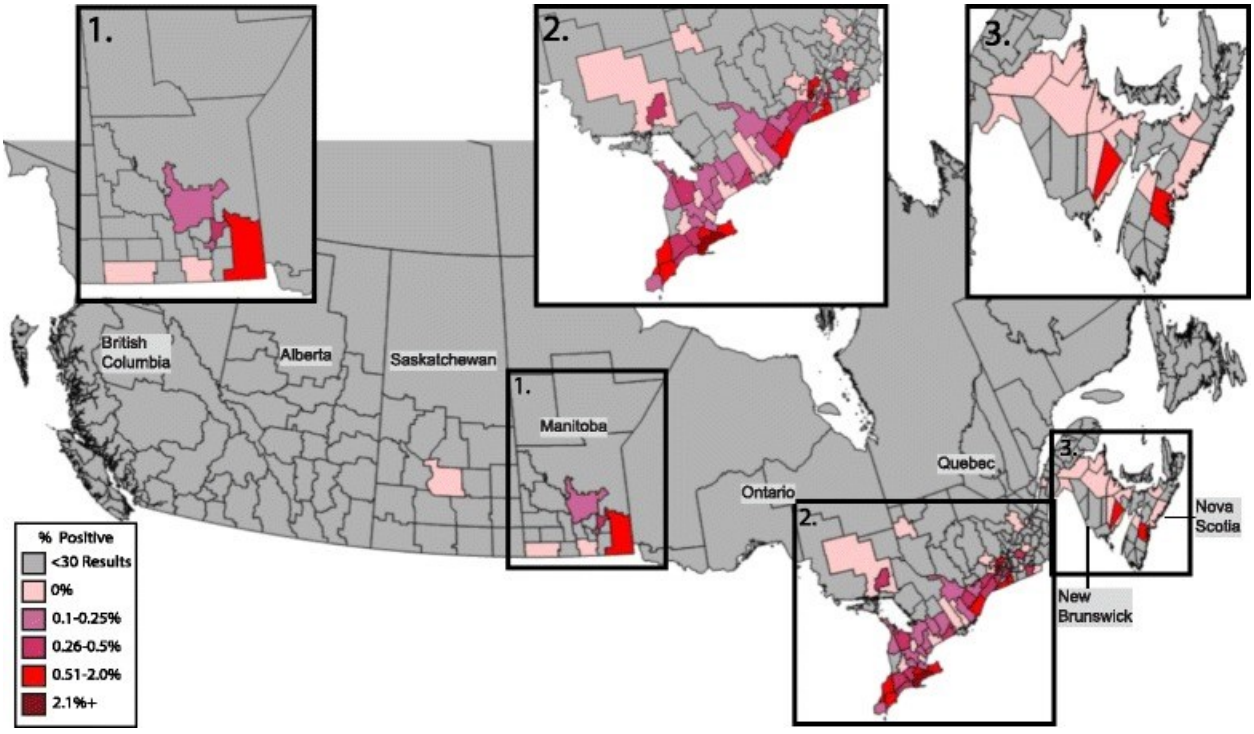


***Figure 1*. Percent positive antigen tests of *D. immitis* in dogs in Canada, 2013-2014.**
Reprinted from Herrin et al., Parasites & Vectors, 2017. [2]

Dirofilaria immitis typically lives in the right ventricle of the heart and the pulmonary vasculature of affected pets. Dogs with mild heartworm infection usually show few clinical signs. However, moderate to severe cases of heartworm disease can lead to a decline in the general health of the animal, including chronic cough, loss of body weight, and dry harsh coat. In serious cases, the parasites can cause permanent damage to the heart, lungs, and arteries, leading to exercise intolerance and circulatory distress. Experiments show that canids are highly susceptible to heartworm infections, with nearly 100% of dogs exposed to mosquitoes carrying infective larvae will develop adult heartworms [4]. Cats, being an atypical host for heartworms, are less likely to become infected because most worms in cats do not survive to the adult stage. Therefore, cats at risk would be those that are repeatedly bitten by infective mosquitoes. It appears, however, that infective larvae often display abnormal migration patterns in cats, resulting in higher chances of ectopic sites for adult worms [5].

1.2. *Dirofilaria immitis* requires two different hosts

Dirofilaria immitis requires an arthropod and a mammalian host to complete its life cycle (Figure 2). The parasites are first taken up by a mosquito as microfilariae, or first stage larvae (L1) that measure around 300 μm in length [6]. A wide range of mosquito species can be exploited by the parasite, many of which are in the genus *Aedes*, *Culex*, *Anopheles*, and *Mansonia* [7, 8]. Upon entering the mosquito, the microfilariae will migrate from the midgut to the Malpighian tubules in the abdomen [9]. There, the parasites will go through two molts, the first of which will cause the stoma to open to the exterior, and the second molt will remove the anal plug; in other words, the gut of the third-stage larvae (L3) is now open from the stoma to the anus [10]. At 27°C and 80% relative humidity, development from the microfilariae to the L3 stage takes about 10 to 14 days [11]. The L3 larvae will then migrate via the body cavity to the mosquito's proboscis where they become infective. When the mosquito takes a blood meal from a canid, the infective larvae emerge with the hemolymph and into the canid through the puncture wound made by the mosquito's mouthparts [12]. The molt from the L3 to the fourth stage larvae (L4) happens in the subcutaneous tissue and begins as early as 3 days post-infection. Both L3 and L4 larvae are the only mammalian stages of *D. immitis* that reside in the soft tissue of the host. Over the next 45 – 65 days, the L4s continue to grow and develop as they travel between the subcutaneous tissues and muscle fibers towards the heart and the lungs of the host [13]. As early as 67 days post-infection, immature adults can be found in the pulmonary vasculature. There, these juvenile adult worms will grow in size and become sexually mature. Fully matured males will grow up to 12 – 20 cm in length, while females can grow up to 25 – 31 cm [14] and will start producing microfilariae around 6-month post infection. The adults can remain fertile over their lifespan of around 5 – 10 years, producing high numbers of microfilariae that circulate in the blood of the canid [15].

It has been estimated that each adult female heartworm is capable of producing more than 11,000 microfilariae per day. Microfilariae can circulate in the blood for up to 2.5 years without any further development until their arthropod vector ingests them [16, 17]. Interestingly, circulating microfilariae exhibit periodicity, with high numbers of microfilariae found in the small vessels of the lungs during the day, and peak levels of microfilaremia occurring from late afternoon through late evening. This periodicity is influenced by the day-night change in the oxygen level,

and could be an evolutionary adaptation that allows high levels of microfilariae to be at the optimal site for transmission by their mosquito vector [18].

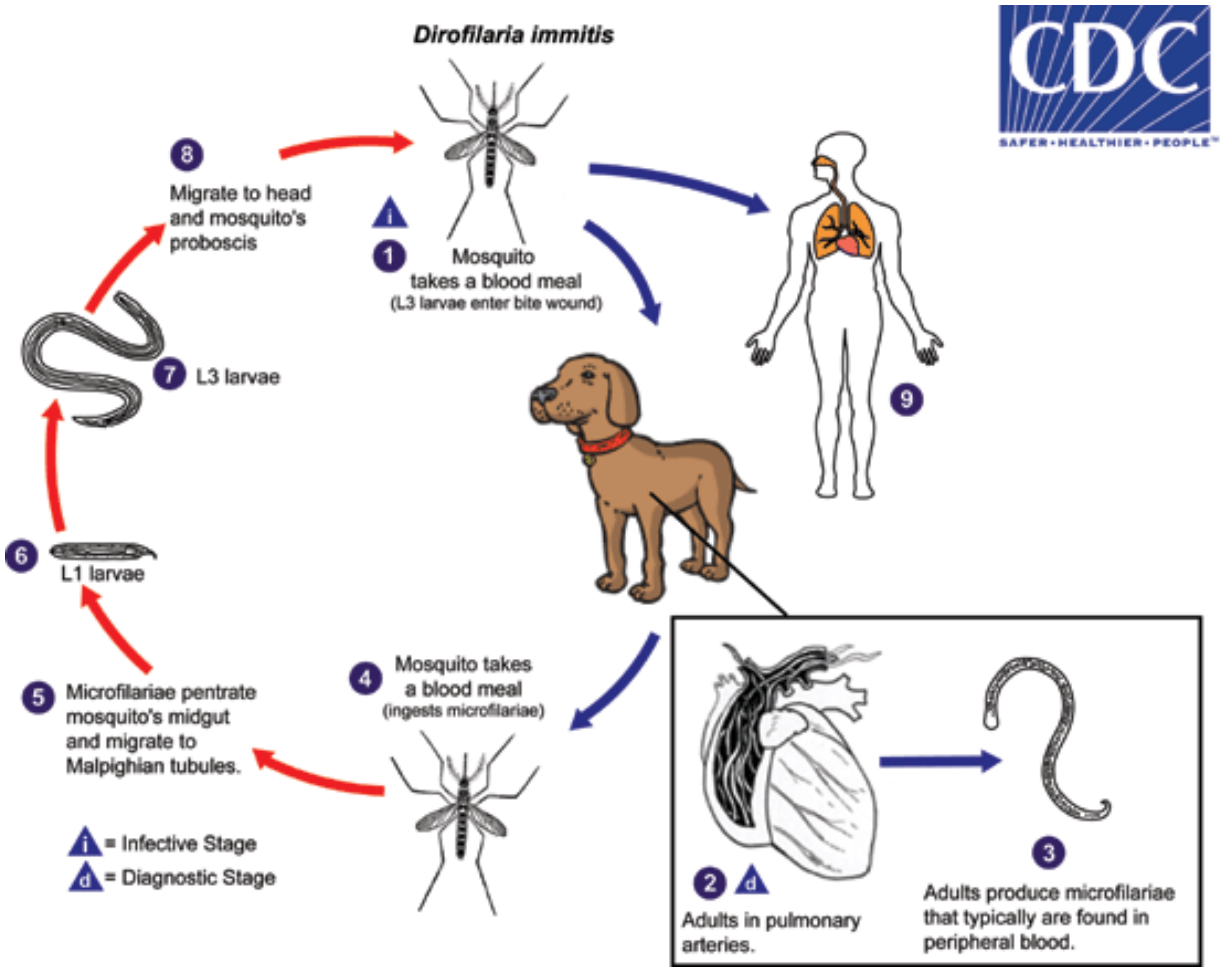


***Figure 2*. Life cycle of *D. immitis*.** Reprinted from Centers for Disease Control and Prevention. [15]

1.3. Heartworm preventive: macrocyclic lactones

Prevention of heartworm disease caused by *D. immitis* in dogs and cats relies on a single class of drugs, the macrocyclic lactones (MLs). MLs (including ivermectin, milbemycin oxime, moxidectin, and selamectin) are structurally similar compounds that are derived from products produced by soil microorganisms belonging to the genus *Streptomyces.* These compounds act by directly opening the glutamate-gated chloride channels (GluCls) or enhancing the effects of the

GluCl agonists, causing the channels to remain in the open-conformational state. The resulting influx of chloride ions causes hyperpolarization of the neuron or muscle, leading to paralysis or starvation of the parasite. Different chloride channel subunits are differentially expressed and show variable sensitivity to macrocyclic lactones, which explains why there could be different paralytic effects to various concentrations of MLs on different neuromuscular systems [19, 20]. Because vertebrates do not have GluCls, MLs are used as broad-spectrum insecticides and parasiticides, targeting many immature nematodes and arthropods.

The goal of canine heartworm preventive therapy has been to prevent *D. immitis* infection by killing the L3 stage deposited by the mosquito vectors, as well as the young L4 larvae [21]. When administered continuously, MLs are also shown to have varying degrees of microfilaricidal activity as well as adulticidal effects, although the use of these agents for eliminating adult heartworm infections is not recommended [22, 23]. It is advised to start a puppy on heartworm prevention no later than 8 weeks of age. The compounds should be given monthly, either orally or topically, for effective chemoprophylaxis. Before prescribing MLs to older dogs, it is necessary to perform primary diagnostic screenings for heartworm infection to avoid any potential harmful complications, which may arise if giving a heartworm preventive to a dog already infected with adult heartworms. If the dog already has microfilariae circulating in its bloodstream, administration of MLs may cause sudden death of the microfilariae, resulting in a shock-like reaction and possibly death of the dog [24].

Several types of diagnostic screenings are performed to detect the presence of *D. immitis*. Microfilaria test screens for the presence of microfilariae in the blood circulation by examining a drop of fresh blood microscopically. This detection would require large numbers of larvae to be present in the blood and so blood samples with low microfilariae counts (50 – 100 microfilariae per mL blood) would be difficult to detect. For subtle infections to be identified, a concentration test known as the Knott's test should be performed, in which a larger amount of blood is centrifuged or filtered to concentrate any microfilariae present prior to quantification [25]. The earliest that microfilariae can be detected in a dog's bloodstream is roughly 6 months post bitten by an infected mosquito. The presence of microfilariae in the blood would indicate infection with adult heartworms. However, the main limitation with microfilaria tests is that a fairly high number of positive infections sometimes have no circulating microfilariae, a condition described as "occult infections". Occult infections can occur in dogs with pre-patent infection, unisexual

heartworm infection, macrocyclic lactone-induced sterility of adult heartworms, or immune-clearance of microfilariae [26]. Another common diagnostic screening is antigen tests, which make use of enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test systems to detect circulating antigens produced by adult worms. This method is more sensitive compared to the microfilaria test; however, the major drawback is that available antigen tests are designed to detect antigens produced by mature female worms, and currently there are no tests capable of detecting infections consisting of only adult male worms. For more reliable results, combination of tests should be done, especially when there is a high degree of suspicion [3].

To treat dogs that have been infected with adult *D. immitis*, melarsomine dihydrochloride is an arsenic-based drug that is approved by the U.S. Food and Drug Administration to use as an adulticide. Treatment for heartworm disease, however, can be potentially dangerous for the dog and can lead to serious pulmonary thromboembolism due to the death of the heartworms. Other medications, including administration of glucocorticosteroids, diuretics, and vasodilators, may be necessary to help control the body's inflammatory reaction [27].

1.4. Confirmed resistance against macrocyclic lactones

In 2005, Hampshire reported the first prophylactic loss of efficacy (LOE) of MLs in *D. immitis* circulating in the Mississippi Delta region of the United States [28]. Over the next few years, a large increase in cases documenting the establishment of adult heartworm infections in dogs given monthly prophylaxis was reported. This prompted Blagburn and his colleagues to collect samples of *D. immitis* microfilariae from areas with heartworm prevention failures to determine their susceptibility to MLs in 2011. Collected field isolates were treated with either ivermectin, milbemycin oxime, moxidectin, or selamectin to determine the 95% lethal concentration (LC_{95}) for the different MLs, and results indicated that microfilariae recovered from dogs undergoing suspected LOE events required higher concentrations of all four MLs to reach a 95% lethal dose, compared to those recovered from susceptible dogs [29]. In the same year, Blagburn and his coworkers also conducted a study to evaluate the efficacy of four commercial heartworm preventive products administered as a single dose against the MP3 strain of *D. immitis*, which was found to be less susceptible to traditionally effective doses of an ivermectin-based preventive. Results reported showed that some products were not 100% effective against the

MP3 strain when applied as a single treatment according to labeled instructions [30].

Molecular analyses also indicated genetic differences between susceptible strains and suspected resistant strains. In 2011, Bourguinat and her coworkers reported two P-glycoprotein (Pgp) single nucleotide polymorphic sites (SNP) at positions 11 and 618 that showed a strong correlation with reduced sensitivity to MLs in an in vitro assay. Pgp is known to be involved in ML pharmacology, and upon investigation, the genotype of GG at both positions on the Pgp fragment was highly correlated with the level of insensitivity to ivermectin, suggesting the high level of homozygosity of the Pgp SNPs is the result of ML-driven genetic selection [31].

In the same year, microfilariae collected from a dog that remained microfilaraemic despite successful adulticidal treatments and multiple treatments with high doses of MLs were reported to have a frequency of 45.3% of the GG-GG genotype in the Pgp gene, which is significantly higher than those of microfilariae known to be sensitive to MLs (0 – 18.5%). This further confirmed the correlation between the Pgp genotype and ML insensitivity described previously [32]. The genetic origin of ML resistance was further demonstrated by the Pulaski et al. (2014) study [33], which showed that resistance is heritable, and the Bourguinat et al. (2015) study, which identified genetic differences that could correlate the evidence of LOE and resistance [34].

1.5. Ecdysteroids in arthropods and the ecdysteroid signaling pathway

Molting is a natural biological process in arthropods that plays an important role in arthropods' survival, development, and reproduction. During the process of molting, the old exoskeleton becomes detached, and a new layer of exoskeleton is formed underneath. The process is completed when the old layer is degraded and shed (ecdysis), and the new cuticle is tanned [35].

Ecdysteroid is a group of steroid hormones that is responsible for molting, development, and to a lesser extent, reproduction. Members of the group include ecdysone, ecdysterone, turkesterone, 20-hydroxyecdysone (20E), ponasterone A (PonA), muristerone A (MurA), and many more, with 20E being the key hormone that mediates the various physiological and behavioral changes that take place during the molting in insects [36]. These compounds are sterol derivatives that bear similar structural features, and those found in arthropods are synthesized from dietary cholesterol upon metabolism by cytochrome P450 enzymes encoded by the so-called 'Halloween' genes. Ecdysone is produced primarily in the prothoracic gland in arthropods and upon being

released into the hemolymph, is converted to 20E in peripheral tissues such as the fat body, ovaries, midgut, and Malpighian tubules by the hydroxylation at C-20 by ecdysone 20-monooxygenase [37-40].

The hormonal action of 20E is exerted through binding to the ecdysone receptor (EcR), a heterodimeric nuclear receptor composed of the EcR protein and the ultraspiracle protein (USP), the insect orthologue of the mammalian retinoid X receptor protein (RXR). Specifically, the hormone-binding function only resides within the EcR protein of the receptor [41-43]. Nuclear receptors (NRs) are a family of ligand-regulated transcription factors that are activated by steroid hormones and other lipid-soluble signals. This group of proteins is characterized by highly similar amino acid sequences in the DNA-binding domain (DBD), the domain that directs sequence-specific DNA-binding of the receptor, and the ligand-binding domain (LBD), the domain that mediates ligand binding, dimerization, and transcriptional activation [44, 45]. Upon activation, NRs will directly regulate the transcription of genes that control a wide variety of biological processes by recognizing a specific DNA sequence termed the hormone response element (HRE) [46]. In the ecdysteroid signaling pathway, the consensus binding site for EcR/USP or the ecdysone-response element (EcRE) was determined as 5′-(A/G) G (G/T) T C A N T G A (C/A) C (C/T)-3′ [47, 48]. According to the Ashburner model [49, 50], the 20E/EcR/USP complex initiates a transcription cascade that will lead to the expression of a small set of early regulatory genes (at least six in *Drosophila melanogaster*, including *E74*, *E75*, *E78*, *HR3*, *HR4*, and *βFtz-F1*) [51], but represses the expression of the late regulatory genes. The proteins of the early genes then repress their own promoters while inducing the expression of the late genes, whose protein products play a more direct role in controlling molting and metamorphosis (Figure 3) [52].

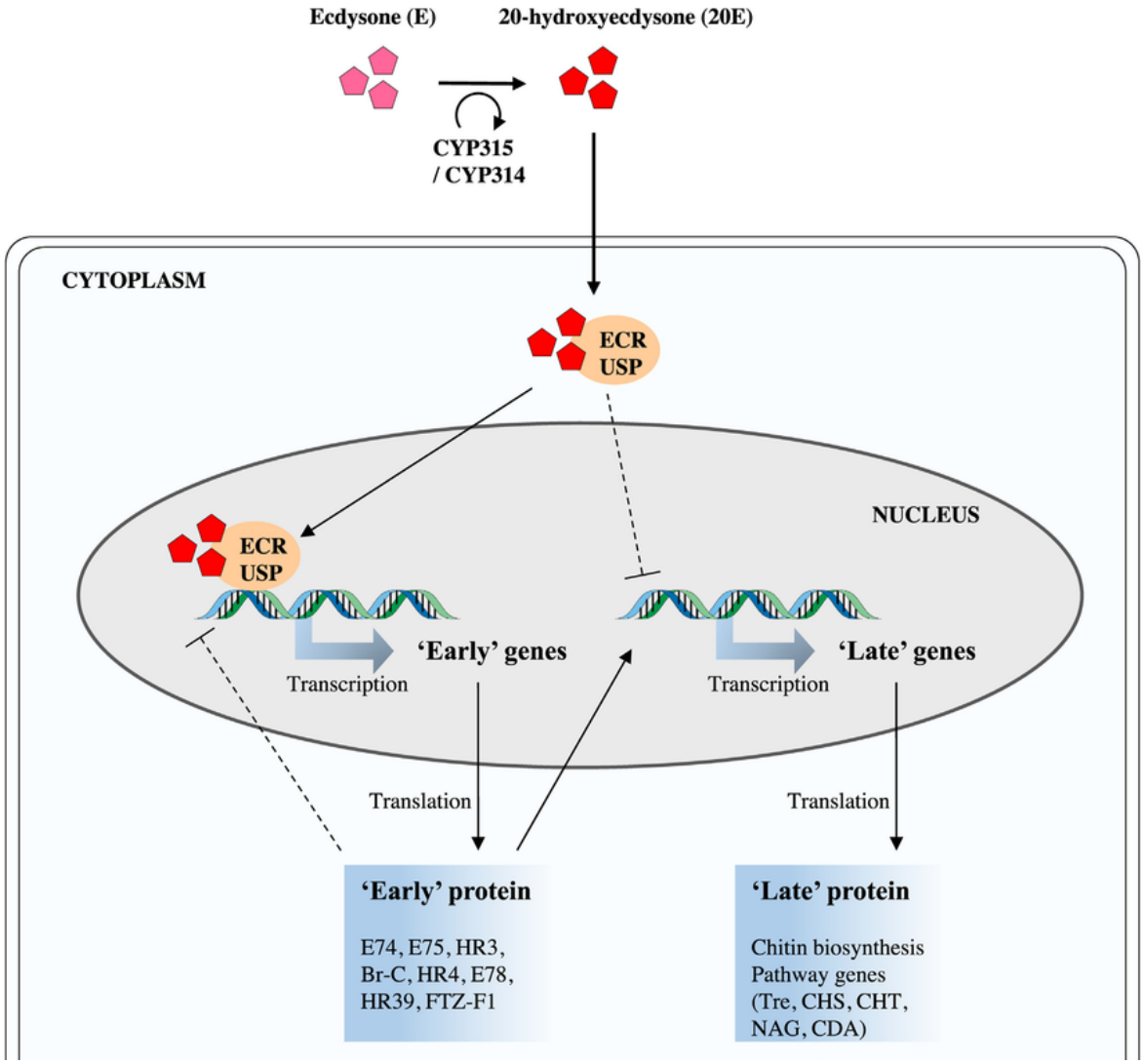


***Figure 3*. Modified Ashburner model of the ecdysteroid signaling pathway.** Adapted from Hwang et al., Aquatic Toxicology, 2016 [52]

1.6. Ecdysteroids in nematodes: Occurrence and origin

The larvae of both insects and nematodes are known to undergo a series of molts before reaching adulthood. Although nematode cuticles are different from that of insects in terms of their nature and composition, this similarity in their development has led to the suggestion that hormones similar to those found in arthropods might also control the molting process of nematodes.

Interestingly, ecdysteroids have also been detected in several species of nematodes, including the larvae of *Anisakis simplex* [53] and *Haemonchus contortus* [54], as well as in the adult stages of

D. immitis, *Ascaris suum* [55], and *Caenorhabditis elegans* [56]. In the studies on *D. immitis* and *A. suum* adults, both free and polar conjugated ecdysteroids have been detected in the total extracts of the female and male parasites. In both species, free ecdysteroids appeared to be concentrated within the reproductive system and the body wall, whereas polar conjugates were mainly detected within the reproductive system and the gut. These polar conjugates are hormonally inactive and possibly serve as storage forms of the hormone [55]. High titers of both free and conjugated ecdysteroids have also been detected in the eggs of *A. suum* and *H. contortus* during early embryogenesis, suggesting the role of the steroids hormone in the early stages of embryo development [57].

The ability of nematodes to metabolize ecdysteroids have been examined by incubating *C. elegans* with radiolabeled ecdysone. After being ingested, the compound was metabolized into putative ecdysone 22-phosphate and a series of apolar conjugates [56]. A similar experiment was performed with *A. suum*, where adults of *A. suum* were injected with isotope-labeled ecdysone. The injected compound was metabolized into different polar and relatively apolar metabolites, including ecdysone 22-phosphate, ecdysonoic acid, and ecdysone 25-glucoside. These results indicate that nematodes are capable of catabolizing ecdysone [58].

Following the detection of ecdysteroids in nematodes, attempts have been made to determine whether or not such steroids are synthesized by the worms or taken up from the environment. Like insects, nematodes cannot synthesize sterols de novo and rely on dietary sources of cholesterol, a precursor of ecdysteroids [59, 60]. Many studies involved either quantification of immunoreactivity after culturing the organism on semi-defined or defined media, or following the incorporation of radioactive ecdysteroid precursors, but there has not been any definitive evidence regarding biosynthesis of ecdysteroids in nematodes. In the study by Mercer and his colleagues, adult *C. elegans* that were cultured on *Escherichia coli* were found to contain 0.47 ng of ecdysteroids per gram dry weight using radioimmunoassay. However, the bacteria that they were grown on also contained 0.59 ng of free ecdysteroids per gram dry weight, suggesting that the steroids could be of dietary origin instead of endogenous synthesis [56]. Although in another study, *C. elegans* cultured on a defined medium containing less than 0.10 ng of ecdysteroids per liter were found to contain similar amount of ecdysteroids (0.52 ng per gram dry weight), the possibility remains that the ecdysteroids detected could have been concentrated from the medium by the worms or possibly hydrolyzed from other forms of conjugates [61]. Radiolabelling

experiments with *C. elegans [62]*, *Litomosoides carinii* [63], *D. immitis*, and *Brugia pahangi* [64] have also been unsuccessful in demonstrating ecdysteroid biosynthesis, so it remains to be proven whether ecdysteroids are truly endogenous nematode hormones. However, the strong biological activity of ecdysteroids is unquestionable and the effects of exogenously applied steroids on molting, development, and reproduction have been evaluated.

1.7. Biological effects of ecdysteroids on nematodes

Various concentrations of exogenously applied ecdysteroids have been shown to exert biological effects on the molting of nematodes. At concentrations as low as 10^{-11} M, 20E was found to stimulate the molting of *Nematospiroides dubius* fourth-stage larvae [65], while in another study, significantly higher proportions of *A. suum* third-stage larvae were molting when cultured in the presence of 1 M 20E [66]. It's important to note that early activation of the EcR can lead to lethal premature or incomplete molting in insects [67], and a similar effect was seen when Warbrick and his colleagues reported premature molting of the third-stage larvae of *D. immitis* when stimulated by 20E at a concentration of 10^{-7} M [68]. These facts suggest that 20E may have a functional role in the ecdysis of nematodes.

In insects, ecdysteroids are also involved in the regulation of meiotic reinitiation [69] and vitellogenesis [70] of adult females, as well as the regulation of embryogenesis [71]. Therefore, the detection of ecdysteroids in the reproductive system and eggs of nematodes raises the possibility that the functions of these compounds in terms of reproduction and embryonic development may be analogous. The possible role of oocyte developmental regulation by ecdysteroids has been demonstrated in *D. immitis*. In a study by Delves and his colleagues, it was shown that *D. immitis* oocytes became developmentally arrest during the pachytene stage of meiosis I [72]. However, when cultured in the presence of 10^{-5} M ecdysone for 24 h in vitro, the number of oocytes that were arrested at the pachytene stage was significantly reduced when compared to the control ovaries maintained in the absence of ecdysone. This effect was not as prominent when cultured in 10^{-6} M ecdysone, indicating the dependence of oocyte development on ecdysone concentration [73]. Another evidence that supports the role of ecdysteroids in the regulation of nematode reproduction and embryogenesis comes from the studies by Barker et al. and Mhashilkar et al. When adults of *B. pahangi* were maintained in vitro in the presence of 2×10^{-6} M ecdysone, there was increase in the number of microfilariae released into the culture

[73]. The same effect was seen when gravid female *Brugia malayi* was cultured in 10^{-5} M 20E; however, many of the microfilariae produced were, in fact, abortive immature progeny [74].

1.8. Functional ecdysone signaling system in filarial nematodes

Factors of the ecdysone pathway and NRs that function downstream of EcR and USP have been identified in *D. immitis*. In the early 2000s, the *D. immitis* ecdysone receptors, DiEcR, and RXR/USP, Di-RXR-1, were identified and characterized as having strong similarity to the insect EcR and USP, respectively, in terms of their amino acid sequences and many of their biochemical properties. Like the insect EcR, DiEcR showed ligand-dependent activation of transcription, while Di-RXR-1 was able to bind to EcR and DHR38 in vitro, both known insect USP partners. Like USP, Di-RXR-1 also activated transcription in *Drosophila* Schneider S2 cells in a 20E dependent manner when interacting with the EcR protein. Northern blot analysis showed that both *DiEcR* and *Di-rxr-1* are expressed in adult females but not in males [75, 76].

In 2002, a putative orthologue of the *Drosophila* ecdysone-regulated early gene *E78* was discovered in *D. immitis* and was named *Di-nhr-7*. Alignment of the amino acid of Di-NHR-7 showed that it has a strong similarity with those of E78, and its expression in adults was also female-specific [77]. A few years later, the *D. immitis* orthologue of another ecdysone-regulated early gene *E75*, *Di-nhr-6*, was also identified. *Di-nhr-6* encodes a canonical nuclear receptor protein that is developmentally regulated, with a DBD that showed significant amino acid sequence similarity to that of E75. Like *Di-nhr-7*, *Di-nhr-6* was detected in a female-specific manner in adults and also detected in the larval stages [78]. The female-specific expression profile of *DiEcR*, *Di-rxr-1*, *Di-nhr-7*, and *Di-nhr-6* suggest their roles in nematode embryogenesis, which is consistent with the study that demonstrated the increase in microfilaria production levels by adult female treated with ecdysone [73, 74].

The evidence for a functional ecdysone signaling system in filarial nematodes was demonstrated in *B. malayi* in a study by Tzertzinis et al. The study reported the cloning and characterization of a *B. malayi* EcR homolog (*Bma-EcR*) and a *Brugia* RXR homolog (*Bma-RXR*). To confirm that Bma-EcR and Bma-RXR interact to form an active heterodimer, the study utilized a mammalian two-hybrid activation assay, and high levels of reporter activity were measured when both NRs were transfected into the cells, as opposed to those that were transfected with either *Bma-EcR* or

Bma-RXR alone. Furthermore, when *B. malayi* embryos were transfected with constructs containing the ecdysone-response element (EcRE), the presence of 1 μM 20E resulted in a strong transcriptional activation, compared to embryos that were only transfected with the vector. When treated with various concentrations of 20E, the results showed a dose-response activation of the reporter, confirming that an ecdysone signaling system operates in *B. malayi,* with 20E functioning as a transcriptional regulatory ligand [79].

1.9. Other environmental cues can govern parasite development

The uptake of filarial nematodes by their arthropod vector requires the detection of environmental changes by the parasite and the adaptation to the new host environment. Aside from the presence of ecdysteroids in the insect midgut that could act as an environmental cue, other major determinants of environmental sensing when moving from a mammalian host to an invertebrate vector could be temperature change [80] and the detection of arthropod-specific stimuli, both of which may upregulate the parasites' signaling cascade for preparation of adaptive mechanism and rapid development [16, 81].

The specific stimuli detected and the exact mechanism employed by filarial nematodes once inside its arthropod host has not been fully elucidated; however, it is known that one common strategy utilized by *Plasmodium* is the initiation of translation of pre-made mRNAs after the sudden temperature drop and rise in the pH inside the arthropod vector, as well as the exposure to xanthurenic acid, a component in the mosquito pigment pathway [82, 83]. In other cases, the presence of host immunomodulatory signals can be utilized by the parasite to regulate their own development, such as the case with *Echinococcus multilocularis*, in which host insulin, epidermal growth factor, and fibroblast growth factor all have positive impacts on the development of this cestode [84, 85].

1.10. Using Droplet digital PCR for absolute quantification of transcript level

Droplet digital polymerase chain reaction (ddPCR) is a recent technology that allows for the absolute quantification of target nucleic acids present in a sample [86]. This technology utilizes *Taq* polymerase in a standard PCR reaction for the amplification of a target DNA fragment. The key difference, however, is that the sample is first randomly partitioned into 20,000 nanoliter-sized droplets following a Poisson distribution so that each partition will contain either no or few target sequences. Because each droplet now acts as an individual PCR reaction, templates would become concentrated in the droplet, allowing for the detection of low expressing targets and permitting higher tolerance to inhibitors present in a sample [87]. Unlike real-time PCR (qPCR), ddPCR detects end-point fluorescent signals and the fraction of amplification-positive droplets is used to determine the target concentration using Poisson's statistics [88]. Also, as ddPCR does not rely on calibrations curves for sample quantification, it avoids the drawbacks associated with varying reaction efficiencies [89].

2. Project Overview

2.1. Rationale and Hypothesis

Dirofilaria immitis causes serious cardiac congestion in pets in many parts of the world, but evidence has confirmed the emerging resistance of macrocyclic lactones, the only class of drug used for prophylaxis that mainly targets the L3 larvae. As a result, a better understanding of the other developmental stages of *D. immitis* may provide an alternative approach to disease intervention. Circulating microfilariae of *D. immitis* are known to live in the blood for several years in a state of developmental arrest; however, upon consumption by its arthropod vector, microfilariae quickly resume development and undergo a rapid molt. This indicates that the developmental cues that trigger the molting of the microfilariae are closely associated with the passage to the new vector. We hypothesize that it is the mosquito host environment and the presence of ecdysteroids in the host midgut that trigger the upregulation of the ecdysone signaling pathway of *D. immitis*, resulting in the development of the microfilarial stage. Inhibition of the molting process would result in the arrest of the life cycle and prevention of the pathology of dirofilariasis. Thus, the study of the molting process of *D. immitis* microfilariae could point to specific targets for new drug development.

2.2. Specific aims

To test the hypothesis, this project will address the following two objectives:

I. Determine the effects of arthropod environment and ecdysteroids (20-hydroxyecdysone, Muristerone A, Ponasterone A) on the development of *D. immitis* microfilariae in vitro.

II. Determine the changes in the transcript level of the ecdysone signaling system in developing microfilariae.

To carry out the objectives, *D. immitis* microfilariae will be cultured in the presence of ecdysteroids and/or insect cells and observed for signs of morphological development. Droplet digital PCR will be used for the absolute quantification of the transcript levels of the key components in the ecdysone signaling pathway.

3. Methodology

3.1. Parasites

Microfilariae of *D. immitis* Missouri (MO) strain used for this study were provided by the NIH/NIAID Filariasis Research Reagent Resource Center (FR3) (University of Georgia, Athens, GA) [90]. The MO isolate is an ML-susceptible strain that was originally isolated in the year 2000 from a dog housed in an animal pound in Missouri.

Blood samples containing the microfilariae were collected in EDTA vacutainer tubes. They were placed in a thermos and shipped on ice to keep the blood sample cool during the shipment. Upon arrival, blood samples were either processed immediately or stored at 4°C until extraction the next morning. In a biological safety cabinet, the blood sample was diluted by 2 fold with 0.22 µm filtered sodium bicarbonate (2 g L^{-1}) (Sigma Aldrich; S8875). Three to five mL of the diluted blood sample was then filtered through a 3.0 µm polycarbonate membrane filter (Sterlitech; PCT3025100), followed by 3 – 5 mL sodium bicarbonate to wash the membrane. The filter was then placed in 2 – 3 mL of PBS (Gibco; 20012), allowing live microfilariae to migrate off the filter and into the PBS. The filters were then gently rinsed with PBS twice and the PBS containing the microfilariae was collected into 15 mL centrifuge tubes (Corning; 430791). All the flow-through from the filtration was collected and the process was repeated two more times to collect any microfilariae that may have passed through the filter. Collected PBS containing the microfilariae was later spun at 1500 x g for 5 min (Sigma; 2-5). The supernatant was discarded and the pelleted microfilariae were resuspended in fresh PBS. To remove larger cellular debris from the microfilariae, the resuspended pellet was centrifuged briefly at 210 x g for 3 sec. The supernatant containing microfilariae was transferred to a new 15 mL centrifuge tube and centrifuged briefly again for 5 – 6 more times to obtain clean parasites.

To quantify the parasites, 10 µL of the well mixed suspension containing microfilariae were placed between a microscope slide (Fisher Scientific; 12-550-A3) and coverslip (Fisher Scientific; 12-542-B) and the number of live microfilariae on the glass slide was counted and multiplied by the dilution factor to determine the total number of parasites in the solution. After quantification, the parasites were concentrated or diluted as needed for further experimentation.

Adult *D. immitis* samples (Kentucky strain, another ML-susceptible strain) used in this study were provided by Zoetis. The adult worms were obtained through necropsy of experimentally-infected dogs and flash-frozen upon collection. The worms were shipped to McGill on dry ice and stored immediately at -80°C before experimentation.

3.2. *Dirofilaria immitis* microfilariae in vitro culture

Extracted microfilariae of *D. immitis* MO strain were cultured at a density of 500 microfilariae in 800 µL of culture media using a 24-well cell culture plate (Corning; 3524). Different culture media tested include Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco; 22400), Dulbecco's Modified Eagle Medium (DMEM) (Gibco; 12430), Ham's F-12 (Gibco; 11765), DMEM and Ham's F-12 mixed at a 1:1 ratio (DMEM/Ham's F-12), and Schneider's Insect Medium (Sigma; S0146). All media were supplemented with 100 U/mL Penicillin, 100 µg/mL Streptomycin (Gibco; 15140), 0.25 µg/mL Amphotericin B (Gibco; 15290), and 0.01 mg/mL Gentamicin (Sigma; G1397) and warmed to 26°C prior to use. The parasites were kept at a controlled atmospheric condition of 26°C, 5% CO_2 (Thermo Scientific, Forma Series II Water Jacket CO_2 Incubator) for 22 days, with/without the presence of fetal bovine serum (FBS) (Gibco; A31607-01), *Anopheles gambiae* cells (cell line 4a-3B), or ecdysteroids. To test the effects of serum, FBS was heat-inactivated at 56°C for 30 min, and 10% (v/v) heat-inactivated FBS was added to each well. For ecdysteroid treatment groups, 20-hydroxyecdysone (Cayman Chemical; 16145), Ponasterone A (Cayman Chemical; 16386), and Muristerone A (Cayman Chemical; 11741) were dissolved in dimethyl sulfoxide (DMSO) (Sigma; D4540) to a stock solution of 20 mM, 10 mM, and 10 mM, respectively, and used at a final concentration of 10 µM, 0.1% (v/v) DMSO.

The media of the cultures were changed daily by allowing the parasites to settle to the bottom of the plate for 5 – 10 min and carefully removing the top 60% of the media without disturbing the parasites.

3.3. *Anopheles gambiae* cell culture

Anopheles gambiae cell line 4a-3B was maintained in Schneider's Insect Medium supplemented with 10% heat-inactivated FBS, 100 U/mL Penicillin, and 100 μg/mL Streptomycin, and cultured in 25 cm^2 cell culture flasks (Corning; 3056) at 26°C with 5% CO_2. When 90 - 100% confluency was reached, cells were scrapped up with a cell scraper (Fisher Scientific; 08-100-241) and passaged at a ratio of 1:10. When co-culturing with *D. immitis* microfilariae, cells were seeded at a density of 5×10^4 cells / cm^2 48 h prior to co-culturing. After 24 h, the cells were washed with PBS and incubated in the culture media intended for culturing the parasite for the remaining 24 h. Cells were re-seeded every 3 days to maintain consistently healthy cells in the culture. For cell-conditioned media groups, cells were seeded at the same density and after 24 h, washed with PBS and incubated in the culture media for another 24 h. The culture media was then collected and centrifuged at 330 x g for 5 min to remove any cells and only the supernatant was used for *D. immitis* culture. *Anopheles gambiae* cells detach from the plates easily so all washing steps had to be done gently.

For cryopreservation, cells were stored in culture medium containing 10% DMSO (Sigma; D2650) and kept in cryogenic vials (Corning; 430289). Cells were replenished after exceeding passage number of 20.

3.4. Morphological studies of *D. immitis* microfilariae

3.4.1. Light microscopy and methylene blue staining

For studying the developmental pattern of the parasite, live microfilariae were observed daily with EVOS XL digital inverted microscope (Life technologies). Morphological changes were noted and motility of the parasite was measured as any movement within a 30 sec timeframe. For measuring the body dimensions, parasite pictures were taken with Nikon Eclipse TE2000-U and body dimensions were analyzed with Nikon NIS-Elements Advanced Research image analysis software.

For better visualization of the cells, microfilariae were fixed in 10 volumes of formalin (2% in H_2O) for 5 min on a shaker at room temperature. The fixed sample was centrifuged for 5 min,

1500 x g, and the supernatant discarded. Thirty µL of methylene blue stain (0.1% in H_2O) was used to stain 200 µL of pelleted sample.

3.4.2. IncuCyte live worm analysis

Live *D. immitis* microfilariae were cultured in 24-well cell culture plates at 26°C, 5% CO_2 (Thermo Scientific, HeraCell 240i CO_2 Incubator) and imaged with IncuCyte ZOOM system (Essen BioScience) at 20X magnification.

3.4.3. Scanning electron microscopy (SEM)

Dirofilaria immitis microfilariae were pre-chilled for 30 min at 4°C to slow down larval movement and placed in fixative consisting of 2.5% glutaraldehyde (Sigma; G5882), 1.5% formaldehyde (Sigma; F8775) in PBS for 2 h at room temperature on an orbital shaker. The samples were then washed with PBS three times for 10 min each, and dehydrated in graded series of ethanol (EtOH) (Commercial Alcohols; P016EAAN) at room temperature: 30% (1 x 10 min), 50% (1 x 10 min), 70% (1 x 10 min), 80% (1 x 10 min), 90% (1 x 10 min), 100% (3 x 30 min). Care was taken to avoid drying of samples in between washes. The dehydrated samples were then critical point dried by CO_2 treatment (Leica EM CPD300) or chemically dried with hexamethyldisilazane (HMDS) (Sigma; 440191) 3 times for 15 min. Dried samples were then sputter-coated with 4 nm of gold and palladium (Leica EM ACE200) to enhance conductivity. Samples were then mounted onto aluminum stubs and examined with a Hitachi TM-1000 scanning electron microscope.

3.5 Quantification of ecdysone signaling pathway transcript level

3.5.1. Sample preparation

To prepare samples for RNA extraction, 3 – 4 x 10^4 microfilariae were cultured in 25 mL DMEM/Ham's F-12 in a 100 mm cell-culture dish (Corning; 430167), with 60% of the media changed daily. On the day of sample collection (every 3 days), the parasites were spun down at the speed of 1500 x g for 5 min, media aspirated, and the pellet washed twice with PBS. Samples were then pooled together in 1.5 mL microcentrifuge tubes (Fisherbrand; FS749520-0090) to

obtain 6 - 8 x 10^4 microfilariae in 50 µL PBS. One hundred and fifty µL of TRIzol™ reagent (Ambion; 15596026) was added and the sample flash-frozen in liquid nitrogen. The parasites were then frozen at -80°C until RNA extraction.

Adult *D. immitis* samples (Kentucky strain) were stored at -80°C prior to RNA extraction. On the day of RNA extraction, adult worms were cut up into 1 cm pieces with a surgical blade (Feather No. 21) and quickly transferred to 150 µL of TRIzol™ reagent in a 1.5 mL microcentrifuge tube.

3.5.2. RNA extraction and DNase treatment

Prior to RNA extraction, all surfaces and materials were wiped down with ELIMINase (Foxwest; 1101) and all dilutions were prepared with nuclease-free UltraPure distilled water (Invitrogen; 10977). Three cycles of flash-freezing in liquid N_2 and crushing of worms in TRIzol™ reagent with plastic pestles (Fisherbrand; FS749520-0090) were performed to break the worms into smaller pieces. An additional 850 µL of TRIzol™ reagent and 200 µL of glass beads (Sigma; G8772) were added and the tubes were wrapped with Parafilm. Five cycles of vortexing (MO Bio, Vortex-Genie 2 with vortex adaptor) at max speed for 40 sec and chilling on ice for 20 sec were performed at 4°C to homogenize the worm extracts. Samples were incubated at room temperature for 3 min to allow complete dissociation of nucleoprotein complexes. Two hundred µL of chloroform (Sigma; C2432) was then added to each tube, samples shaken vigorously for 30 sec and incubated at room temperature for 3 min. The mixtures were then centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase containing the RNA was transferred to fresh tubes and mixed with 500 µL ice-cold isopropanol (Fisher Chemical; A415-4). Tubes were incubated for 15 – 20 min at -20°C for RNA precipitation and centrifuged at 12,000 x g for 10 min at 4°C. Supernatants were discarded and pellets were washed twice with 1 mL 75% ice-cold EtOH, with a centrifugal step of 7,500 x g, 4°C, 5 min in between each wash to remove the supernatant. After the second EtOH wash, the samples were allowed to air dry for 5 – 10 min in a flow-hood to ensure complete evaporation of EtOH. For microfilariae samples, 15 µL of nuclease-free UltraPure distilled water was added to the pellet for resuspension; for adult samples, 50 µL of nuclease-free UltraPure distilled water was used.

Samples were treated with DNase to remove contaminating DNA using an Invitrogen DNA-free Kit (Thermo Fisher Scientific; AM1906). In short, 0.1 volume of 10X DNase buffer and 1 µL

rDNase I were added to the RNA sample and incubated at 37°C for 30 min with the shaker on. 0.1 volume of resuspended DNase inactivation reagent was then added and the tube vortexed every 15 sec for 2 min. The tubes were then centrifuged at 10,000 x g for 3 min to precipitate the inactivation reagent and the supernatant containing the RNA was transferred to a new tube. The concentration and quality of the RNA was assessed by spectrophotometry (Thermo Scientific, NanoDrop One^{C}) and by running an aliquot of the RNA sample on a native agarose gel. RNA samples were stored at -80°C or reverse transcribed immediately.

3.5.3. Reverse transcription

RNA was reverse transcribed using Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific; 18080-051). The reaction was carried out using oligo $(dT)_{20}$ primers, with thermocycler setting of 65°C for 5 min (disruption of RNA secondary structures), 50°C for 50 min (cDNA synthesis), 85°C for 5 min (reaction termination), and 37°C for 20 min (RNA template removal). Minus reverse transcriptase (-RT) control samples were prepared at the same time as a negative control to monitor genomic DNA contamination. cDNA was stored at -20°C or used for PCR immediately.

3.5.4. PCR

Conventional PCR was performed to ensure primer specificity. Primers were designed for four genes of interest: *Dim-EcR*, *Dim-rxr-1*, *Dim-nhr-7*, *Dim-nhr-6*, as well as three internal controls: *Dim-GAPDH*, *Dim-Actin*, *Dim-β-tubulin* using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Primer sequences listed in Table 1). All primers were checked to confirm low-homology with other sequences in the *D. immitis* genome using BLAST. Reactions were carried out in 20 µL-reaction volumes containing 2 µL 10X PCR Buffer (Invitrogen; 18038), 200 µM dNTPs (Invitrogen; 10297018), 1.5 mM $MgCl_2$ (Invitrogen; 18038), 500 nM of each forward and reverse primer, 0.4 units of *Taq* DNA polymerase (Invitrogen; 18038), nuclease-free UltraPure distilled water, and cDNA sample in 0.2 ml PCR tubes (Diamed; AD0210-FCN). PCR was run with the following program: 94°C for 3 min, 40 cycles defined as 94°C for 45 sec, 52 - 57°C for 30 sec, 72°C for 1 min, followed by 72°C for 10 min. Amplicons were checked on a 2% agarose gel (Invitrogen; 15510-027) with SYBR Safe

DNA gel stain (Invitrogen; S33102) in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Primers that gave a single product at the predicted size with no background were used for qPCR.

3.5.5. qPCR

qPCR was performed for melt curve analysis and generation of standard curves to evaluate PCR efficiency. The reactions were carried out in 20 µL-reaction volumes containing 10 µL 2X SYBR Select Master Mix (Applied Biosystems; 4472908), 200 nM final concentration of each forward and reverse primer, nuclease-free UltraPure distilled water, and cDNA sample in MicroAmp Fast Optical 96-well reaction plates (Applied Biosystems; 4346907). Plates were sealed with optical adhesive covers (Applied Biosystems; 4360954) and ran in an StepOnePlus™ Real-Time PCR System (Applied Biosystems; 4376592) using the following program: 50°C for 2 min, 95°C for 2 min, 40 cycles defined as 95°C for 15 sec, 60°C for 15 sec, 72°C for 1 min, followed by a melt curve. Primers that gave a single-peak melt curve with no shouldering and a standard curve with R^2 value $\geq$ 0.97 were used for ddPCR.

3.5.6. ddPCR and data interpretation

ddPCR mix comprised of 10 µL of 2X QX200 ddPCR EvaGreen Supermix (Bio-Rad; 1864033), 250 nM final concentration of each forward and reverse primer, cDNA sample, and nuclease-free UltraPure distilled water of up to 20 µL. The PCR mix was pipetted into the compartments of the Droplet Generator DG8™ Cartridge (Bio-Rad; 1864008) and 70 µL of the Droplet Generation Oil (Bio-Rad; 1864006) was added to the appropriate wells. The cartridges were covered with the Droplet Generator DG8™ Gaskets (Bio-Rad; 1863009) and placed in a QX200™ Droplet Generator (Bio-Rad) to generate the droplets. Forty µL of droplets generated were gently transferred to a semi-skirted 96-well plate (Bio-Rad; 12001925). The PCR plate was heat-sealed with a pierceable foil (Bio-Rad, 1814040) using a PX1™ PCR Plate Sealer (Bio-Rad). The PCR plate was then placed in a C1000 Touch™ Thermal Cycler (Bio-Rad) for PCR amplification with the following program: 95°C for 5 min, 50 cycles defined as 95°C for 30 sec, 48 – 63°C for 1 min, 72°C for 30 sec, followed by signal stabilization period of 4°C for 5 min and 90°C for 5 min. The droplet reading was done with the QX200™ Droplet Reader (Bio-Rad) using ddPCR™ Droplet Reader Oil (Bio-Rad; 1863004).

Results were analyzed by normalizing the copy number of the gene of interest against the geometric mean of the copy numbers of three reference genes. The fold change in transcript level was then calculated by comparing the normalized value to the baseline day 1 transcript level.

4. Results

4.1 Microfilariae develop into pre-sausage, sausage, and late sausage stages

To determine the effects of arthropod environment and ecdysteroids on the development of *D. immitis* microfilariae, the parasite had to be classified into different stages of growth. Development was categorized as microfilaria, pre-sausage, sausage, or late sausage stage based on their morphological differences (Figure 4A-D).

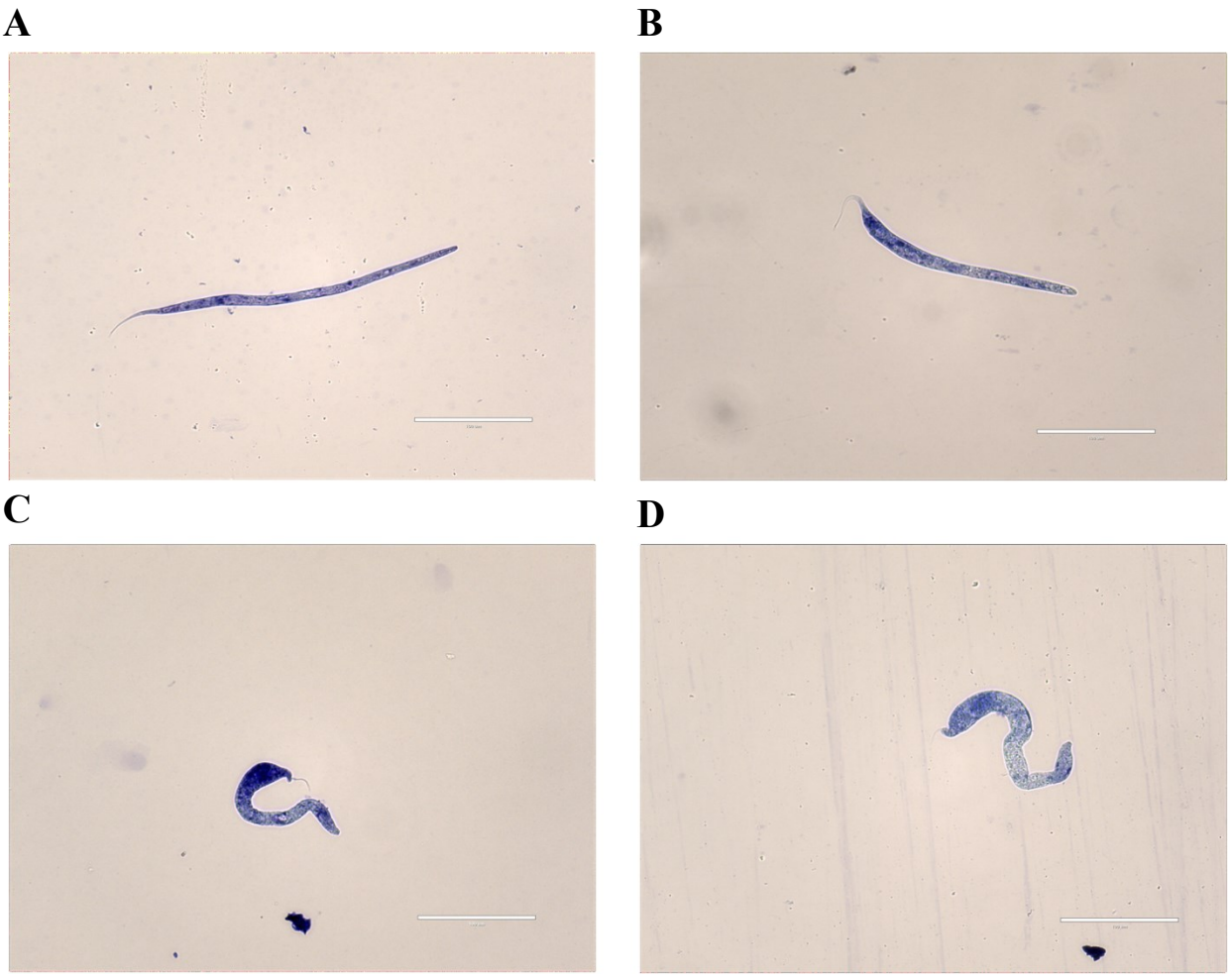
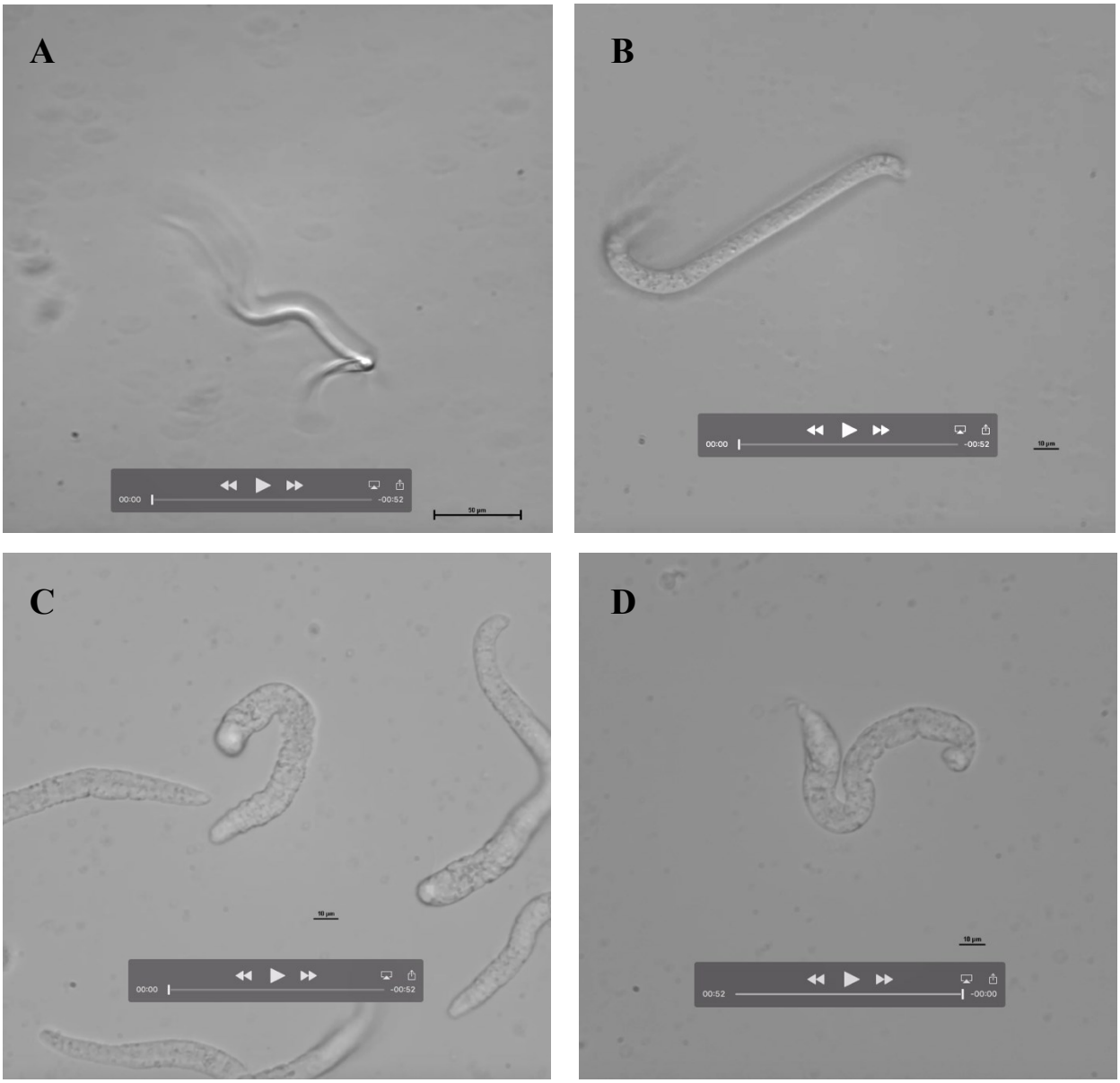


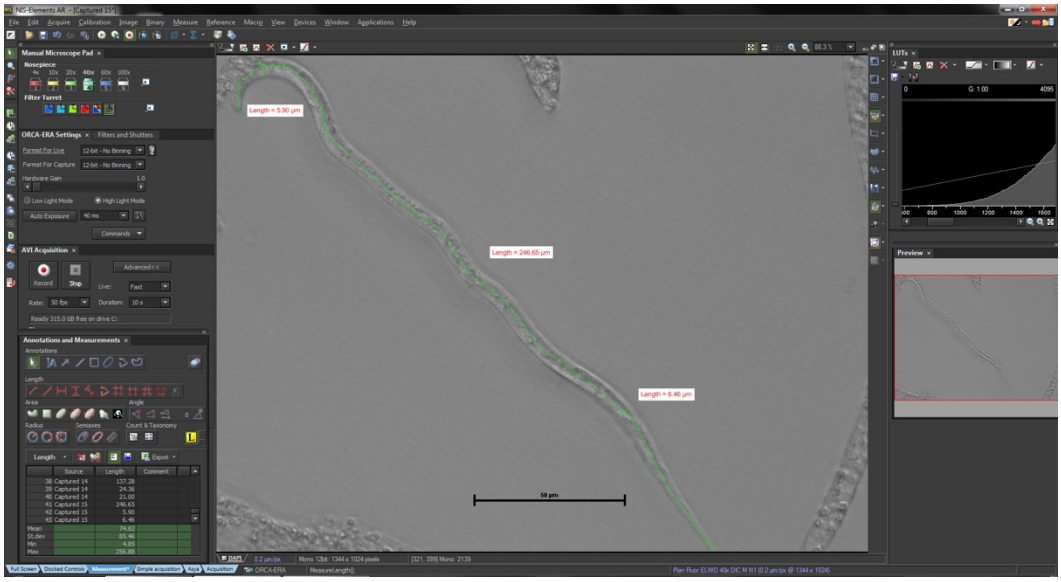
***Figure 4*. Morphological development of *D. immitis* microfilariae.** Light microscopy images of methylene blue-stained microfilariae at 40X magnification. (A) Microfilaria (B) Pre-sausage (C) Sausage (D) Late sausage

The microfilaria stage is what the parasite looks like after being extracted from dog blood samples. At this stage, the parasite appeared elongated and slender and moving with vigorous serpentine movements (Video 1A). After a few days, the body shortened and the posterior region began to enlarge, making the tail more distinct; this stage was classified as the pre-sausage stage. Compared to the microfilaria stage, the pre-sausage stage was more granular in appearance and movement was slowed (Video 1B). Over the next few days, the pre-sausages continued to grow shorter in length, resulting in a stumpy appearance with a fine tail at the tip, approaching the sausage stage. The sausage stage was more basophilic than the microfilaria, with heavy internal granulation and movement was sluggish (Video 1C). At the late sausage stage, movement continued to decline (Video 1D) and at the same time, the anterior region of the sausages started to enlarge while the body became more elongated. The length and width of the parasites were measured (Figure 5A) to determine the mean body dimensions for the different stages (Figure 5B).



Video 1. Movement of *D. immitis* microfilariae. (A) Microfilaria (B) Pre-sausage (C) Sausage (curved parasite in the center of the video) (D) Late sausage. Video of the microfilaria stage was taken at 40X magnification, while those of pre-sausage, sausage, and late sausage stage larvae were taken at 60X magnification. Hyperlinks have been added to the images.

A



B

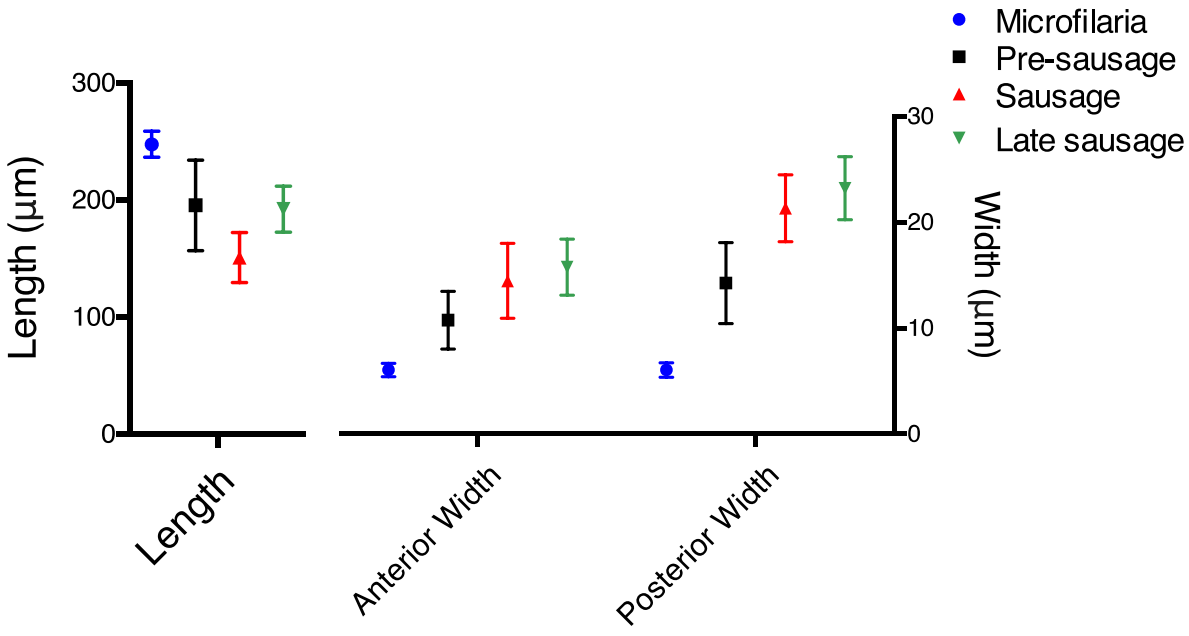


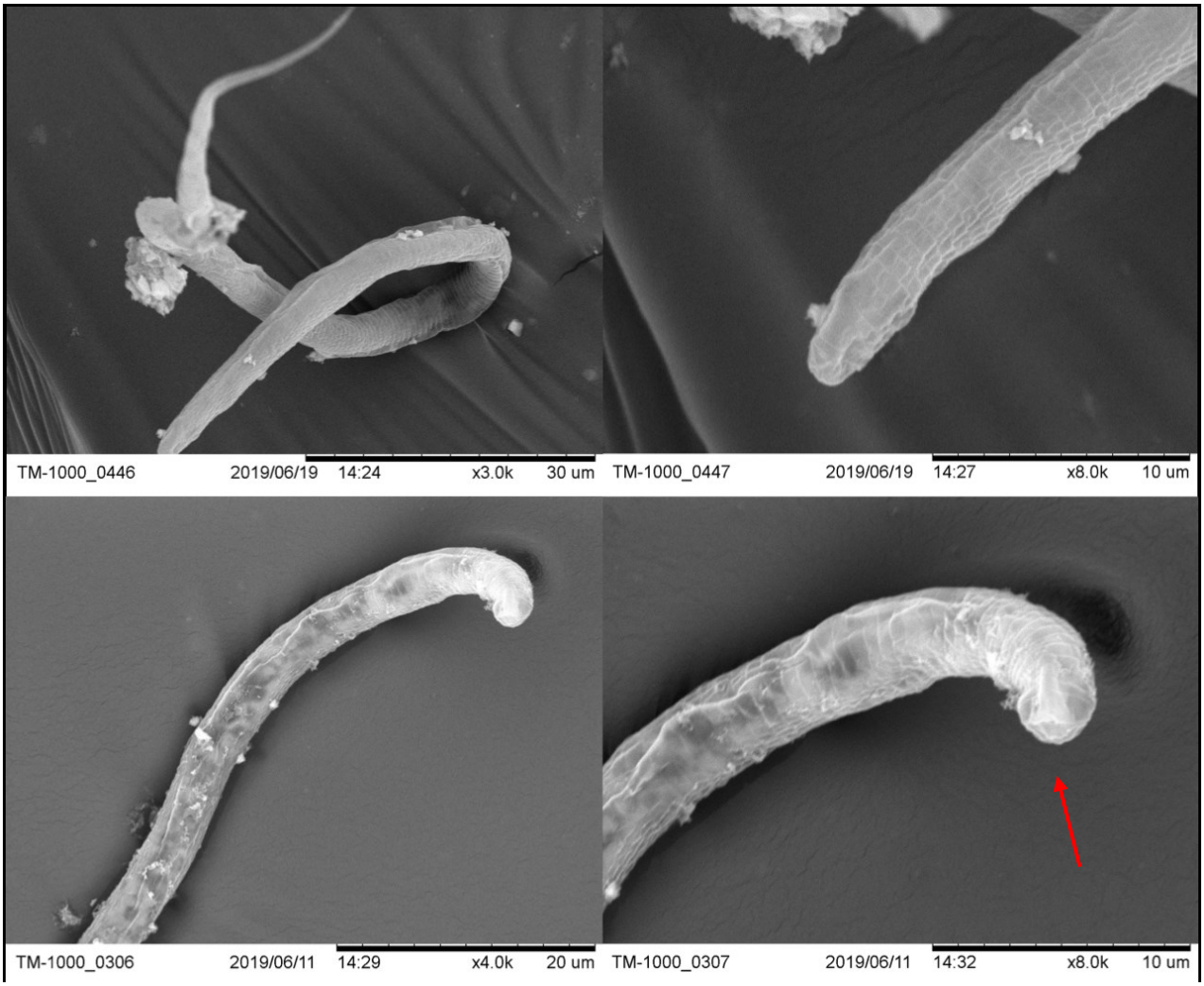
Figure 5. **Body dimensions of *D. immitis* microfilariae developmental stages.** (A) Representative measurement analysis of microfilaria with Nikon NIS-Elements Advanced Research image analysis software. (B) Mean body dimensions of microfilariae developmental stages. Data reported as means with standard deviation (SD). $n = 10$.

4.2 Pre-sausage, sausage, and late sausage stages are L1 larvae

To determine if pre-sausage, sausage, and late sausage stages were L2 larvae, SEM analysis was performed to see if the stoma of the parasites were visible. Numerous transverse grooves in the cuticle were evident in all the developmental stages, giving the parasite a striated appearance, as shown in Figure 6A-D. The grooves seemed to become more noticeable in the pre-sausage, sausage, and late sausage stages. These developed larvae also had more enlarged posterior regions compared to the microfilariae, with the sausage form appearing more "stumpy" in morphology, all of which are consistent with the light microscopy images in Figure 4. All the stages had a circular mass of tissue located at the tip of the head and layers of folded tissue that surrounded it.

Because the high magnification of the SEM analysis impacts the resolution of the pictures, it was difficult to discern the details of the anterior region and so a definite conclusion regarding the opening of the stoma could not be drawn. To overcome this problem, live microfilariae were imaged over time with the IncuCyte ZOOM system to observe if any shedding occurred between each stage. Older literature has shown that sausage stage microfilariae are still L1 larvae [91], and results from the IncuCyte ZOOM system showed that the change from a pre-sausage stage to the late sausage stage was not accompanied by any shedding (Video 2), although a separation of the outer cuticle could be seen.

A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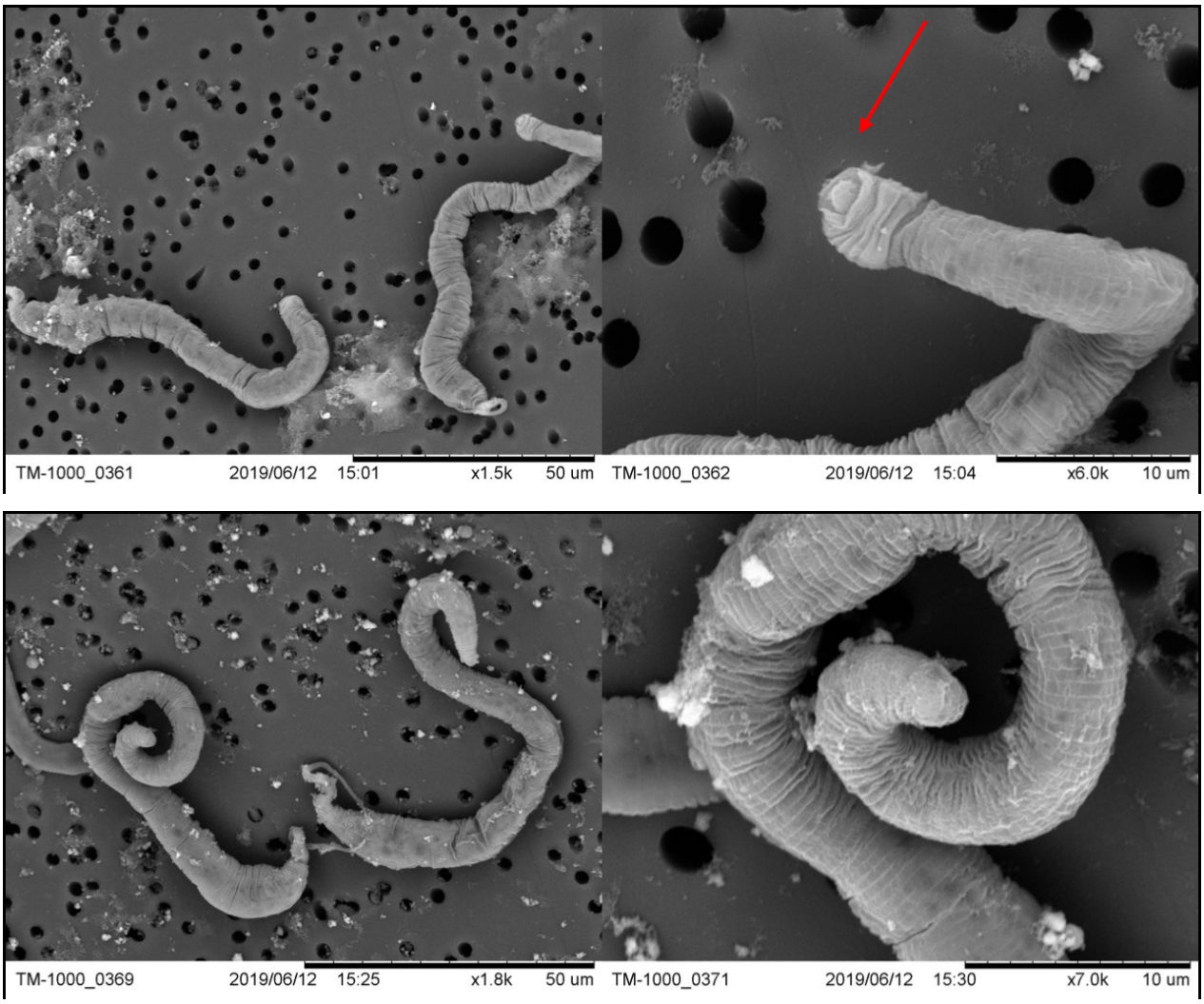
TM-1000_0447 2019/06/19 14:27 x8.0k 10 um
TM-1000_0306 2019/06/11 14:29 x4.0k 20 um
TM-1000_0307 2019/06/11 14:32 x8.0k 10 um

B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TM-1000_0362 2019/06/12 15:04 x6.0k 10 um
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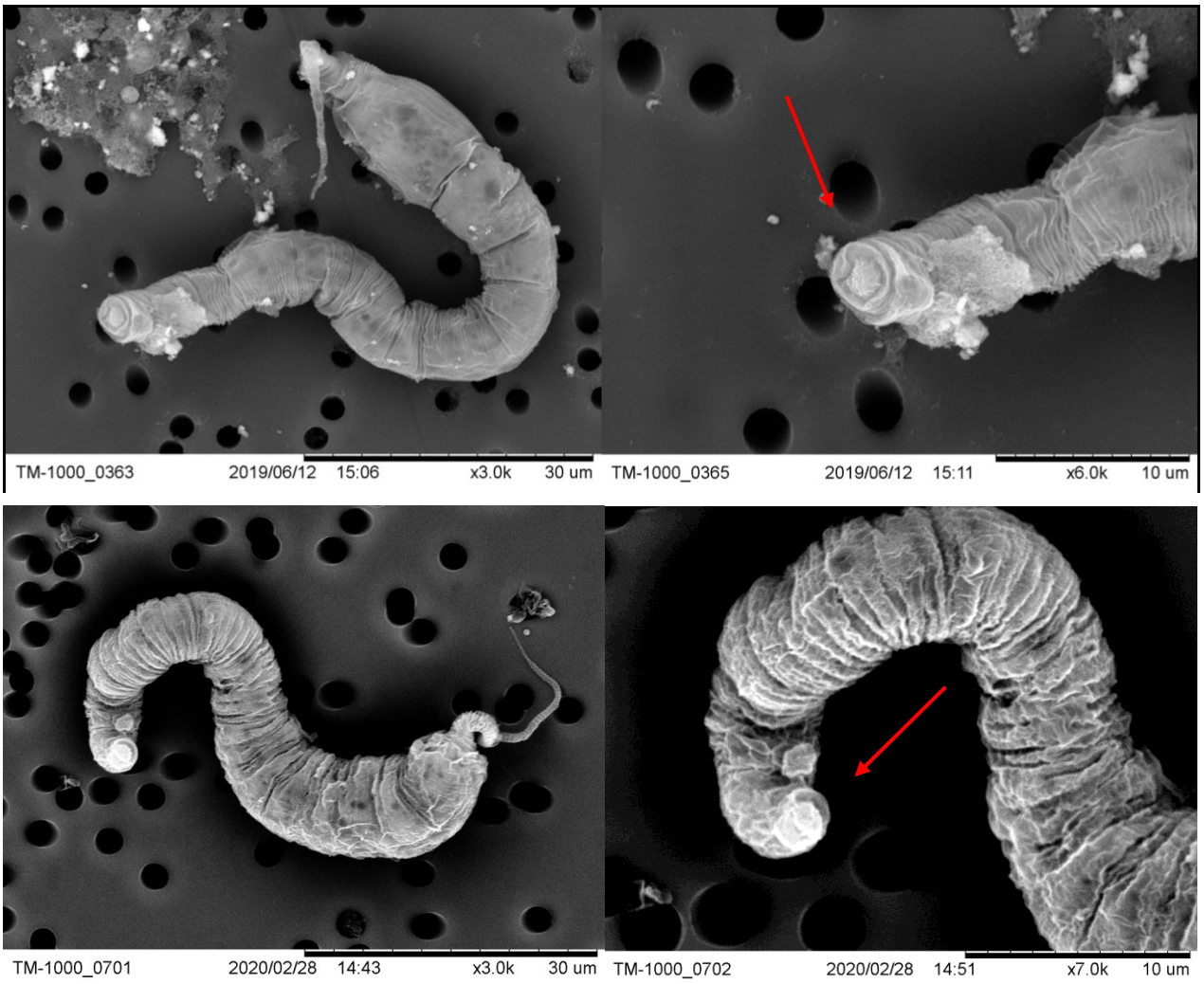
TM-1000_0371 2019/06/12 15:30 x7.0k 10 um

C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TM-1000_0365 2019/06/12 15:11 x6.0k 10 um
TM-1000_0701 2020/02/28 14:43 x3.0k 30 um
TM-1000_0702 2020/02/28 14:51 x7.0k 10 um

D

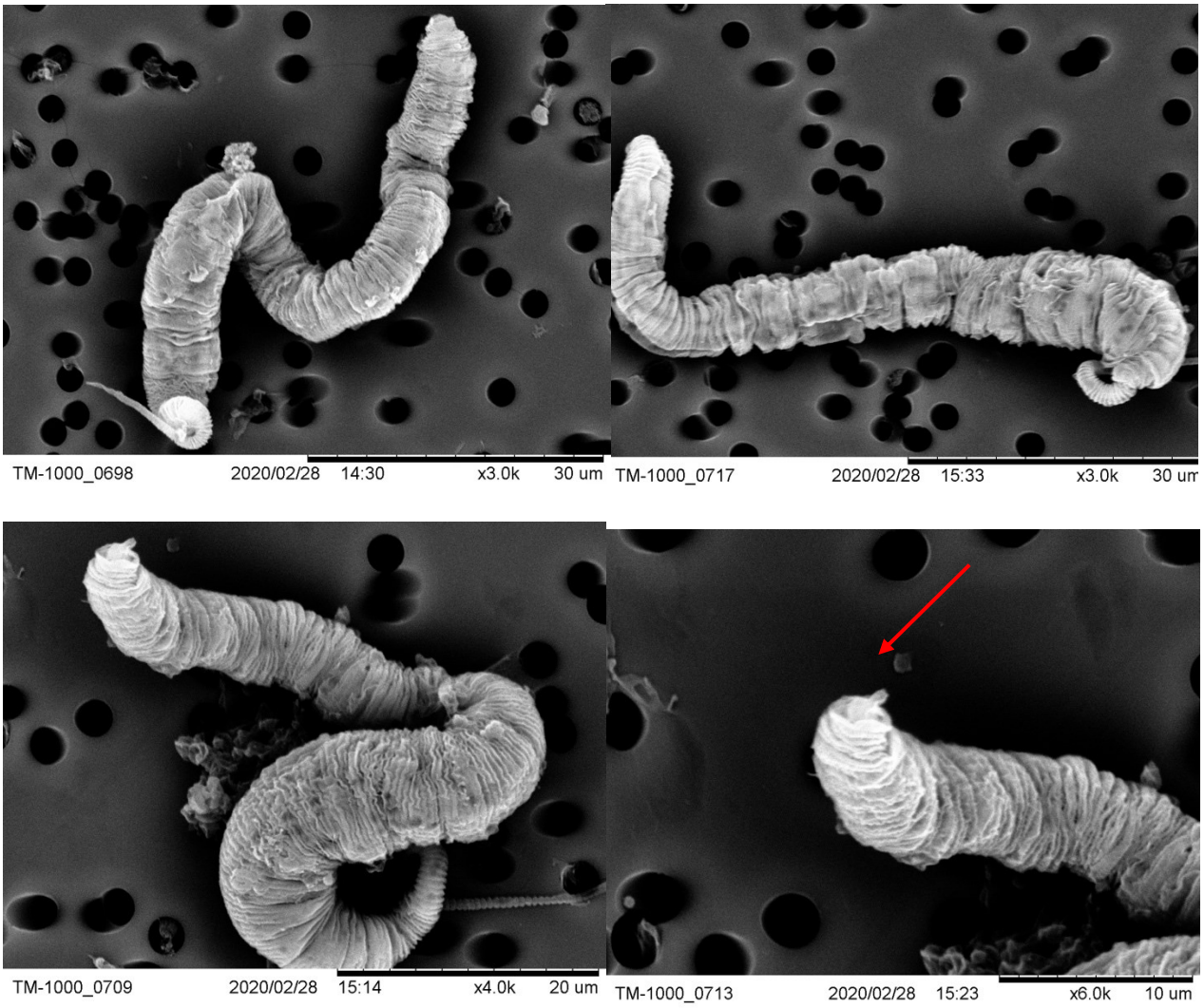
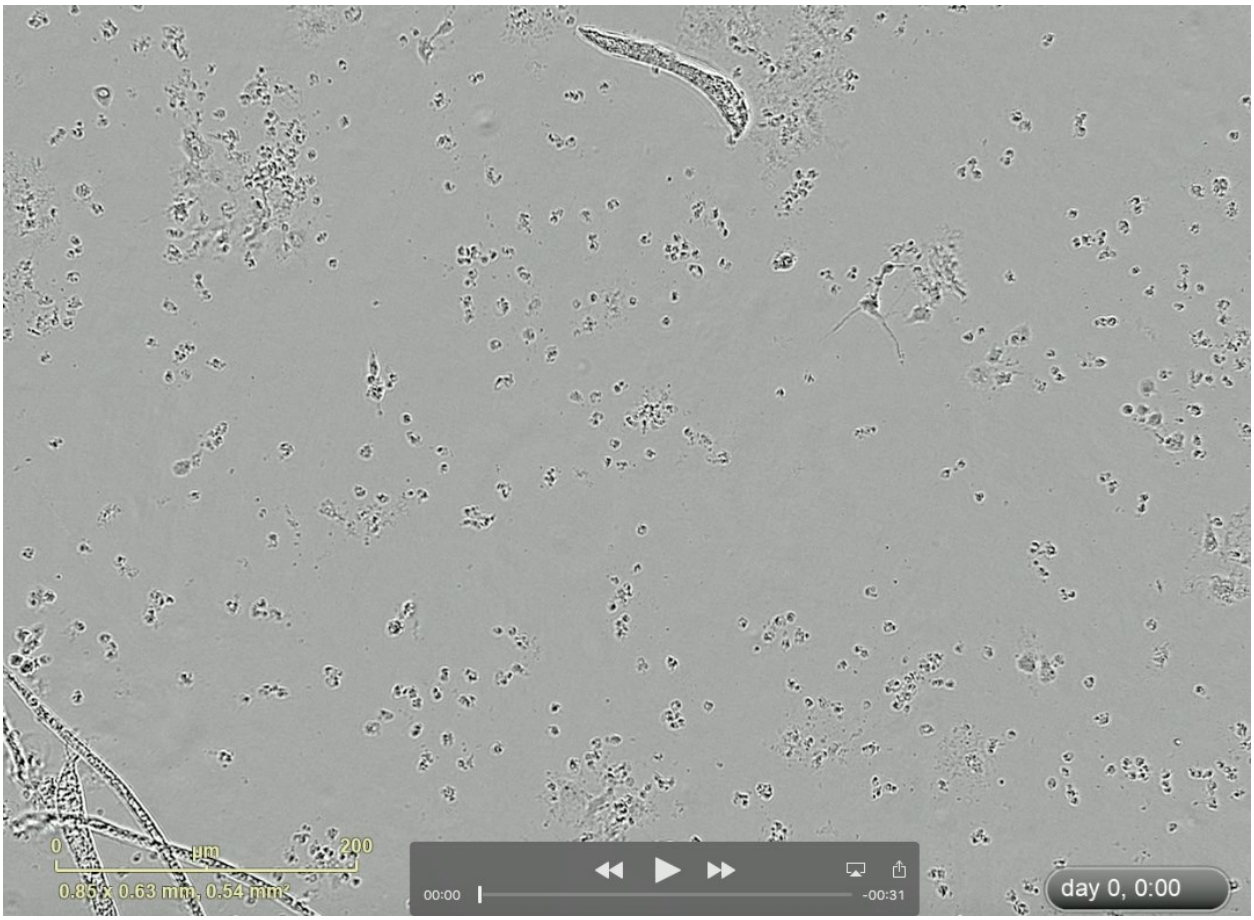


Figure 6. **SEM analysis of *D. immitis* microfilaria, pre-sausage, sausage, and late sausage stages.** (A) SEM analysis of microfilariae that were critical point dried and mounted on a double-sided tape showing whole microfilaria (top left) and the enlarged head region (top right), as well as the ventral anterior view of the microfilaria (bottom left) and the enlarged head region (bottom right). (B) SEM analysis of pre-sausages that were critical point dried and mounted on a 3.0 µm polycarbonate membrane filter. The pictures show whole pre-sausages (top left and bottom left) and enlarged views of the head region (top right and bottom right). (C) SEM analysis of sausage staged larvae that were critical point dried (top left and top right) and dried with HMDS (bottom left and bottom right) and mounted on a 3.0 µm polycarbonate membrane filter. The pictures show whole sausages (top left and bottom left) and enlarged views of the head region (top right and bottom right). (D) SEM analysis of late sausage staged larvae that were dried with HMDS

and mounted on a 3.0 μm polycarbonate membrane filter. The pictures show whole late sausages (top left, top right, and bottom left) and an enlarged view of the head region (bottom right). Red arrows show where the stoma opening would be.



Video 2. Imaging of live larva taken with the IncuCyte ZOOM system. Pre-sausage stage starts from 0:00; sausage stage starts from 0:19; late sausage stage starts from 0:24. Background shows *An. gambiae* 4a-3B cells that gradually died off. Hyperlink has been added to the image.

4.3 DMEM/Ham's F-12 provides a relatively favorable condition for in vitro cultivation of *D. immitis* microfilariae

To determine the suitable condition for the in vitro cultivation of *D. immitis* microfilariae, extracted microfilariae were incubated in various commercially available culture media at 26°C, 5% CO_2 for 22 days and the number of motile parasites as well as the number of pre-sausage and sausage stages were counted. Initially, RPMI 1640 medium and Schneider's insect medium were used because the former has been used in our lab as a basal medium for short-term *D. immitis* culture, and the latter is designed for insect tissue culture. However, results showed that both RPMI 1640 and Schneider's insect medium were poor at sustaining the development and motility of the microfilariae (Figure 7A-B). DMEM, Ham's F-12, and a mixture of both at a 1:1 ratio were next tested as Sneller and Weinstein reported observing some development after culturing microfilariae in media designed for mammalian cell culture for 10 days [92]. DMEM maintained microfilariae motility but was unable to initiate development into the pre-sausage stage (Figure 7C), while Ham's F-12 was beneficial to their development but motility was compromised (Figure 7D). When used at a 1:1 ratio, DMEM/Ham's F-12 provided a favorable condition for the microfilariae and supported the development of the larvae to the pre-sausage and sausage stage (Figure 7E). As motility reflects the survival of the parasites, only DMEM/Ham's F-12 was used for consecutive experiments.

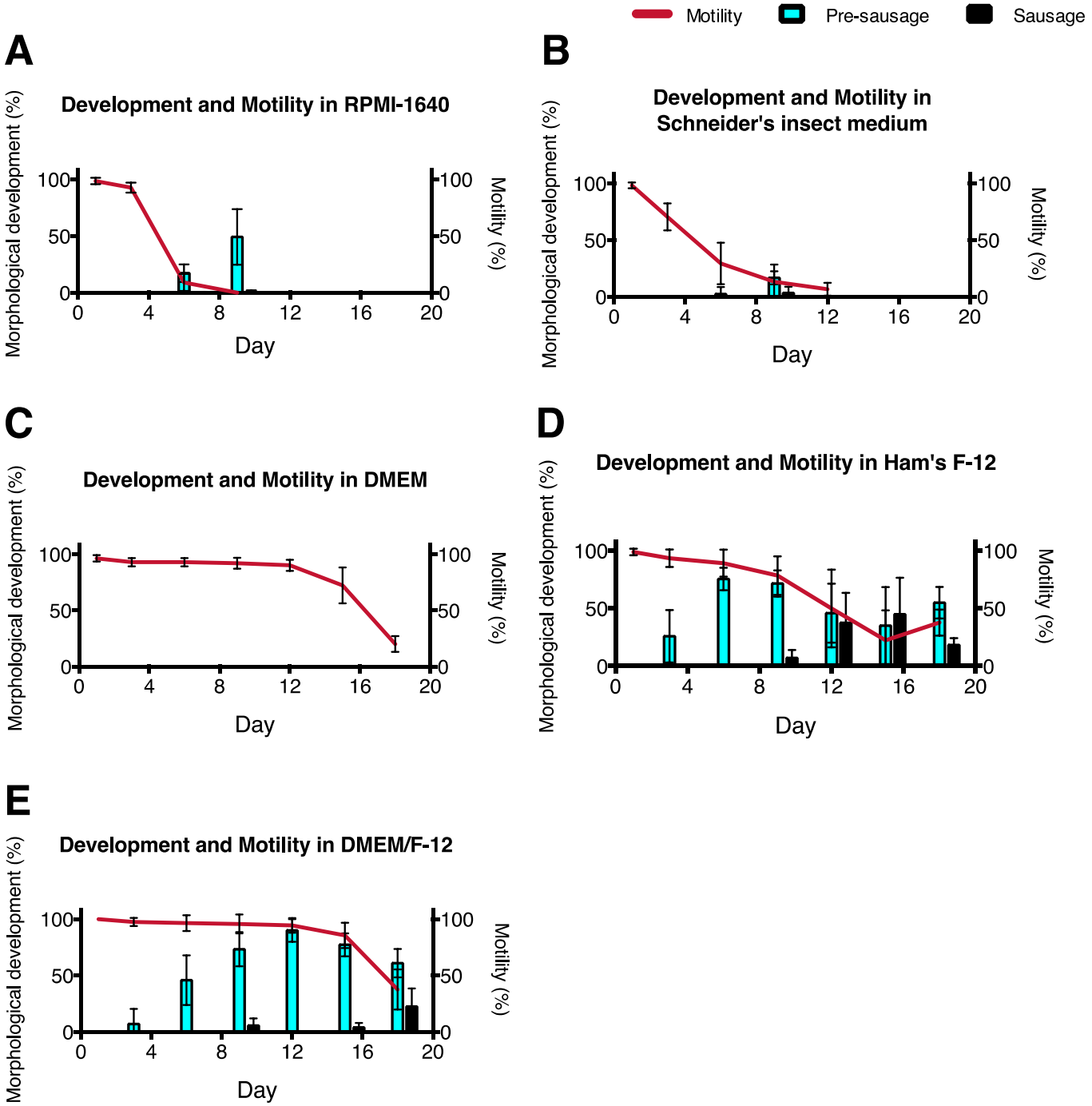


***Figure 7*. Development and motility of *D. immitis* microfilariae in several commercially available media.** (A) RPMI-1640 medium. A blood sample of microfilariae MO strain was collected from canine #1 and four individual replicates were performed. (B) Schneider's insect medium. Two blood samples of the MO strain were collected from canine #1 and shipped on

different days, with four individual replicates performed on the microfilariae from the first blood sample and one replicate performed on the microfilariae from the second blood sample. (C) DMEM. A blood sample of microfilariae MO strain was collected from canine #1 and four individual replicates were performed. (D) Ham's F-12. Two blood samples of the MO strain were collected from canine #1 and shipped on different days, with four individual replicates performed on the microfilariae from the first blood sample and one replicate performed on the microfilariae from the second blood sample. (E) DMEM/Ham's F-12. Three blood samples of microfilariae MO strain were collected from canine #1 and one blood sample was collected from canine #2, also infected with the MO strain, with one replicate performed on the microfilariae from each of the blood samples of canine #1 and three individual replicates performed on the microfilariae from the blood samples of canine #2; each blood sample was shipped on a different day. All data reported as means with SD, using all of the replicates described for each procedure.

4.4 FBS is detrimental to the motility and development of the microfilariae

The effect of FBS on the growth of microfilariae was next examined by the addition of 10% FBS to the culture media and incubating the microfilariae at 26°C, 5% CO_2 for 22 days. Again, the number of motile parasites, as well as the number of pre-sausage and sausage stages, were counted. After FBS was added to the culture media, development and motility of the microfilariae were both drastically lowered, and microfilariae appeared more lethargic and degenerated, with little growth to the pre-sausage stage observed in most groups (Figure 8A, B, D, E). However, an exception was seen in those that were incubated in DMEM and 10% FBS (Figure 8C); although development was initiated in this group and some pre-sausages developed into sausages, most of the parasites appeared degenerated and motility was greatly reduced compared to the group without 10% FBS. Therefore, FBS was not added to the culture media in later studies.

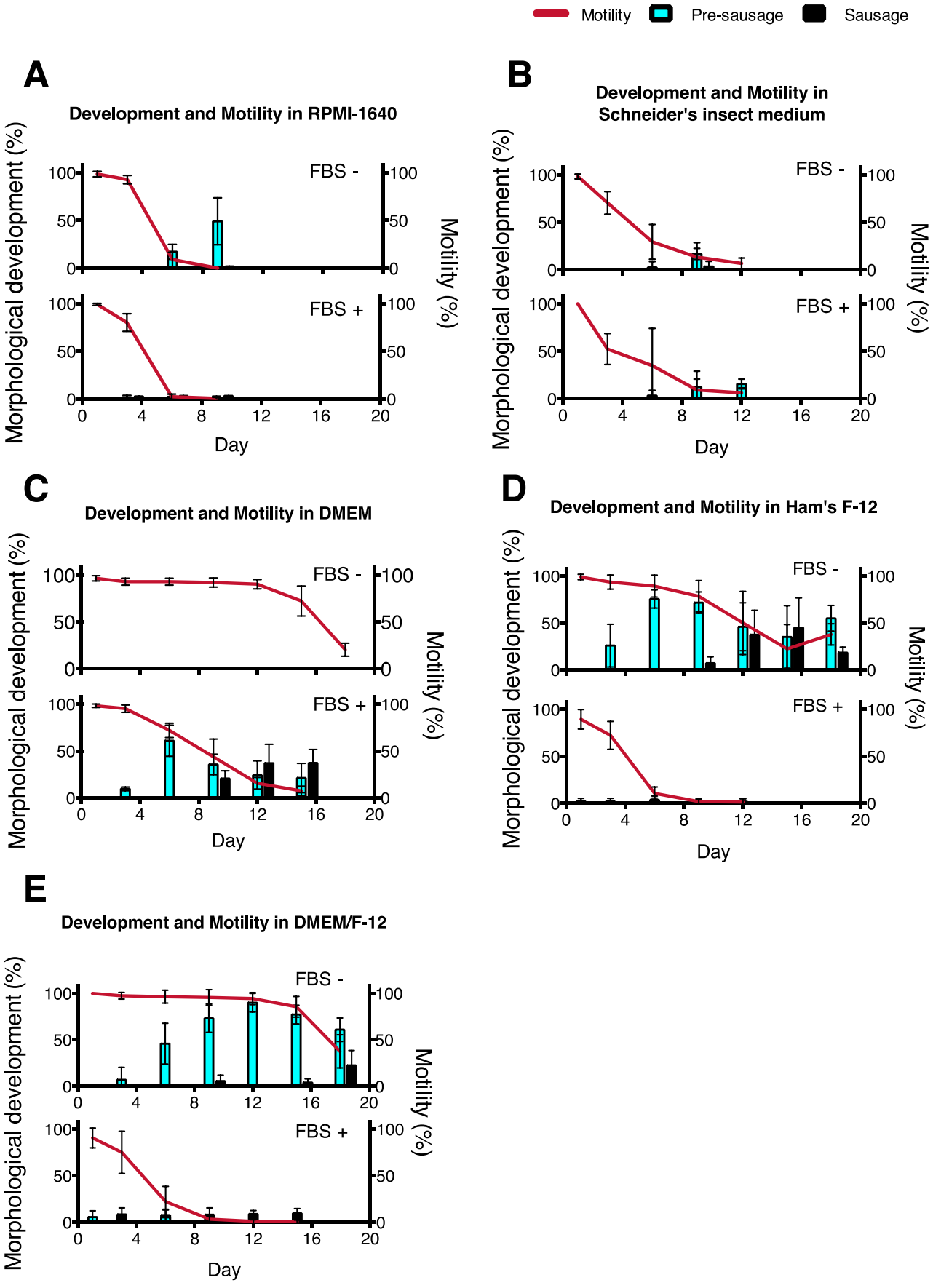

Motility
Pre-sausage
Sausage
A
Development and Motility in RPMI-1640
B
Development and Motility in Schneider's insect medium
C
Development and Motility in DMEM
D
Development and Motility in Ham's F-12
E
Development and Motility in DMEM/F-12
Morphological development (%)
Motility (%)
FBS -
FBS +
Day
0
4
8
12
16
20
50
100

***Figure 8*. Development and motility of *D. immitis* microfilariae in the presence of FBS.** Results from Figure 7 are placed together as the no FBS (FBS -) comparison. (A) RPMI-1640 medium. One blood sample of microfilariae MO strain was collected from canine #1 and four individual replicates were performed. (B) Schneider's insect medium. Two blood samples of the MO strain were collected from canine #1 and shipped on different days, with four individual replicates performed on the microfilariae from the first blood sample and one replicate performed on the microfilariae from the second blood sample. (C) DMEM. One blood sample of microfilariae MO strain was collected from canine #1 and four individual replicates were performed. (D) Ham's F-12. Two blood samples of the MO strain were collected from canine #1 and shipped on different days, with four individual replicates performed on the microfilariae from the first blood sample and one replicate performed on the microfilariae from the second blood sample. (E) DMEM/Ham's F-12. Two blood samples of the MO strain were collected from canine #1 and shipped on different days, with four individual replicates performed on the microfilariae from the first blood sample and one replicate performed on the microfilariae from the second blood sample. All data reported as means with SD, using all of the replicates described for each procedure.

4.5 *Anopheles gambiae* cells and cell-secreted factors promote *D. immitis* microfilariae development

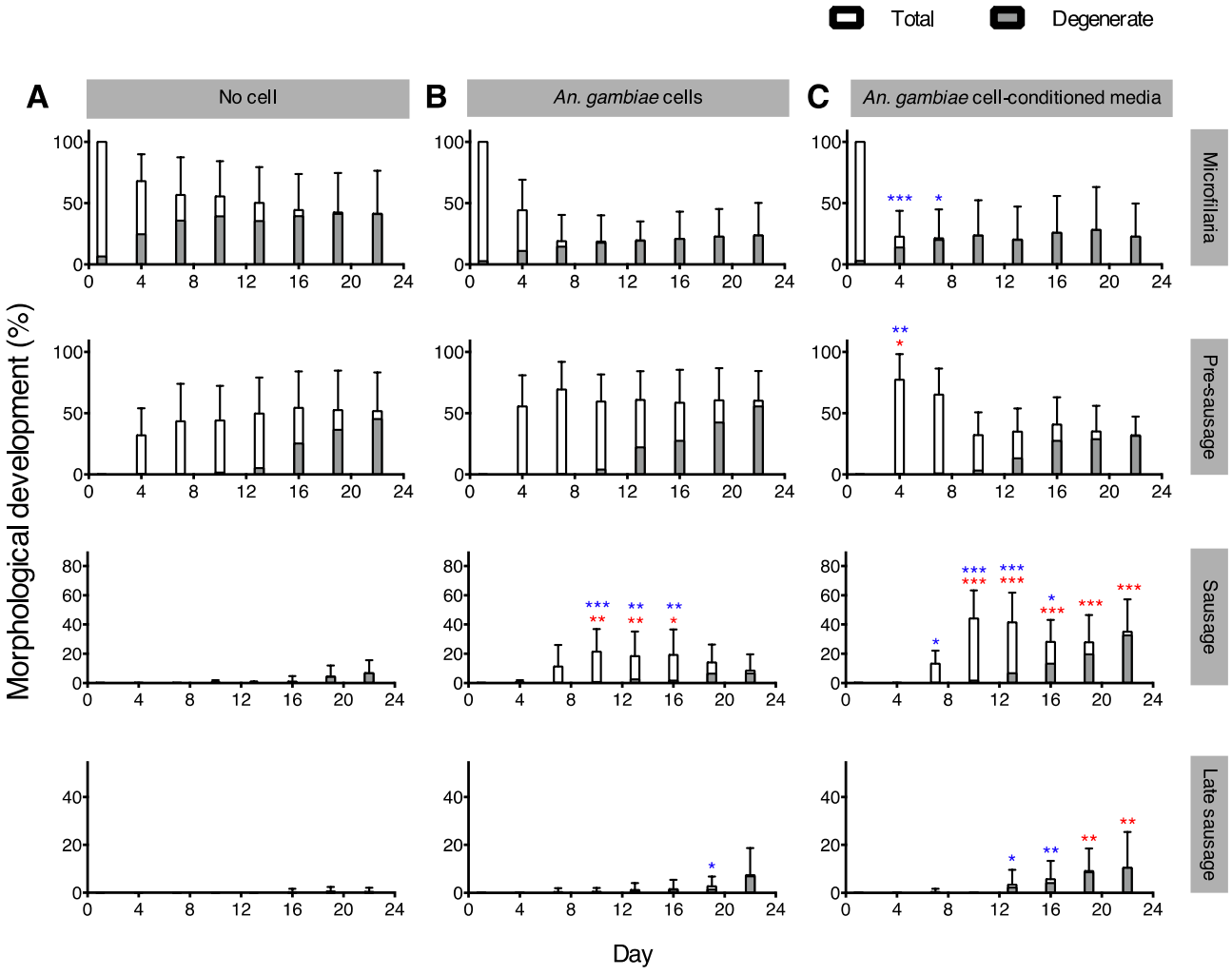
Anopheles gambiae cells were introduced to the culture of microfilariae incubated in DMEM/Ham's F-12 for 22 days to test the effect of insect cells on the development of the microfilariae. For this, the percentages of microfilaria, pre-sausage, sausage, and late sausage stage parasites were noted. Consistent with results in Figure 7, larvae incubated in DMEM/F-12 in the absence of mosquito cells developed into the pre-sausage stage around day 4, with few that developed into the sausage stage throughout the 22-day incubation period. It is evident from Figure 9A-B, however, that the presence of *An. gambiae* cells initiated faster growth and significantly higher levels of sausage stages were observed as early as day 8. Also, some parasites were able to develop into the late sausage stage.

When medium conditioned with *An. gambiae* cells was used instead of having physical cells in the culture, a similar effect in the development of microfilariae was also seen. In fact, there was a

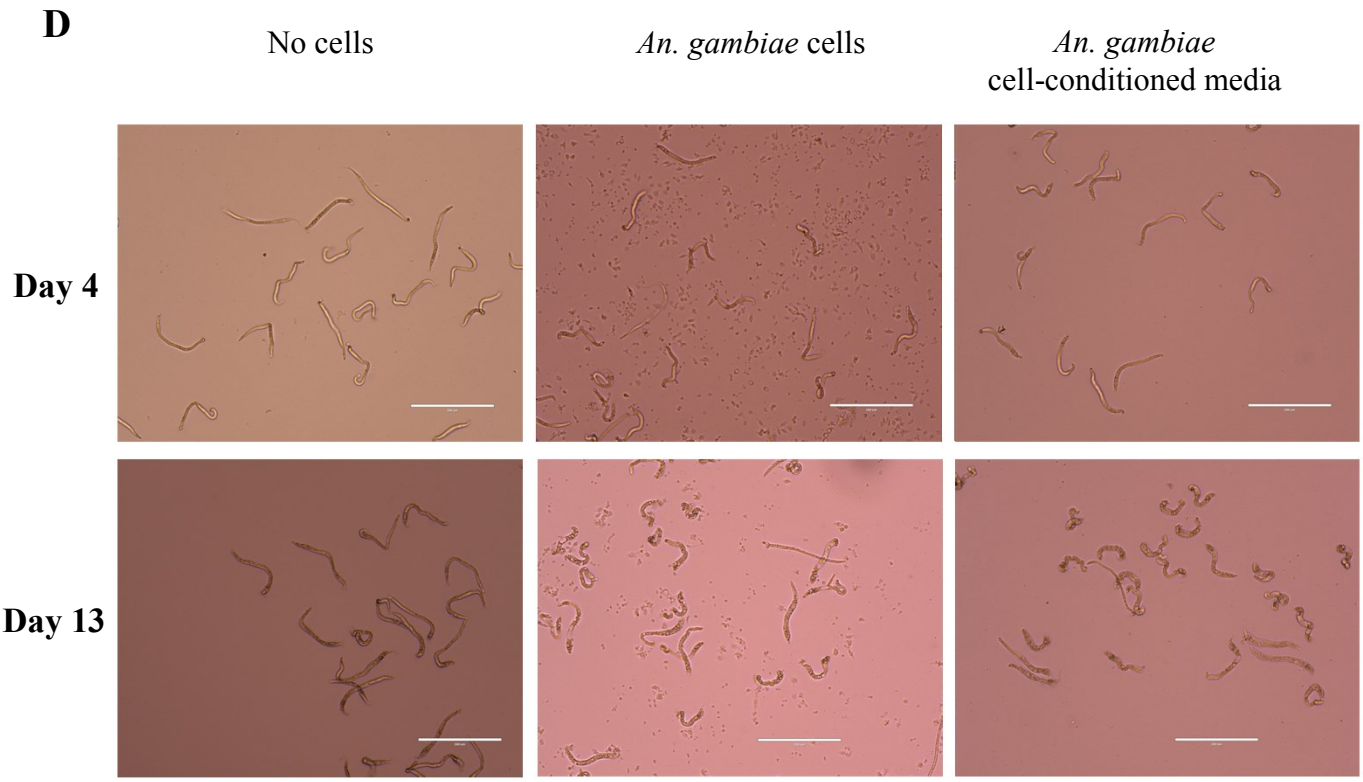
significant decrease in the percentage of microfilaria and an increase in the number of pre-sausages during early incubation. In addition, more sausages were observed when the cell-conditioned medium was used compared to when microfilariae were in physical contact with the insect cells (Figure 9C). This is also shown in Figure 9D, where development into the sausage stage was more noticeable at day 13 in both groups cultured in the presence of cells and cell-conditioned media, with slightly higher proportions of sausages found in the cell-conditioned group.

In terms of motility, the presence of cells and cell-conditioned media seemed to improve and maintain parasite activity, whereas in the absence of cells, motility dropped at a faster rate and the parasites appeared more sluggish. The presence of physical cells in the culture also appeared to slow the drop in motility compared to parasites cultured in cell-conditioned media (Figure 9E).

It is important to note that microfilariae that were co-cultured with insect cells do not interact with the cells, but instead occupy the space above the layer of cells. Also, towards the end of the 22-day incubation, higher percentages of the parasites became degenerated in each experimental group (as indicated by the gray bars). In addition, larvae that did not develop into the next stage eventually died off, and those that have gone on to the next stage tend to remain motile.


Total
Degenerate
A
No cell
B
An. gambiae cells
C
An. gambiae cell-conditioned media
Microfilaria
Pre-sausage
Sausage
Late sausage
Morphological development (%)
Day

D


No cells
An. gambiae cells
An. gambiae cell-conditioned media
Day 4
Day 13

E

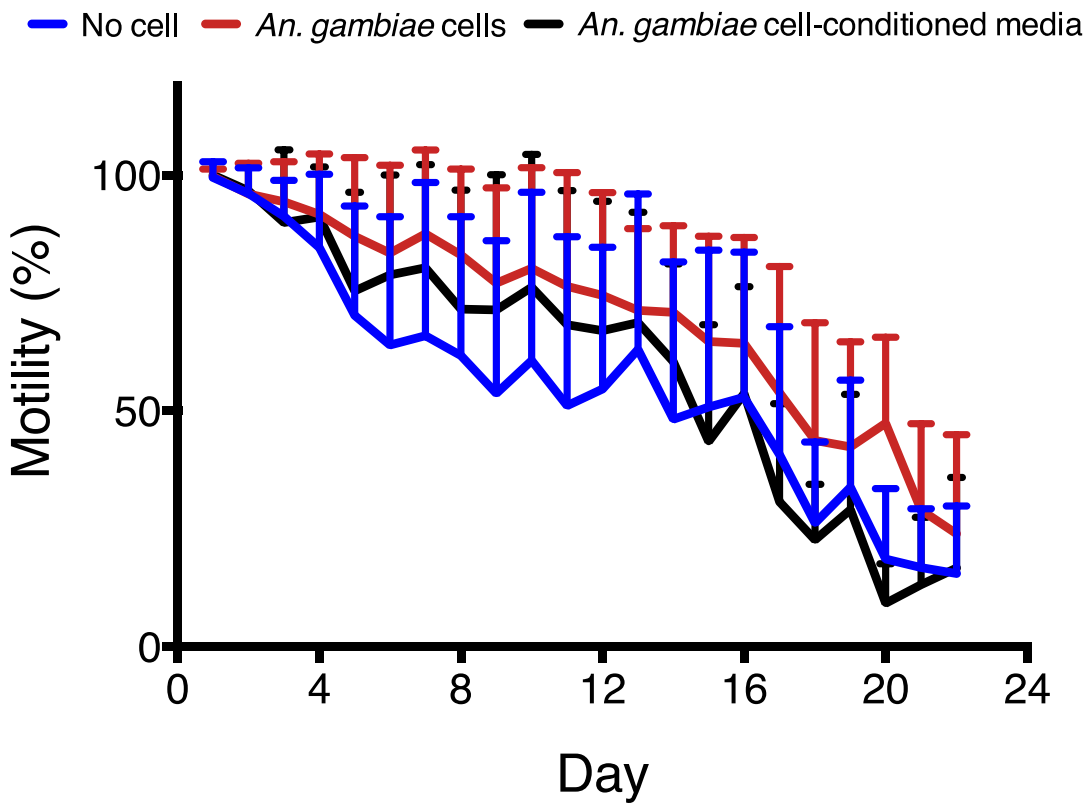

No cell
An. gambiae cells
An. gambiae cell-conditioned media
Motility (%)
100
50
0
0
4
8
12
16
20
24
Day

***Figure 9*. Development of *D. immitis* microfilariae cultured in the presence of *An. gambiae* cells or cell-conditioned media.** Results show percentages of larvae at the microfilaria, pre-sausage, sausage, and late sausage stages cultured (A) in the absence of *An. gambiae* cells. One blood sample of microfilariae MO strain was collected from canine #3 and two blood samples of the MO strain were collected from canine #2, with nine individual replicates performed on the microfilariae from the blood samples of canine #3 and three individual replicates performed on the microfilariae from each of the blood samples of canine #2; each blood sample was shipped on a different day. (B) With *An. gambiae* cells. A blood sample of MO strain was collected from canine #1, canine #2, and canine #3, and shipped on three different days; four individual replicates were performed on the microfilariae from the blood samples of canine #1, three individual replicates were performed on the microfilariae from the blood samples of canine #2, and two individual replicates were performed on the microfilariae from the blood samples of canine #3. (C) With *An. gambiae* cell-conditioned media. One blood sample of microfilariae MO strain was collected from canine #3 and two blood samples of the MO strain collected from canine #2, with three individual replicates performed on the microfilariae from each of the blood samples of canine #2 and canine #3; each blood sample was shipped on a different day. All data reported as means with SD, using all of the replicates described for each procedure. Asterisks denote statistical difference between total (*) and healthy (*) parasites compared to the no-cell group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Statistical analysis performed using 2-way ANOVA and Dunnett's multiple comparison test, with a 95% confidence interval. (D) Light microscopy of *D. immitis* microfilariae cultured with no cells, *An. gambiae* cells, and *An. gambiae* cell-conditioned media at day 4 and day 13. (E) Motility of microfilariae cultured in the absence of cells, with *An. gambiae* cells, or with *An. gambiae* cell-conditioned media.

4.6 Ecdysteroids have no or negative effect on *D. immitis* microfilariae development

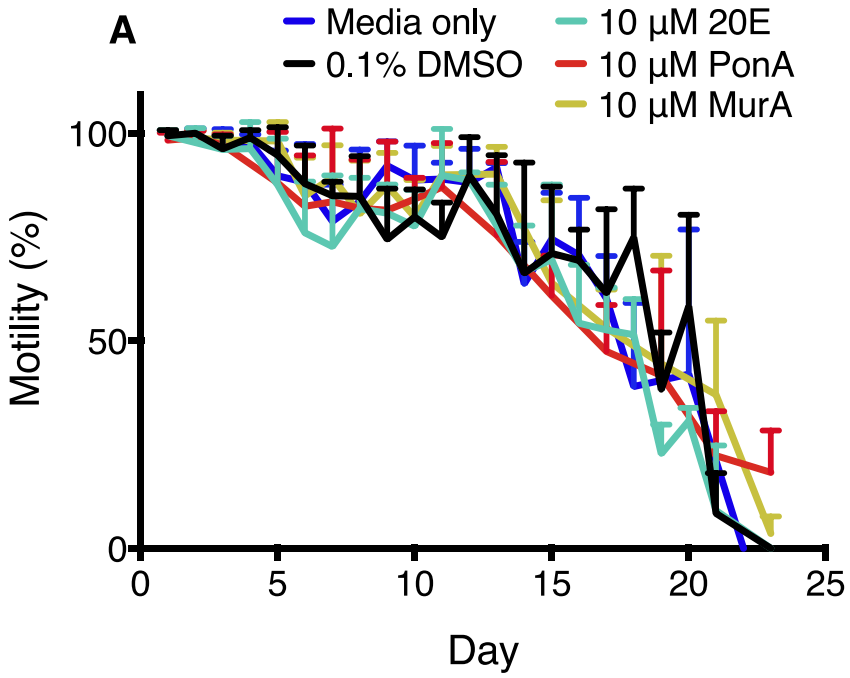
20-hydroxyecdysone (20E), Ponasterone A (PonA), and Muristerone A (MurA) were chosen to examine the effects of ecdysteroids on the growth of *D. immitis* microfilariae because 20E is the known active hormone that controls the molting process in arthropods, and PonA and MurA are both analogs of ecdysone and found to be strong agonists of the ecdysone receptor.

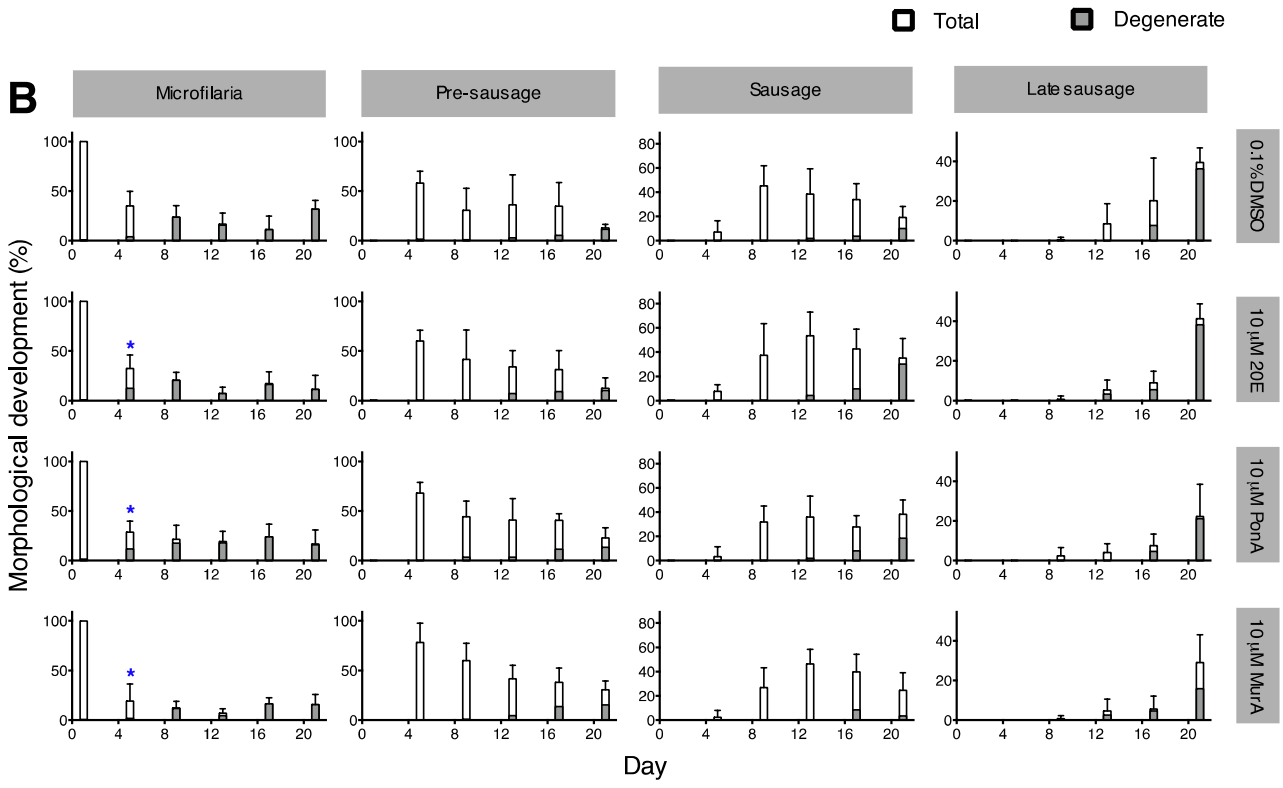
Parasites were treated with either 0.1% DMSO, 10 μM 20E, 10 μM PonA, or 10 μM MurA. Motility of the microfilariae was measured (Figure 10A) and was found to be comparable to those that were not treated, indicating that 0.1% DMSO and 10 μM of ecdysteroids had little effect on the movement and activity of the parasites and so were used for later experiments.

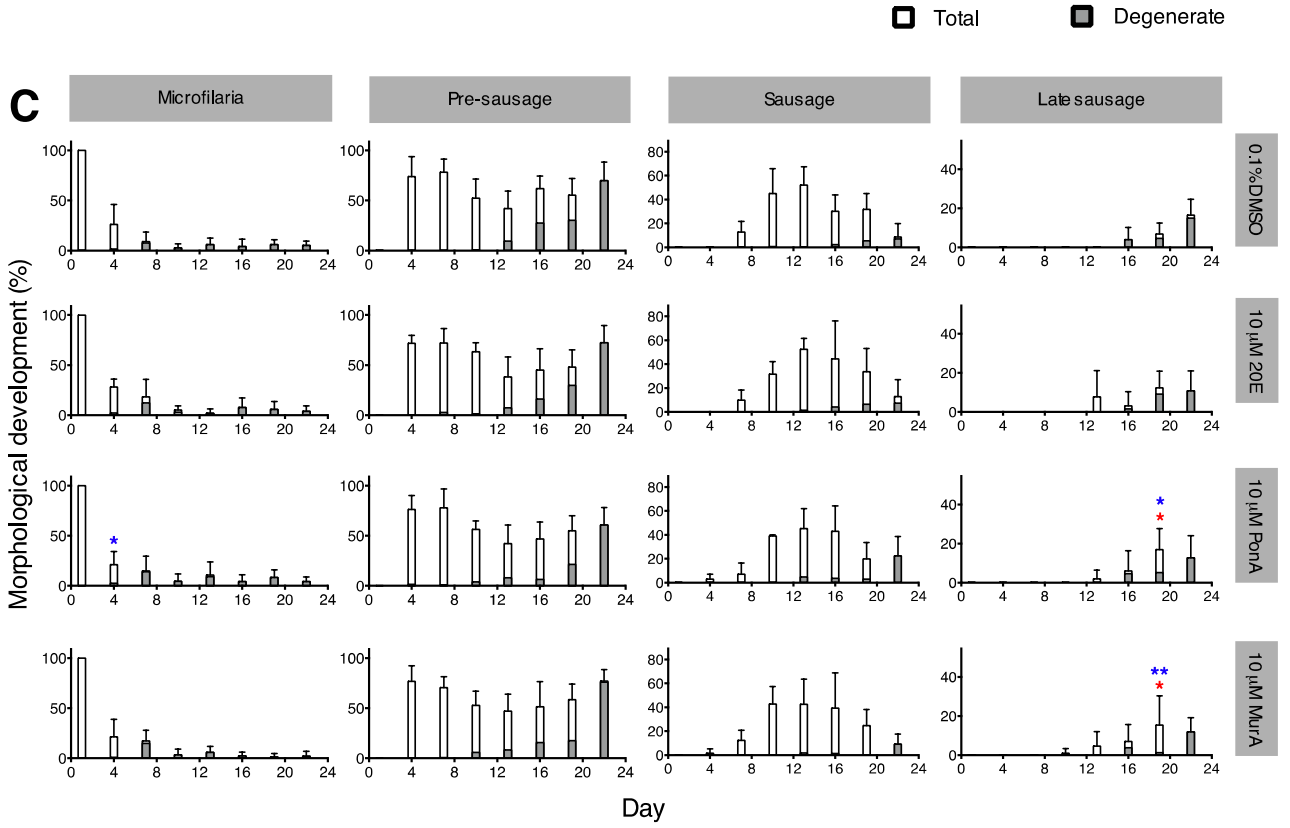
When microfilariae were treated with 10 μM ecdysteroids (20E, PonA, MurA) every 3 days, starting from day 0, there was very little difference in the developmental pattern compared to the group that was not treated (0.1% DMSO), as shown in Figure 10B. In fact, there seemed to be a slower development to the late sausage stage in the presence of ecdysteroids, although the difference was not significant. Literature research indicated that ecdysteroids and other steroid hormones have short half-lives, with 20E having a half-life of 4 – 9 h in humans [93], and PonA having a half-life of 48 min in mouse models [94]. This led to the hypothesis that the level of ecdysteroids may not remain consistently high throughout the experiment. The experiment was thus repeated with 10 μM ecdysteroids added every day to maintain higher concentrations in the culture media.

When 10 μM ecdysteroids were added every day, there was still no significant difference in the growth of the microfilariae (Figure 10C). Even though there were slightly more late sausages at day 19 in groups treated with 10 μM PonA and 10 μM MurA compared to the 0.1% DMSO control group, the effect was not as dramatic as previously published reports on the effects of ecdysteroids on the development of the L3 stage [68]. It was then hypothesized that early treatment with ecdysteroids may be detrimental to the early development of the microfilariae. It has been shown that early activation of the ecdysone receptor in insects can cause premature or incomplete molting [67], and that exposure of female *B. malayi* to 20E can lead to the production of increased levels of immature progeny [74]. Therefore, the next investigation was to delay the treatment with ecdysteroids.

Instead of adding ecdysteroids to the microfilariae culture on day 0, the treatment was delayed to day 10 (Figure 10D), which is when the level of the sausage stage is usually at its highest. However, there was still no significant difference between the treated group and the control group. Again, there seemed to be some negative effects on the growth of the late sausages with the addition of ecdysteroids.


A
Media only
0.1% DMSO
10 µM 20E
10 µM PonA
10 µM MurA
Motility (%)
100
50
0
0
5
10
15
20
25
Day


Total
Degenerate
B
Microfilaria
Pre-sausage
Sausage
Late sausage
0.1%DMSO
10 µM 20E
10 µM PonA
10 µM MurA
Morphological development (%)
Day


C
Total
Degenerate
Microfilaria
Pre-sausage
Sausage
Late sausage
0.1%DMSO
10 µM 20E
10 µM PonA
10 µM MurA
Morphological development (%)
Day

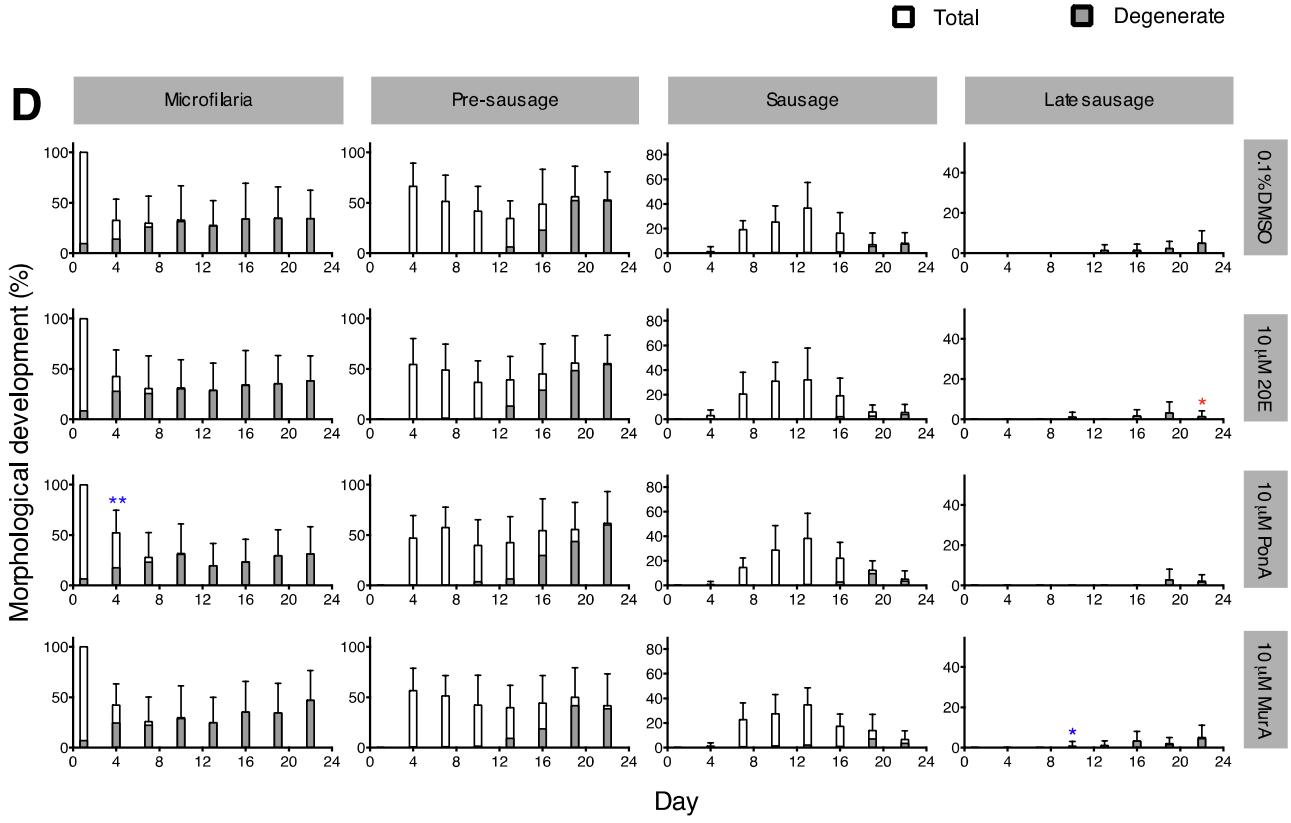

D
Total
Degenerate
Microfilaria
Pre-sausage
Sausage
Late sausage
0.1%DMSO
10 µM 20E
10 µM PonA
10 µM MurA
Morphological development (%)
Day

***Figure 10*. Motility and development of *D. immitis* microfilariae cultured with *An. gambiae* cells and treated with ecdysteroids.** (A) Motility of microfilariae when treated with 0.1% DMSO and 10 μM ecdysteroids. (B) Percentages of larvae at the microfilaria, pre-sausage, sausage, and late sausage stages when treated with ecdysteroids every three days from day 0. Starting from the first row, 0.1% DMSO (two blood samples of microfilariae MO strain were collected from canine #1 and shipped on two different days, with one replicate performed on the microfilariae from each blood sample), 10 μM 20E (two blood samples of microfilariae MO strain were collected from canine #1 and shipped on two different days, with one replicate performed on the microfilariae from each blood sample), 10 μM PonA (one blood sample of the MO strain was collected from canine #1, with two individual replicates performed on the sample), and 10 μM MurA (one blood sample of the MO strain was collected from canine #1, with two individual replicates performed on the sample). (C) Percentages of larvae at the microfilaria, pre-sausage, sausage, and late sausage stages when treated with ecdysteroids every day from day 0 (for each treatment group, a blood sample of microfilariae MO strain was collected from canine #1, with two individual replicates performed on the sample). (D) Percentages of larvae at the microfilaria, pre-sausage, sausage, and late sausage stages when treated with ecdysteroids every day from day 10 (for each treatment group, a blood sample of microfilariae MO strain was collected from canine #2 and canine #3 and shipped on two different days, with two individual replicates performed on the microfilariae from each sample). All data reported as means with SD, using all of the replicates described for each procedure. Asterisks denote statistical difference between total (*) and healthy (*) parasites compared to the 0.1% DMSO group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis performed using 2-way ANOVA and Dunnett's multiple comparison test, with a 95% confidence interval.

4.7 *EcR*, *rxr-1* and downstream early regulatory genes are upregulated in developing microfilariae

Microfilariae were collected every 3 days and the transcript levels of the *EcR*, *rxr-1*, and downstream early regulatory genes were measured to determine if the ecdysteroid signaling pathway may be up or down regulated during the developmental changes that occur in the cultured larvae. Downstream early regulatory genes that were chosen include *Dim-nhr-7* and *Dim-nhr-6*, both of which are orthologues of *E78* and *E75*, respectively. Putative EcRE were found in the upstream promoter regions of both *Dim-nhr-7* and *Dim-nhr-6*, indicating that *Dim-nhr-7* and *Dim-nhr-6* can potentially be regulated by EcR directly (Figure 11).

Primer sequences for *Dim-EcR*, *Dim-rxr-1*, *Dim-nhr-7*, *Dim-nhr-6*, *Dim-GAPDH*, *Dim-Actin*, and *Dim-β-tubulin* are listed in Table 1. All the primers were optimized with conventional PCR, qPCR, and ddPCR. With the conventional endpoint PCR, primers were optimized for specificity and only primer pairs that gave a single sharp band at the predicted size on the agarose gel (Figure 12A) went on to qPCR. With qPCR, primers were tested for specificity and efficiency and primer pairs that went on to ddPCR all had a single-peak melt curve and a standard curve with R^2 value ≥ 0.97 (Figure 12B). The cycle threshold (Ct) values from qPCR were used to estimate template concentrations for use on the ddPCR. With ddPCR, the annealing temperatures for the primers were optimized for better reaction specificity, and the temperature that generated results with the largest fluorescence amplitude difference between the positive and negative droplets, as well as little or no nonspecific amplification, was chosen (Figure 12C).

After normalizing the genes of interest to three reference genes *Dim-GAPDH*, *Dim-Actin*, and *Dim-β-tubulin*, results in Figure 13 show that *Dim-EcR*, *Dim-rxr-1*, *Dim-nhr-7* and *Dim-nhr-6* were all upregulated to different magnitudes in developing L1 larvae. In both no-cell and cell-conditioned media groups, transcript levels of all four genes consistently increased during the first few days of development. In no-cell groups, transcript levels peaked around day 7 for all four genes before incurring a drop, with a low level seen at day 13-16, after which a rise was seen again at day 19. In groups cultured in the cell-conditioned medium, a gradual increase throughout the 22-day incubation period was seen for *Dim-EcR*, *Dim-rxr-1*, and *Dim-nhr-6*, with a sharp rise at day 22. On the other hand, transcript level of *Dim-nhr-7* in the cell-conditioned medium group remained relatively constant during the early incubation period, but a sharp rise

could be seen on day 22. Overall, developing larvae in both no-cell and cell-conditioned medium groups expressed relatively similar levels of transcript expression during early incubation, but gene expression levels of those cultured in cell-conditioned medium rose a lot more quickly, compared to the no-cell group, after day 10. It is important, however, to note that insert figures in the top left corner of each graph showed that the transcript levels at day 1 for both media were different and so the results of the two groups were compared to different baselines. Transcript levels of female and male adults were also included as a comparison, and results showed that the transcript level of the ecdysteroids signaling system components was higher in females than in males for all four genes.

Figure 11. **Putative EcRE in the promoter region upstream of *Dim-nhr-7* and *Dim-nhr-6* start codon**. Start codons are marked with the blue box, putative EcREs are highlighted in yellow, and nucleotides that differ from the canonical EcRE are highlighted in red.

Table 1.

Primer sequences for genes of interest.

Gene name	Function	Primer sequence (5' to 3')	Amplicon size
Dim-EcR	Ecdysone receptor	F1: AGCAACAGCAACAGGCTCA R1: ACCAGATGCTTTATCACCGCA	170
Dim-rxr-1	USP orthologue	F1: TCAAACGAACAGTGCGCAAG R1: TCTTCTTGTACCGCTTCTCTTTTC	148
Dim-nhr-7	E78 orthologue	F1: TGCGTTATGGACGAACACCA R1: ACTGCAGCAGGTCGTATGAG	170
Dim-nhr-6	E75 orthologue	F1: TGATTACACCGCCACAACGA R1: TTTTTGCTGAATGGAGCGGC	130
Dim-GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F1: TCATTCCAGCAAGCACTGGT R1: GTTGGCACACGAAAAGCCAT	94
Dim-Actin	Actin	F1: GCGTCTTGATTTGGCTGGTC R1: ACAATCTCACGTTCCGCAGT	99
Dim-β-tubulin	Beta-tubulin	F1: TTTGCTCCTCTCTCTGCACG R1: TTGCTCGTCTACTTCTCGCA	180

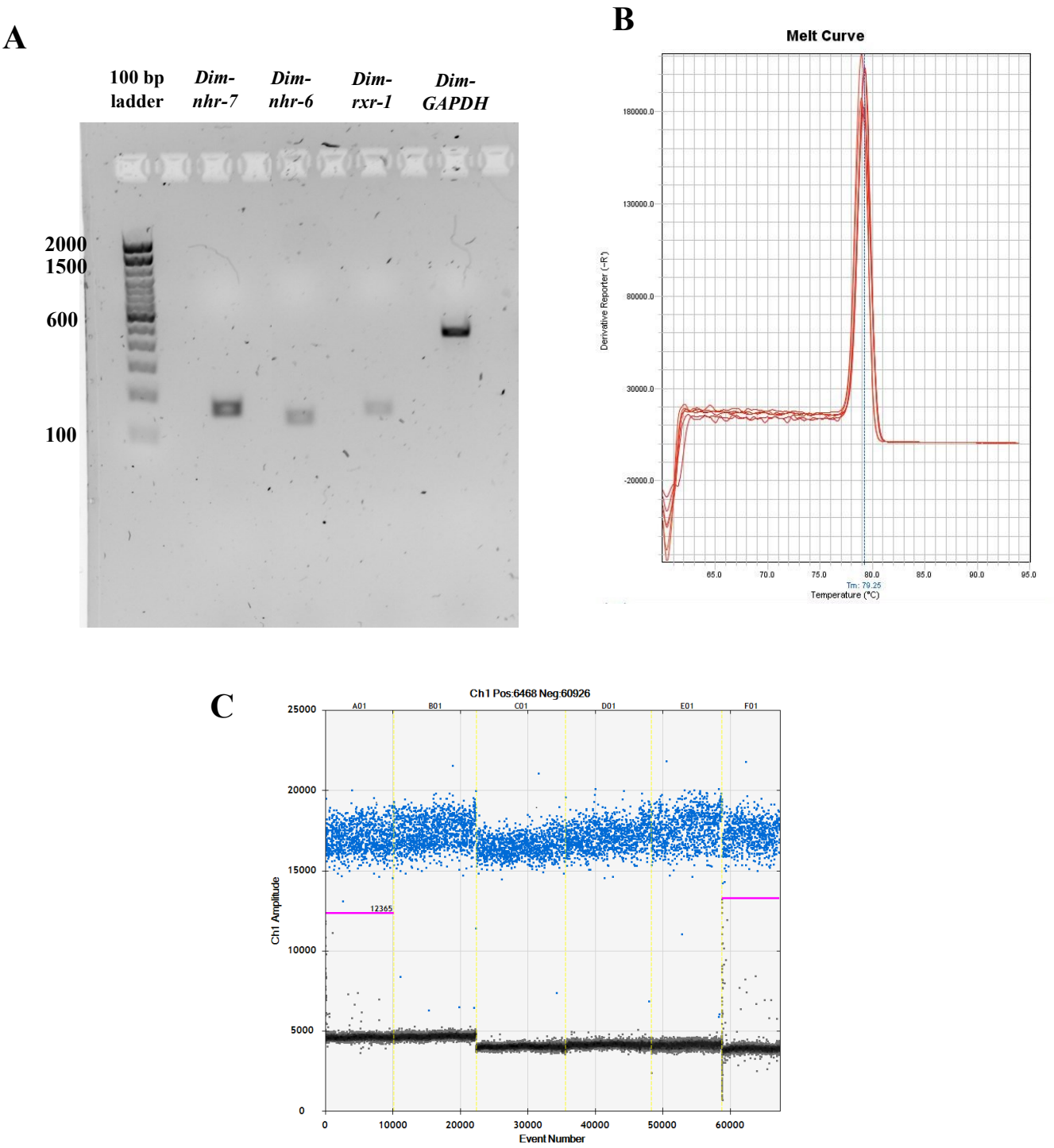


***Figure 12*. Primer optimization with conventional PCR, qPCR, and ddPCR.** (A) Example of conventional PCR products for optimized primers analyzed by 2% agarose gel electrophoresis. Results show (left to right) Invitrogen™ 100 bp DNA ladder, PCR products of *Dim-nhr-7*, *Dim-nhr-6*, *Dim-rxr-1*, *Dim-GAPDH*. (B) Example of qPCR melt curve of optimized primers, shown

here is the melt curve of *Dim-nhr-7* primers. (C) Example of ddPCR optimization with thermal gradient showing droplets plotted as fluorescence intensity vs. droplet number. Shown here is the results generated with *Dim-GAPDH* primers and running at 6 temperatures: from A01 to F01, the temperature are as follows: 63°C, 62.1°C, 60.1°C, 57.2°C, 53.9°C, 51°C. All positive droplets are colored blue, and all negative droplets are colored black. The threshold line for wells A01 and F01 had to be set manually because the software either could not auto-analyze due to the presence of non-specific amplification or there was unusually low droplet counts.

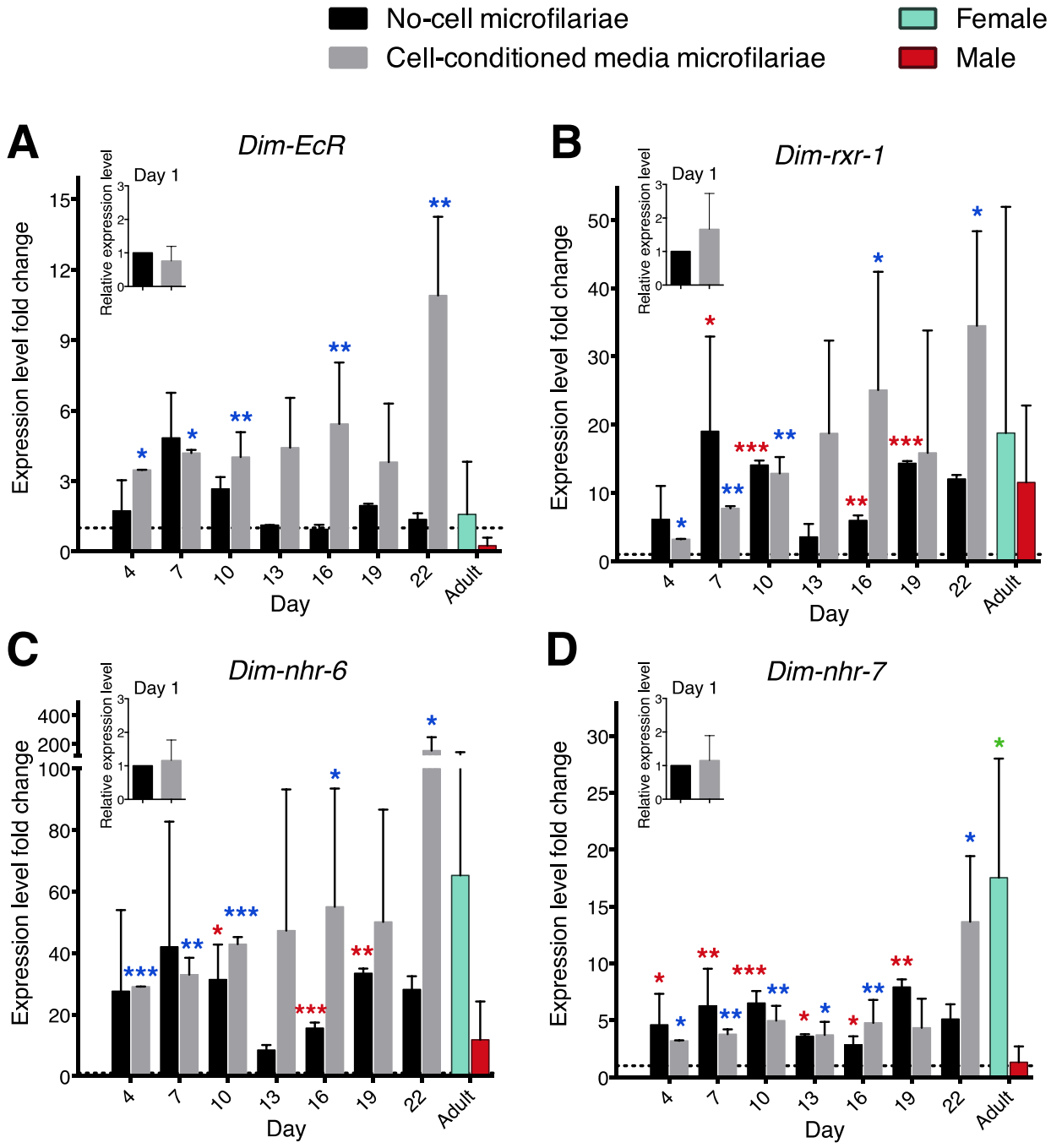


***Figure 13*. Transcript level of the ecdysone signaling system in *D. immitis* using ddPCR.** Fold changes for both no-cell and cell-conditioned media groups were calculated relative to day 1 of each medium. Fold changes for adults were calculated relative to day 1 of the cell-conditioned media group. Insert figures show transcript level at day 1 for both media. All transcript levels were normalized to three reference genes: *Dim-GAPDH*, *Dim-Actin*, and *Dim-β-*

tubulin. (A) *Dim-EcR* (B) *Dim-rxr-1* (C) *Dim-nhr-6* (D) *Dim-nhr-7*. For days 1, 4, and 7 of no-cell groups and days 1 and 16 of cell-conditioned media groups, three different blood samples were collected from canine #2 (MO strain) and shipped on different days, with two individual ddPCR replicates performed on each sample. For days 10, 16, and 22 of no-cell groups and days 7, 10, 13, 19, 22 of cell-conditioned media groups, two different blood samples were collected from canine #2 (MO strain) and shipped on different days, with two individual ddPCR replicates performed on each sample. For days 13, 19 of no-cell groups and day 4 of cell-conditioned media group, one blood sample was collected from canine #2 (MO strain), with two individual ddPCR replicates performed. Three individual parasites were obtained and analyzed for both female and male adult groups (Kentucky strain) and two individual ddPCR replicates were performed on each sample. All data reported as means with SD, using all of the replicates described for each procedure. Asterisks denote statistical difference between no-cell microfilariae (*), cell-conditioned media microfilariae (*), adults (*) compared to Day 1 groups (---). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis performed using unpaired t-test with Welch's correction, two-tailed p value, and 95% confidence interval.

5. Discussion

As very little literature has reported the in vitro development of *D. immitis* microfilariae, the current project sought to understand the developmental regulation underlying the growth of *D. immitis* microfilariae under in vitro conditions that sought to promote development as similar as possible to that in its mosquito vector. This study hopes to find an alternative way to truncate the life cycle of *D. immitis* so that eventually the increasing problem with drug resistance and the spread of dirofilariasis could be addressed by new approaches.

Extracted microfilariae were found to undergo significant morphological changes when cultured at 26°C in some of the commercially available culture media. Initially, Schneider's insect medium was hypothesized to provide the best in vitro condition for the parasites as the medium is designed to mimic the body fluid of insects, specifically for the culture of *Drosophila* cells and tissues. It was thus a surprise to see that the media was poor at sustaining both the development and motility of *D. immitis* microfilariae. Upon inspection of the media formulation, Schneider's insect medium was found to contain higher levels of intermediate compounds found in the citric acid cycle, including α-ketoglutaric acid, malic acid, fumaric acid, and succinic acid, while lacking in B vitamins like biotin, niacinamide, riboflavin, thiamine, and cobalamin. As some filarial nematodes have low levels of activity of enzymes in the citric acid cycle [5, 6, 95], it is possible that intermediates of the citric acid cycle do not contribute significantly to the overall energy metabolism of the parasite, and that the lack of B vitamins is detrimental to the growth and motility of the microfilariae. RPMI 1640, like DMEM and Ham's F-12, is a medium suitable for mammalian cell culture. However, compared to DMEM and Ham's F-12, RPMI 1640 is comparatively lower in the variety of inorganic salts and lacks in sources of certain ions, such as copper, iron, and zinc. This could possibly explain why *D. immitis* microfilariae did not grow or remain motile during the in vitro incubation in this medium.

It is interesting to note that when microfilariae were incubated in DMEM, parasite motility was maintained but there was no initiation of development, while incubation in Ham's F-12 was beneficial to development but motility was compromised. When comparing their formulations, DMEM was found to be high in the levels of glucose, amino acids, and vitamins, while Ham's F-12 contains a wide variety of components not found in other culture media, including putrescine, polyunsaturated fatty acid (linoleic acid), organosulfur compound (lipoic acid),

purine (hypoxanthine) and pyrimidine (thymidine). The difference in the composition of DMEM and Ham's F-12 may be what causes the difference seen with the in vitro culture results. When mixed at a 1:1 ratio, DMEM's high concentration of glucose, amino acids, and vitamins, as well as Ham's F-12's diversified components together provided a condition that supported early development and motility of *D. immitis.*

One of the limitations of the experiment was that the FBS that were used were not tested for endotoxins. However, the fact that the presence of FBS in the culture inhibited the growth of the parasite can be understood, as the addition of FBS would imitate a mammalian environment and since microfilariae are known to circulate in the dog blood for up to 2.5 years in a state of developmental arrest, no growth would be anticipated. Similar results were seen when serum was collected from infected dog blood and added to the microfilariae cultures (data not shown). Together these show that components of the serum could be inhibitory to the initiation of the parasite development. A possible inhibitory component of the serum could be mammalian cholesterol derivatives, such as cholestenoic acid, as the presence of mammalian cholesterol metabolites would imitate the mammalian host environment, and also because cholesterol derivatives have been found to contribute to the molting of *D. immitis* L3, a stage that develops inside the mammalian host (Long, T. et al., in press). However, it remains unclear why the motility of the parasites in the presence of FBS was reduced, and whether or not this is reflective of reduced vitality still needs further evaluation.

As mosquitoes in the genus *Anopheles* are also possible vectors of *D. immitis* [7, 8, 15], cells of *An. gambiae* were introduced to microfilariae cultured at 26ºC to mimic the environment of the arthropod host. The *An. gambiae* cell line 4a-3B was established from neonate larvae of the *An. gambiae* 4a r/r strain, although the tissue of which these cells originated from is unknown. The presence of cells as a monolayer underneath the parasites greatly increased the number of sausage and late sausage stage parasites found in the culture. When only *An. gambiae* cell-conditioned media was used, the number of sausage and late sausage stage larvae was also significantly higher than those that were not cultured with cells. This suggests that factors secreted by the mosquito cells are important to the developmental regulation of the parasite. Also, it is possible that the parasites cultured in the presence of physical cells developed into fewer numbers of sausage stage compared to those cultured in cell-conditioned media because the uptake of components by cells diminishes the available nutrients that could be utilized by the

parasites, thus affecting the number of parasites that developed into the later stages. To determine which component is responsible for the initiation of growth of the microfilariae, the next approach would be to do a fractionation of the protein, lipid, and metabolite content of the cell-conditioned media using liquid chromatography mass spectrometry-based proteomics, lipidomics, and metabolomics.

Due to the limitation of the number of *D. immitis*-infected dog blood samples that could be obtained every week, a shortcoming of this study is the number of replicates performed for each experiment. The conditions upon which the parasites were shipped was also a critical determinant that affected the number of experiments that could be done. Because there had been some delays in some of the shipments, due to weather constraint, some of the parasites came in undesirable conditions and could not be used for experimentation. In addition, even though the presence of *An. gambiae* cell-secreted factors enhanced the growth of *D. immitis* microfilariae in vitro, the low percentage of late sausages and the increased level of degenerated larvae at the end of the incubation period indicate that the optimal condition for in vitro culture of *D. immitis* microfilariae still requires further assessment. The effects of mosquito gastric juice and fluctuation in pH have been considered for future studies to better understand how the parasite senses a change in the environment as it travels from the mosquito pharynx to the midgut, and later reaching the Malpighian tubules [9]. In addition, it would be interesting to see if media conditioned with mammalian cells would still have the same developmental effects seen with the results of this study.

It is important to note that from the in vitro culture results, only parasites that developed into the next stage remained healthy looking and went on to develop into more advanced stages, a pattern that could be seen in all experimental conditions. To determine if the larvae that became more developed were early L2 larvae, SEM analysis was used to examine whether the late stage larvae had visible stoma openings, however, the resolution of the images was not high enough to make a definite conclusion. A major issue that was encountered during the SEM imaging of the samples was sample loss during the dehydration process with the critical point dryer, because the increase in pressure during the drying process often caused sample loss. As late sausages are usually present in the culture at lower levels, chemical drying with HMDS was used instead as an alternative dehydration approach to increase sample recovery rate. Critical point drying has been the established method used for drying of delicate samples for SEM analysis because it is

based on the physical principle of critical temperature and pressure. When water in the biological sample is replaced by liquid CO_2, and the temperature and pressure is raised above the critical point, liquid CO_2 changes to the gaseous state without a change of density. As a result, it bypasses the surface tension effects during the phase change and is able to preserves the surface structure of a specimen [96]. However, images in this study show that samples dried with HMDS were also well-formed and there seems to be little differences in terms of the ultrastructure between images taken with the two methods [97].

To overcome the problem of low resolution with the SEM analysis, live microfilariae were imaged over time with the IncuCyte ZOOM system to observe any possible ecdysis during development. From the IncuCyte video, it is visible that there was a separation of the outer cuticle as the parasite transitioned from the pre-sausage to sausage stage; however, there was no actual shedding or ecdysis of the outer cuticle, and so it is concluded that the late sausage stage parasites are still considered to be in the late L1 stage. An alternative approach to determine if the late sausage stage parasites could be in the early L2 stage would be to incubate the parasites in the late sausage stage in dyes such as Trypan blue and to observe if the parasites could actively uptake Trypan blue by the oral route [98], which would indicate the presence of an open stoma that is characteristic of the L2 larvae [10]. Because microfilariae and early L1 stages do not possess a functional digestive tract, uptake of nutrients and/or dyes would probably be via the cuticular route [5].

Although ecdysteroids were found to have significant effects on the molting of L3 larvae of *D. immitis* in previous literature, no effects were seen when the microfilariae were treated with a high concentration of ecdysteroids in the present study. No significant morphological change was observed even after increasing the frequency of ecdysteroid treatment to account for the short half-life of the steroid hormones, nor was any significant development detected after delaying the treatment to day 10 of incubation. The rationale for delaying the ecdysteroid treatment to day 10, which happens to be when the level of sausage stage was at its highest, was that early treatment could be detrimental to the growth of the parasite, and because inducing growth beyond the sausage had been difficult, a delayed treatment could possibly be beneficial to the parasite's development and help it bypass the sausage stage. The same results were seen when ecdysteroids were added to the microfilariae culture at day 6 of incubation, which happened to be when sausage stage parasites started to appear (data not shown). Together these

indicate that ecdysteroids do not seem to play an important role in the development of the *D. immitis* microfilariae.

Surprisingly, when microfilariae were collected for ecdysone signaling system transcript-level analysis, the transcript level for the four target genes, *Dim-EcR*, *Dim-rxr-1*, *Dim-nhr-6*, *Dim-nhr-7*, were all upregulated in developing L1 larvae. A reasonable explanation could be that the ecdysteroid treatment condition carried out in this study was not optimal and that the chosen concentration, as well as the treatment timing were not enough to induce significant changes. *Anopheles gambiae* cells, on the other hand, may have secreted potent non-steroidal ecdysone agonists [99, 100] that could activate Dim-EcR, resulting in the upregulation of the transcript level that was observed. Another possibility is that Dim-EcR may require an activated Dim-RXR to form an active dimer and to bind to ecdysteroids, as a study on *D. immitis* EcR showed that Dim-EcR seems to form an active dimer with RXR only in the presence of 9-*cis* retinoic acid, an RXR-specific ligand [75]. It is thus possible that *An. gambiae* cells secreted factors that activated both Dim-EcR and Dim-RXR and so significant morphological changes were observed when parasites were cultured with *An. gambiae* cells or cell-conditioned media, and a significant increase in the transcript levels of the ecdysone signaling cascade components in developing microfilariae was noted. For future research, it would thus be fascinating to see if the addition of ecdysteroids with RXR-specific ligand could induce the development of the parasites to more advanced stages.

A closer look at the transcript level for all four target genes shows that the transcript expression of the ecdysone signaling cascade tend to be higher in microfilariae cultured in *An. gambiae* cell-conditioned media, especially towards the end of the 22-day incubation period. This can be understood because in the presence of cell-secreted factors, higher numbers of microfilariae could be found advancing to later stages at the end of the incubation, and so a greater upregulation of the ecdysone signaling system indicates that activation of the EcR may play an important role in the development of the microfilarial stage. In terms of the pattern of the transcript expression for all four genes, a gradual increase in the first few days could be seen for both microfilariae cultured in the absence of cells and those cultured in *An. gambiae* cell-conditioned media. A peak expression for all four genes was observed at day 7 for microfilariae cultured in the absence of cells, corresponding to peak levels of pre-sausages stage parasites in the culture. A drop at day 13 was then observed before a second peak at day 19, coinciding with

the emergence and peak levels of sausage stage parasites observed when microfilariae were incubated with just DMEM/Ham's F-12.

On the other hand, transcript level of microfilariae cultured in *An. gambiae* cell-conditioned medium rose gradually over time, with a final sharp peak observed on day 22 for all four genes, corresponding to the gradual development of the parasites into late L1 stages observed in vitro. The fact that the largest peak was seen when the number of late sausage stage parasites was at its highest suggests that the upregulation of the ecdysone signaling system is required for the development of the parasite into later stages.

Another interesting thing to note is that even though all four genes of the ecdysone signaling cascade targeted in this study displayed the same pattern of expression over time, the overall transcript level of *Dim-nhr-6* in developing microfilariae was significantly higher than those of the other three genes. This suggests that the EcR/RXR complex possibly upregulates the mRNA expression level of *Dim-nhr-6* to a much higher extent than *Dim-nhr-7* and that *Dim-nhr-6* might be a crucial downstream target of EcR.

Older literatures mentioned that expression of the ecdysone cascade members displays a sex-specific pattern in the adult stage because they were only detectable in female parasites. However, results from this study showed that even though mRNA levels of adult male *D. immitis* were lower than those of the females for all the components of the ecdysone signaling cascade, the transcript expression levels in males were still detectable [75-78]. This could be due to the high sensitivity of ddPCR, compared to the Northern blot analysis employed by previous studies. In addition, the fact that the expression level of *Dim-EcR* and *Dim-rxr-1* in females were relatively lower than the levels of *Dim-nhr-6* and *Dim-nhr-7* detected suggests that in adult females, low expression of EcR/RXR can induce a high transcription level of the downstream genes.

Published literature on *Drosophila* mentions that EcR has three isoforms and that the isoforms displayed distinctive spatial expression patterns during the onset of metamorphosis [101], with EcR-A found mostly in larval tissues, and EcR-B1 found mainly in cell clusters that will eventually generate adult tissues. As *Dim-EcR* and *Dim-nhr-7* have also been reported to have three isoforms [75, 77], it would thus be interesting for future studies to see if the transcript level of the different isoforms shows any stage-specific pattern in developing microfilaria.

6. Conclusion

As the problem with drug resistance is posing a great obstacle to the continued prevention of heartworm infection, a better understanding of the microfilaria stage of *D. immitis* might provide new opportunities to disrupt the life cycle of the parasite. This study sought to understand the developmental regulation of *D. immitis* microfilariae as well as how the mosquito host environment and ecdysteroids play a role in the growth of the larvae.

In this study, *D. immitis* microfilariae developed into late L1 parasites that could be classified as pre-sausage, sausage, and late-sausage stages. These late L1 stages differed considerably in their morphological appearances. Among the commercially available culture media tested, DMEM and Ham's F-12 mixed at a 1:1 ratio provided a relatively favorable condition for the in vitro cultivation of microfilariae, although the presence of FBS in the media was shown to impede parasite growth and motility. The addition of *An. gambiae* cells to the microfilariae culture further induced the early appearance of the sausage and late sausage stage parasites. On further testing, the media conditioned with *An. gambiae* cells also had a similar effect, indicating that factors secreted by the mosquito cells offer conditions similar to the environment in the mosquito and promote faster microfilarial growth. Ecdysteroids, or insect molting hormones, were found to exert biological effects on the molting and embryogenesis of the third-stage larvae and adults in previous studies, but were found to have no effects or negative effects on the development of the microfilariae in the present research. Transcript-level analysis of the ecdysone signaling system showed that components of the ecdysone cascade were upregulated in developing microfilariae and particularly in those cultured in *An. gambiae* cell-conditioned media.

The results from the present study demonstrate that factors from insect cells play a role in the development of *D. immitis* microfilariae, and that the ecdysone cascade may contribute to the morphological changes seen with cultured microfilariae. However, they also bring out many unanswered questions. For future follow-up research, we would like to determine (1) the component(s) in the insect cell-conditioned medium which promote microfilariae development, (2) component(s) in the FBS which inhibit development of the larvae, and (3) whether the *Nhr-8*/*Daf-12* nuclear hormone pathway, which is also present in *D. immitis,* (Long, T. et al., in press) may influence development of the microfilaria stage.

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