Elucidation of *Dirofilaria immitis* microfilarial development and its regulation using an *in vitro* model

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Abstract

Dirofilaria immitis heartworm infection is a serious and potentially fatal disease that affects companion animals. Macrocyclic lactone (ML) preventives target infective larval stages, preventing disease; however, ML resistance has been demonstrated, prompting a search for novel ways to interrupt the *D. immitis* life cycle.

As *D. immitis* microfilariae (mf) undergo developmental arrest inside the mammalian host but commence development and molting once ingested by the mosquito vector, this study aimed to understand the developmental regulation of *D. immitis* mf in the arthropod and mammalian hosts, which could lead to potential new targets for disease prevention. Previous *in vitro* studies showed that culture media conditioned with *Anopheles gambiae* cells provides a favourable growth environment, whereas Fetal bovine serum (FBS) inhibited mf development. Mf treated with 20-Hydroxyecdysone, a known activator of the ecdysteroid system, upregulated genes involved in the ecdysteroid pathway. In our study, the addition of charcoal-stripped FBS and 20-Hydroxyecdysone to *An. gambiae* conditioned media accelerated the early growth of mf to the late sausage stage. This suggests that the ecdysteroid pathway may play a role in the early development of the mf and a lipid factor in FBS may inhibit development because charcoal stripping removes lipids from FBS. A DAF 12 orthologue receptor with high sensitivity for dafachronic acids was recently discovered in *D. immitis.* The addition of 10 µM Δ4-dafachronic acid, a metabolite of cholesterol, significantly increased early microfilarial degeneration and prevented development.

Using untargeted UPLC-Fourier transform mass spectrometry, we analysed metabolomic changes in Mosquito conditioned media (MSCM) and MSCM supplemented with FBS (MSCMFBS). We found 174 metabolites in MSCM/MSCMFBS to be statistically significantly

different. Pathway analysis showed that Arachidonic acid pathways, amongst several others, were differentially enriched in MSCM. Various Vitamin D analogues were identified to be differently enriched in MSCM compared to MSCM supplemented with FBS. Several metabolites related to either the development or degeneration of *D. immitis* mf were identified to be differentially enriched in MSCM and MSCM supplemented with FBS, respectively.

This study advances our understanding of the interaction between *D. immitis* mf and its hosts, which may lead to the development of novel heartworm control strategies in the future.

Résumé

L'infection par le ver du cœur *Dirofilaria immitis* est une maladie grave et potentiellement mortelle qui affecte les animaux de compagnie. Les préventifs à base de lactone macrocyclique (LM) ciblent les stades larvaires infectieux, prévenant ainsi la maladie; cependant, la résistance aux LM a été démontrée, incitant la recherche de nouveaux moyens pour interrompre le cycle de vie de *D. immitis.*

Comme les microfilaires de *D. immitis* (mf) subissent un arrêt de développement à l'intérieur de l'hôte mammifère mais commencent à se développer et à muer une fois ingérées par un moustique vecteur, cette étude visait à comprendre la régulation du développement des mf de *D. immitis* chez leurs hôtes arthropodes et mammifères, ce qui pourrait mener à de nouvelles cibles potentielles pour la prévention de la maladie. Des études *in vitro* antérieures ont montré que les milieux de culture conditionnés avec des cellules d'*Anophèles gambiae* fournissent un environnement de croissance favorable, tandis que le sérum bovin fœtal (SBF) inhibe le développement du mf. Les mf traitées avec le 20-Hydroxyecdysone (20E), un activateur connu des ecdystéroïdes, ont régulé à la hausse les gènes impliqués dans la voie de signalisation des ecdystéroïdes. Dans notre étude, l'ajout de 20-E et de SBF préalablement traité avec du charbon au milieu conditionné d'*A. gambiae* a accéléré la croissance précoce de mf jusqu'au stade de « saucisse tardive ». Cela suggère que la voie de signalisation des ecdystéroïdes peut jouer un rôle dans le développement précoce du mf et qu'un facteur lipidique dans le SBF peut inhiber le développement parce que la déplétion du SBF avec du charbon élimine les lipides du milieu. Un orthologue de DAF 12, un récepteur très sensible aux acides dafachroniques, a été récemment découvert chez *D. immitis*. L'ajout de 10 μM d'acide Δ4 dafachronique, un métabolite du cholestérol, a augmenté de manière significative la dégénérescence microfilarienne précoce et a empêché le développement.

En utilisant la spectrométrie de masse à transformée de Fourier UPLC non ciblée, nous avons analysé les changements métabolomiques dans le milieu conditionné des moustiques (MCM) et le MCM supplémenté en SBF (MCMSBF). Nous avons trouvé 174 métabolites dans le MCM/MCMSBF qui sont statistiquement significativement différents. Cette analyse a démontré que les métabolites impliqués dans le métabolisme de l'acide arachidonique, entre autres, étaient différentiellement enrichis dans les MCM. Divers analogues de la vitamine D ont été identifiés comme étant différemment enrichis dans les MCM par rapport aux MCM supplémentés en SBF. Plusieurs métabolites liés au développement ou à la dégénérescence des mf de *D. immitis* ont été identifiés comme étant différentiellement enrichis dans les MCM et les MCM supplémentés en SBF, respectivement.

Cette étude nous permet de mieux comprendre l'interaction entre *D. immitis* mf et ses hôtes, ce qui pourrait conduire à l'avenir au développement de nouvelles stratégies de lutte contre les vers du cœur.

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Contribution to original knowledge

This study significantly contributes to the existing knowledge on the developmental regulation of *Dirofilaria immitis* microfilariae and the factors influencing their growth. By investigating the impact of culture media composition, including the conditioning with mosquito cells and the presence of fetal bovine serum, valuable insights are gained for optimizing the *in vitro* culturing of microfilariae. The identification of metabolomic profiles and differentially expressed metabolites provides a deeper understanding of the underlying mechanisms governing microfilariae development and sheds light on the stimulatory and inhibitory effects of specific components, such as hormones derived from 7-dehydrocholesterol, metabolites from porphyrin metabolism etc.

These findings shed light on the effects of the intricate interplay between ecdysteroids, Dafachronic acids and their signalling pathways on microfilariae growth. Thus, advancing our knowledge and contributing to the development of innovative strategies for combating heartworm infection and addressing the challenges posed by drug resistance.

Contribution of authors

All parts of this manuscript were written by Priyal. Revisions and corrections were provided by supervisor Dr. Roger Prichard.

All experiments and analysis presented in this manuscript were performed by Priyal.

Abbreviations

20-E	20-Hydroxyecdysone
AHS	American Heartworm Society
ARA	Arachidonic acid
CA	Cholestenoic acid
CDC	Center for Disease Control and Prevention
COX	Cyclooxygenase
CS-FBS	Charcoal-stripped Fetal bovine serum
DA	Dafachronic acid
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EcR	Ecdysone receptor
EcRE	Ecdysone-response element
FAs	Fatty acids
FBS	Fetal bovine serum
FC	Fold change
FR3	Filariasis Research Reagent Resource Center
FTMS	Fourier transform mass spectrometry
GluCls	Glutamate-gated chloride channels
GSEA	Gene Set Enrichment Analysis
HP-β-CD	2-Hydroxypropyl-β-cyclodextrin
HREs	Hormone response elements
IVM	Ivermectin

KEGG	Kyoto encyclopedia of Genes and Genomes
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
LBD	Ligand-binding domain
LM	Lactone macrocyclic
LOE	Loss of effectiveness
LOX	Lipooxygenase
LXR	Liver X Receptor
MCM	Milieu conditionné des moustiques
MCMSBF	MCM supplémenté en SBF
MD	Melarsomine dihydrochloride
MF	Microfilariae
ML	Macrocyclic lactone
MO	Missouri
MSCM	Mosquito conditioned media
MSCMFBS	MSCM supplemented with fetal bovine serum
MSCMFBSCS	MSCM supplemented with charcoal-stripped fetal bovine serum
NCM	Non-conditioned media
NCMFBS	NCM supplemented with fetal bovine serum
NCMFBSCS	NCM supplemented with charcoal-stripped fetal bovine serum
NHR	Nuclear hormone receptor
NRs	Nuclear Receptors
PBS	Phosphate-buffered saline
PC	Principal component

Pgp	P-glycoprotein
PIX	Protoporphyrinogen IX
PLA_2	Phospholipase A2
PLS-DA	Partial least square discriminant analysis
RXR	Retinoid X Receptor
SBF	Sérum bovin fœtal
SNP	Single nucleotide polymorphism
UPLC	Ultra Performance liquid chromatography
USP	Ultraspiracle protein
VDR	Vitamin D Receptor
VIP	Variable importance in projection

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1 Introduction

1.1 Heartworm infection: *Dirofilaria immitis*

Cardiac dirofilariasis is caused by the parasitic worm *Dirofilaria immitis* in both domestic and wild hosts. All around the world, cases of heartworm infection have been reported. Most of the world's temperate, tropical, and subtropical regions are home to this parasitic illness [1]. Although there are other *Dirofilaria* species, *D. repens, D. tenuis,* and *D. immitis* are the three species that most frequently cause infection. Their primary natural hosts are canines and other wild canids like foxes and wolves. Cats are often hosts and sometimes produce mf.

Dirofilaria causes heartworm infection in canids, where the parasite is spread through a plethora of mosquito species, which host an intermediate stage to complete its life cycle. Some of the mosquito genera include *Aedes spp, Culex spp and Anopheles spp* [1]. Heartworm infection is a potentially fatal illness. Adult worms obstruct pulmonary circulation, harming the lung and, finally, the right chambers of the heart [2]. Heartworm infections are very likely to affect canids, irrespective of their age. Most infected animals show no obvious clinical symptoms or aberrations in lab tests. Canids suffering from moderate to severe heartworm disease show respiratory distress, nosebleeds, hemoptysis, ascites, intolerance to physical activity, loss of weight, cough, exhaustion, dry coat and, in severe cases, fatality [3]. Feline infection is identified more commonly in areas of high prevalence, but the parasite takes longer to show in cats than in dogs. In addition, a lot of cats tolerate the illness with no obvious clinical signs or with symptoms that only last a short period, and occasionally unexpected death can occur without warning [4] [5].

The presence of a climate providing optimal temperature and humidity levels is a key need for transmitting heartworms. This climate is necessary for mosquito populations to remain viable and for the infective third-stage larvae (iL3) to develop from microfilariae in the intermediate host. The spread of the parasite is aided by the movement of microfilariae-carrying dogs and the movement of microfilariae-carrying wild canids into new areas, both locally and internationally. This spread is aided by the availability of competent mosquito vectors, which are found in areas with favourable climatic circumstances. Changes in any of these factors, such as changes in climatic patterns or the addition of new reservoirs, can have a significant impact on the potential for disease transmission in certain geographic areas [6].

According to new statistics from the American Heartworm Society (AHS), heartworm prevalence is increasing in several parts of the United States **(see Figure 1).** The organization's recently updated 2022 Heartworm Incidence Map demonstrates rising patterns in previously identified heartworm "hot spots" and locations where these parasites were once uncommon [6, 7].

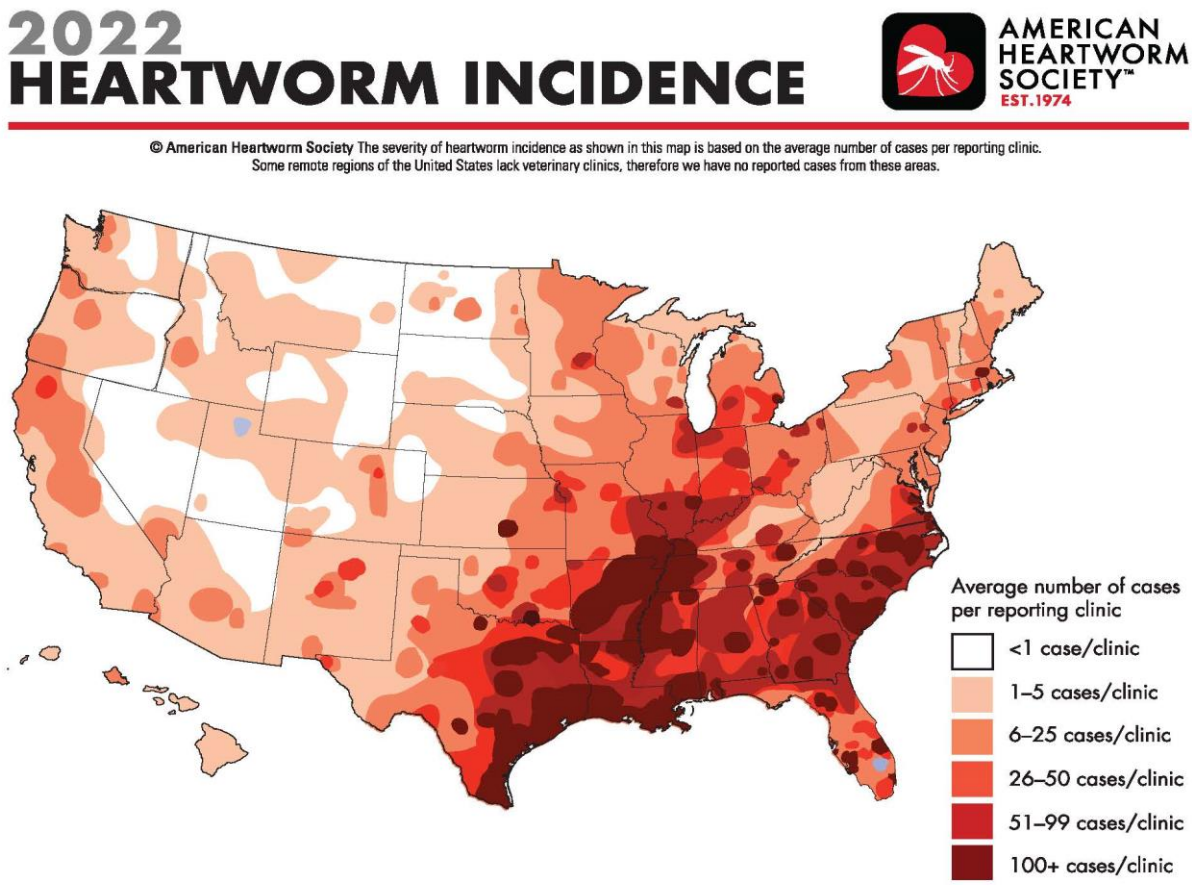


Figure 1. **Heartworm incidence survey 2022.** Reprinted from American heartworm society (AHS) [6].

1.2 *D. immitis* Biology and Life Cycle

Compared to most parasitic nematodes, *Dirofilaria immitis* has a rather extended life cycle (often 7 to 9 months from infection to production of microfilariae) [6]. This life cycle requires a mammalian host that is susceptible to infection from an arthropod vector; domestic dogs and some wild canids have a proclivity to have high parasitaemia, due to which they serve as a reservoir for heartworm infections. These are also the most common definitive hosts for heartworms. Low-level, transient microfilaremia may occasionally develop in unsuitable hosts, such as cats and ferrets, and may serve as a source of mosquito infection for an insignificant period of time [5].

When mosquitoes take a blood meal from a *D. immitis*-infected mammal, the microfilariae mature into third-stage larvae (L3) in their Malpighian tubules [6]. It is critical to realise that before microfilariae develop into infectious stages, they must first molt through larval stages L1 to L3 in the mosquito [7].

The mosquito's mouth and head are where the iL3 larvae are subsequently transferred for transmission after passing through its body cavity. Temperature has an impact on how long microfilariae take to reach the infectious stage within a mosquito. At 80% relative humidity and temperature of 27°C, the early L1 stage larvae molt into iL3 stage larvae in two weeks; maturation of the parasite slows when temperatures are lower [6, 7].

Infection is spread when an infected mosquito takes another blood meal when the iL3 stage larvae are in the mosquito's proboscis, allowing transmission of the parasite. These larvae enter the mammal’s body through the mosquito's puncture site just after the blood meal. The iL3 larvae molt into fourth-stage larvae (L4) between days 9-12. Inside the infected mammal, they

migrate between the subcutaneous tissues and muscle fibres. Juvenile/immature adults go through their last molt. As they pass through the body between days 50 and 70, they eventually enter the circulatory system and travel to the heart and lungs [8, 9].

These juvenile worms start entering the pulmonary vasculature by day 67 and, after approximately 90-120 days, have all reached the location. When these young heartworms initially enter the pulmonary arteries, they are 25 to 38 mm long. Female heartworms grow to be 25 to 31 cm in length, and 1 to 1.3 mm in diameter as they mature [12,13]. Sexual maturity develops around day 120 after infection. Female adult worms begin to produce microfilariae in the host's blood; they can produce over 11,000 microfilariae each day. These microfilariae can remain latent or in an arrested state of development in the host peripheral system for more than two and a half years [8, 9].

Patent infections (i.e., having circulating microfilariae) in dogs can develop as soon as 6 months after infection [8-11]. Juvenile heartworms are forced into the tiny pulmonary arteries when they first enter the lungs by the blood flow [12]. The worms eventually take up more and larger portions of the arteries as they mature until they are fully developed. The size of the dog and the number of worms will ultimately determine where the mature worms are located. In a medium-sized dog (such as a Beagle) with a low worm burden, the main pulmonary artery and lobar arteries are generally where the majority of worms are located. Worms can be identified in the right ventricle as the worm burden rises. When worms reach the right ventricle, right atrium, and vena cava and disrupt blood flow and valve function, a dog is more prone to develop caval syndrome, as a result of which hemolysis, liver and renal disease, and heart failure occur [10, 13, 14].

D. immitis in the adult form are thin and whitish in appearance, with a disk-like mouth shape that is encircled by six ambiguous papillae. Males have the typical filarioids' loose spiral tail and are 12-20 cm in length and 700-900 µm broad [8, 9]. The left and right spicules are of different lengths, measuring 300–375 µm and 175-229 µm, respectively [12]. There doesn't seem to be a gubernaculum present. The front end of the vulva is 2.5 mm long and has an obtuse posterior in female worms.

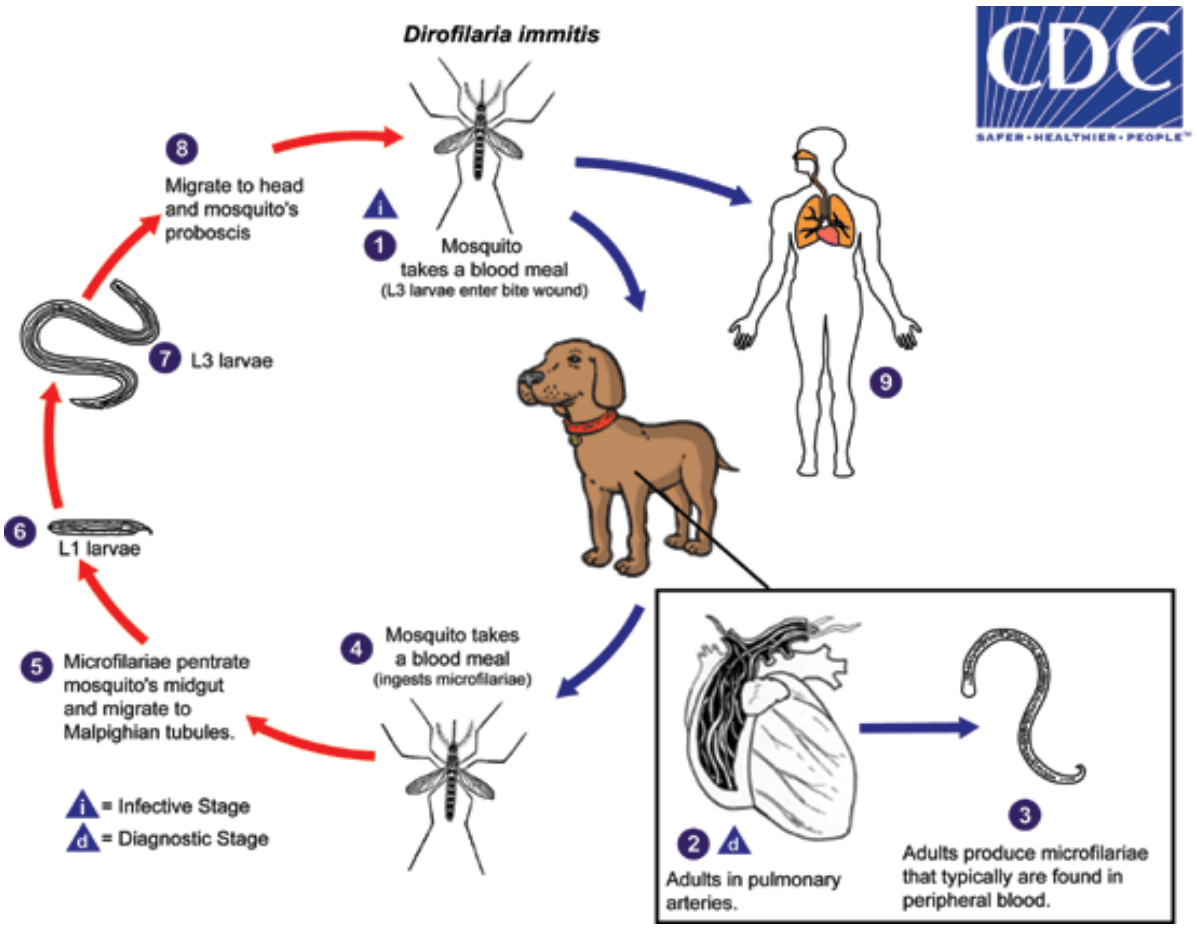


Figure 2. **Life cycle of *Dirofilaria immitis*.** Reprinted from Centers for Disease Control and Prevention (CDC) [15].

1.3 Prevention against dirofilariasis: Macrocyclic Lactones

Heartworm infection is a potentially lethal parasite disease that affects both pets and wild canids [16-19]. Macrocyclic lactone (ML) endectocides, which are FDA-approved heartworm preventives, have been utilized for more than 30 years to keep dogs and cats free of heartworm disease. MLs include ivermectin (IVM), milbemycin oxime, moxidectin, and selamectin, and they are thought to function in tandem with the dog's immune system to destroy sensitive larval stages [20-22].

Heartworm prevention is advocated year-round by the American Heartworm Society (AHS) and FDA, and it is recommended to commence no later than eight weeks of age [6]. These medications target third- and fourth-stage larvae, impeding their transformation into adult parasites as well as microfilariae. They are typically administered orally or topically on a monthly basis to dogs [22]. However, their efficacy against late fourth-stage larvae diminishes and becomes unpredictable beyond this timeframe [23]. Juvenile worms, which can appear as early as 52 days after infection, exhibit lower susceptibility to preventive measures. As a result, longer-term treatment becomes increasingly necessary to obtain a high level of protection as the worms age [24].

All dog breeds can safely use ML preventives at recommended dose rates [16]. Monthly preventives are administered orally or topically. Continuous year-round prophylaxis is recommended in the USA. In other jurisdictions, seasonal treatment is recommended beginning one month prior to the heartworm transmission season. Treatment duration can extend up to 6 months after the transmission ends. Topical products align with monthly oral preventives. For long-lasting protection, a single subcutaneous injection of slow-release moxidectin provides 6 months of continuous efficacy. Additionally, a novel formulation of moxidectin allows for administration every 12 months, enhancing compliance and convenience [6].

The recommended dose rate for IVM as a preventive measure against heartworm infection in dogs is typically 6-12 µg/kg body weight [25]. At slightly higher dose rates, MLs also have microfilaricidal effects and reduce the reproductive capacity of adult parasites [25]. Milbemycin oxime (MO) at a dose of 0.5 mg/kg orally is approved for use in dogs, including heartworm prevention [25]. In 2000, selamectin received approval as a topical treatment for heartworm prevention, flea control, and the treatment of other parasites in dogs and cats. When applied topically, the recommended dose for selamectin is 6 mg/kg [25]. To eliminate adult

heartworms, melarsomine dihydrochloride (MD) is commonly used. It is given via intramuscular injection at a dose of 2.5 mg/kg body weight, with two additional doses given 24 hours apart at the same dose rate, effectively killing approximately 90% of adult parasites. Pre-treatment with doxycycline to remove Wolbachia is now recommended [6].

MLs exert their action by binding to glutamate-gated chloride channel receptors (GluCls). The channels continue to be in an open conformation as a result of this interaction, allowing chloride ions to enter. Against *D. immitis* larvae, it is thought that MLs act on the excretory pore, affecting the release of immune modulators [21, 25]. This leads to the immune mediated death of the larval worms.

It is crucial to conduct diagnostic screening for heartworm infection in older dogs prior to prescribing MLs. This is because administering MLs to dogs already infected with adult heartworms can potentially lead to the demise of the dog [24].

In order to confirm the presence of adult heartworm infection, microfilariae can only be discovered in a dog's circulation about six months following the infected mosquito bite. To detect *D. immitis* infection, the most common test used by veterinarians is a filarial antigen test. This test is simple to perform but requires the presence of a mature female worm, as the antigen detected is only produced by mature female worms. The microfilarial count and Knott's tests are more time-consuming and require some experience and thus are usually used to confirm the antigen test [26]. It is important to note that false-negative antigen test results can occur due to antigen blocking caused by antigen-antibody complexes in some infected dogs. Studies have reported a 7.1% occurrence of this phenomenon in shelter dogs, where they test negative for antigens but may still be positive for microfilariae [20]. ELISA and immunochromatographic tests are available for detecting heartworm antigen and have

demonstrated high clinical utility. However, a limitation of microfilaria testing is the occurrence of "occult infections," where some cases may test positive despite the absence of circulating microfilariae [26]. The latest generation of antigen tests can detect most "occult" infections involving mature female worms [21-23]. However, sensitivity can vary in cases with low worm burdens or antigen levels. Currently, there are no validated assays available to specifically identify infections with mature male worms.

In conclusion, a combination of preventive medications, regular testing, and appropriate diagnostic techniques is crucial in managing heartworm disease in dogs. Adhering to recommended protocols and seeking confirmation when necessary help ensures accurate diagnosis and effective treatment.

1.4 ML resistance

In 2005, Hampshire reported cases of ML efficacy loss in *D. immitis*. In the following years, more investigations revealed ML resistance [19, 24, 27]. Genetic variations between susceptible and resistant strains were found through molecular analysis. The P-glycoprotein (P-gp) family of ABC transporters, specifically, has single nucleotide polymorphisms (SNPs) at positions 11 and 618 that have been identified as potential causes for the loss of effectiveness (LOE) of these MLs. Investigation revealed a strong correlation between the GG genotype at both Pgp fragment locations and the degree of IVM sensitivity, supporting the theory that ML-driven genetic selection could be responsible for Pgp SNPs having a high level of homozygosity. Pgp is recognised to play a role in the ML pharmacology [28, 29]. Additionally, it was established that these genetic alterations linked to resistance were heritable It is now

clear that the cause of ML resistance in *D.immitis* is not pgp alteration; these SNPs are associated with the trait but not casual [26].

For cases of LOE in heartworm treatment, owner non-compliance has been suggested as a possible alternative explanation. Researchers have suggested utilizing a 2–4-week microfilariae suppression test to distinguish between instances resulting from true ML resistance and those deriving from inadequate compliance [30, 31]. However, particularly in extensive epidemiological surveys, this test is not ideal for routine diagnosis of resistant heartworm patients.

To clearly confirm ML resistance, phenotypic resistance must be determined under controlled laboratory circumstances, and significant genetic differences from known susceptible isolates must be demonstrated. This method helps to establish ML resistance without any hint of uncertainty. In addition, creating a test based on molecular markers related to the resistant phenotype will substantially assist larger investigations in determining the geographic breadth of ML resistance. It would also guide the use of alternate heartworm preventive methods [26].

1.5 Developmental regulation-Nuclear hormone receptor (NHR)

The emergence of ML resistance has required the investigation of alternative heartworm infection control techniques. Understanding how *D. immitis* develops allows us to understand how the parasite grows through several developmental stages and maybe find treatment targets to disrupt its lifecycle.

Insects and nematodes, including *D. immitis*, exhibit developmental regulation controlled by nuclear receptors (NR), also known as nuclear hormone receptors (NHRs). The fundamental physiological processes of growth, development, ageing, reproduction, metabolism, and the detoxification of endo- and xenobiotics all depend on these receptors, also known as transcription factors [32-35]. Typically, they bind small lipophilic molecules, activating them and allowing co-regulators to be brought in to control the activation of particular genes [36]. Because of their critical function in vital metabolic processes such as development, NRs are appealing drug discovery targets [33].

NHRs typically comprise a conserved DNA-binding domain (DBD) at the N-terminal and a ligand-binding domain (LBD). They can bind to co-repressors and co-activators. The number of NHR-encoding loci can vary significantly among different genomes, with species like *D. melanogaster* having 18 NHRs, 48 NHRs in humans, and 248 NHRs in *C. elegans* [37-40]. Steroid hormones and other lipid-soluble signals activate NRs. The DBD of these proteins has substantially similar amino acid sequences, responsible for specific DNA binding and the various functions of the LBD, including the transcriptional activation, dimerization, and ligand binding [41, 42]. By detecting DNA sequences called hormone response elements (HREs), NRs regulate the transcription of genes that control a number of biological activities when they are activated [43]. While ligands have been identified for many NHRs, especially those in vertebrates, several receptors are considered "orphan" receptors as their ligands remain unknown. Moreover, some NHRs can function without ligands [37, 44].

D. immitis is a clade III filarial nematode and is a bit different from insects and clade V nematodes. But they all seem to have a cholesterol-dependent regulatory pathway, like the

ecdysone signalling pathway present in *D. melanogaster* or the DAF-12 signalling pathway in *C. elegans* [45], studies have shown their presence in *D. immitis* as well [46, 47].

1.5.1 Ecdysone signalling pathway

Arthropod development, survival, and reproduction depend on molting, a fundamental biological process. During molting, the old exoskeleton is split apart, and a fresh layer of the exoskeleton develops beneath. When the newly produced cuticle tans and the newly degraded outer layer sheds by ecdysis, the process is complete [41].

Ecdysteroids belong to the group of steroid hormones. They play a role in various developmental, molting, and reproductive processes. Among them, 20-hydroxyecdysone (20-E) is an ecdysteroid that plays a significant role in mediating physiological and behavioural changes during insect molting [42]. Ecdysteroids are derived from dietary cholesterol and are metabolized by the Halloween genes encoded enzymes called cytochromes P450. Among arthropods, the prothoracic gland is where ecdysone is primarily produced. It is then transformed to 20-E by the activity of 20-monooxygenase in the fat body, midgut, malpighian tubules, and ovaries. 20-E binds to the ecdysone receptor, a nuclear receptor consisting of two subunits: the EcR protein and the USP protein (known as ultraspiracle). In insects, the USP protein analogous to the mammalian retinoid X receptor protein (RXR), forming a complex with EcR for hormone binding [41-43]. According to the Ashburner model, the hormone receptor complex activation leads to the activation of early regulatory genes (activation of at least six genes in *D. melanogaster*) [51]. It does, however, suppress late regulatory gene expression.

Then, as the late regulatory genes are induced to express, proteins produced by early regulatory genes inhibit the activity of their own promoters. The late genes create proteins that are directly involved in the regulation of molting and metamorphosis [52]. The ecdysone signalling system is significantly upregulated during the developmental changes in *D. immitis* microfilaria [48].

Various helminth species, such as *Brugia pahangi, Anisakis simplex,* and *Ascaris suum,* as well as cestodes such as *Echinococcus granulosus Moniezia expansa,* and *Hymenolepis diminuta,* and trematodes such as *Schistosoma mansoni* and *Fasciola hepatica,* produce ecdysteroid hormones [41-46]. In *D. immitis,* ecdysteroids were found to be concentrated in the reproductive system of both sexes, while in *S. mansoni,* they were detected in eggs [47]. While ecdysteroids have been widely investigated in insect molting, their function in parasites is poorly understood.

1.5.2 DAF-12 signalling pathway

DAF-12, a receptor, shares homology with the vertebrate VDR (vitamin D receptor) and LXR (liver X receptor). It regulates growth and physiological functions in response to environmental factors in a variety of ways. These roles include effects on longevity, reproductive maturation, metabolism, and lifespan [49-53]. The DAF-12 pathway has been extensively studied in the nematode *C. elegans*, where it was initially identified as a key regulator of dauer formation, a developmentally arrested and stress-resistant larval stage.

Several studies have investigated the DAF-12 signalling pathway in parasitic nematodes to identify potential targets for therapeutic intervention; modulating this pathway could disrupt

the parasite's ability to survive and reproduce, offering new strategies for controlling parasitic infections.

Under favourable conditions, the DAF-12 pathway promotes reproductive development, allowing to complete its life cycle. However, under unfavourable conditions such as nutrient scarcity or host immune response, the pathway induces the formation of infective larvae or dauer larvae. This enables nematodes such as *C.elegans* to enter a dormant and stress-resistant state until conditions improve.

The activation of DAF-12 is controlled by various factors, including environmental cues, pheromones, and endogenous hormones. The development of nematodes is governed by a hormonal signalling cascade activated by insulin/IGF-I and TGF- β. This eventually results in the stimulation of DAF-12, which is a nuclear receptor. In nematodes, such as the case of *C. elegans*, the TGF-β pathways and insulin/IGF-I get stimulated under favourable environmental conditions, which, in turn, trigger the synthesis of daf-9, an enzyme that produces steroid-like hormones known as dafachronic acids (DAs) [54-56]. These DAs act as ligands for DAF-12, binding to and activating it [55-57]. Activation of DAF-12 then promotes reproductive growth in nematodes, allowing them to complete their lifecycle. The TGF-β and insulin/IGF-I pathways, on the other hand, are inactive under unfavourable circumstances and impede the synthesis of DAs. As a result, DAF-12 interacts with DIN-1, a potent co-repressor necessary for the formation of a dormant stage known as the dauer stage [55, 56, 58]. This repressor activity is absent in *C. elegans* larvae lacking DAF-12, resulting in a faulty dauer phenotype that presumably lowers viability under unfavourable environments [49, 54]. The insulin/IGF-I/DAF-12 signalling pathway that regulates development is believed to be conserved in parasitic nematodes as well [59-64]. In parasitic nematodes, similar to *C. elegans*, the absence

of DAF-12 ligands is necessary for dauer-like stages in iL3 larvae, whereas activation of DAF-12 is necessary for reproductive growth and development [64].

Although studies have recently discovered multiple DAF-12 ligands and started to explain the biosynthesis and impact on dauer formation, DAF-12 was previously an orphan NHR with unknown ligands. Enzymes needed for ligand synthesis are encoded by a number of genes that function upstream of DAF-12: the homologs of Niemann-Pick type C1 (NPC), ncr-1 and ncr-2 in *C. elegans*, which are crucial for sterol homeostasis and cholesterol trafficking [55, 65, 66] Daf-36, a Rieske-like oxygenase encoded by the cytochrome P450 (CYP450) gene [45], hsd-1, a member of the 3-hydroxysteroid dehydrogenase family [51, 67, 68], and daf-9, a similar steroidogenic hydroxylase, are also included [69]. These enzymes are components of a biosynthetic pathway that results in the production of two 3-keto bile-acid-like steroids, Δ4-DA and Δ7-DA, which bind and activate DAF-12 [55]. Additionally, it has been discovered that the similar bile acid 25S-cholestenoic acid (CA) binds to DAF-12 [42]. Application of CA and DA restores daf-9 mutants, while DA rescues dauer and other traits of ncr-1, ncr-2, daf-9, and daf-36 mutants [53, 55, 56, 70, 71]. DAF-12 is commonly recognized as the central regulator of the life cycle in *C. elegans*, functioning as the master coordinator at the hormonal level [72, 73]. Recent studies have found a filarial ortholog of the DAF-12 receptor in *D. immitis* , called *Dim*DAF12, which is highly sensitive to DA and CA [46, 47]

Recent findings in nematodes and fruit flies, which were discussed above, strongly imply that the steroid hormone pathways represented by DA/DAF-12 and 20-E/EcR have similar impacts on life history, particularly on their longevity [45].

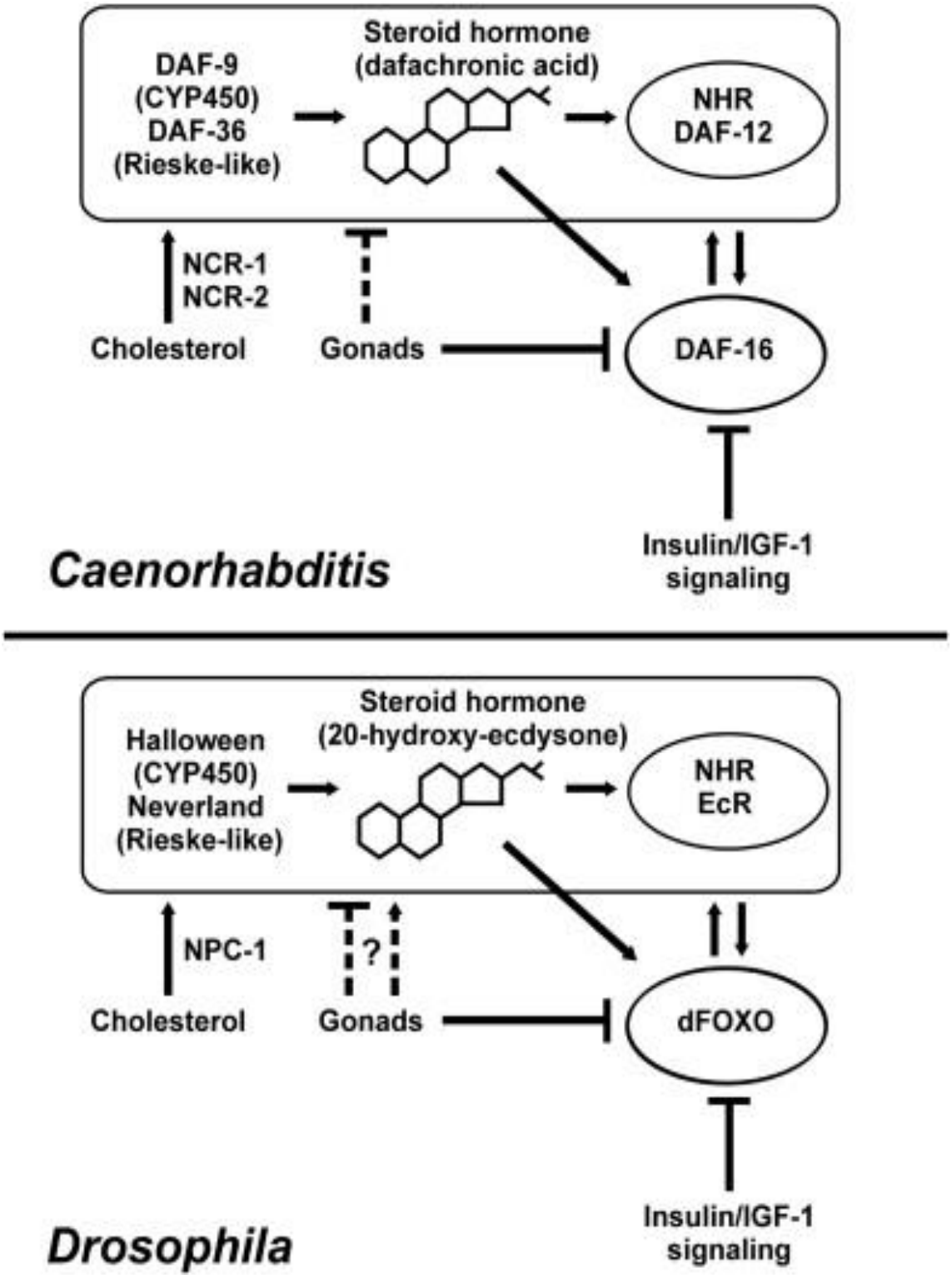


Figure 3. **Functional endocrine analogues** in the control of life history variables by steroid hormones, particularly lifespan, between (A) *C. elegans* and (B) *D. melanogaster*. Reprinted from Gáliková, M. et al., Exp Gerontol, 2011 [45].

1.6 Biological effects of these hormones in insects and filarids

In several nematodes, the biological effects of 20-E have been noted. It has been demonstrated that 20-E can induce molting in fourth-stage *Heligmosoides polygyrus* larvae at doses that are as small as 10^{-11} M [74]; similarly, in *A. suum*, a high proportion of third-stage larvae underwent molting when exposed to 1 M of 20-E. [75]. It's crucial to remember that early EcR activation in insects might cause fatal premature or partial molting [76]. Third-stage *D. immitis* larvae that are stimulated by 20-E at a concentration of 10^{-7} M molt prematurely [77], suggesting the role of 20-E in nematode ecdysis.

Ecdysteroids play a role in the control of meiotic reinitiation [78], vitellogenesis [79], as well as embryogenesis [80] in adult female insects. Ecdysteroids have been found in nematode eggs and the reproductive system, suggesting that they may have similar activities in terms of reproduction and embryonic development. Developmental arrest has been shown in *D. immitis* oocytes [81], but the number of oocytes was reduced compared to control (ovaries without ecdysteroid treatment) when they were in the presence of ecdysone (10^{-5} M) for 24 hours *in vitro*. This effect was less pronounced at a concentration of 10^{-6} M ecdysone, showing how ecdysone concentration affects how oocytes develop [82].

Further studies showed the supporting nature of ecdysteroids for regulating growth and reproduction in nematodes; it was found that maintaining adult *B. pahangi in vitro* in 2 x10^{-6} M ecdysone resulted in an increased number of released microfilariae [82]. Similarly, culturing female *B. malayi* in medium containing 10^{-5} M 20-E resulted in an increase in microfilariae production, although many of them were abortive immature progeny [83].

Exogenous application of DAs and other bile acids also demonstrated biological effects on nematodes. In *Haemonchus contortus*, an economically important parasitic nematode, DA encouraged larval exsheathment and growth via the DAF-12 NR. Both dose and timing were factors in this stimulatory effect and likely involved dauer-like signaling modulation through a negative feedback loop. The activation of *Hc*DAF-12 by (25S)-D7-DA exhibited an EC50 of 12.54 nM, which was identical to the activation observed in *Ac*DAF-12., according to luciferase reporter experiments. Moreover, in *H. contortus*, exogenously applied (25S)-Δ7-DA stimulated larval growth and development in a time and dose-dependent manner [84]. Incubating *S. stercoralis* in Δ7-DA encouraged post-parasitic L1 to mature into adults capable of living on their own and stopped iL3 from experiencing developmental arrest [62].

The DAF-12 homologue in the clade III nematode *D. immitis* was recently demonstrated to have a significantly greater affinity for DAs than those reported for DAF-12 in other nematodes. Activation of *Dim*DAF-12 at 10 µM Δ4- and Δ7-DAs accelerated molting of *D. immitis* from the iL3 to the L4 stage, suggesting that this parasite's development may be significantly influenced by these isoforms. The fact that *D. immitis* can only molt from iL3 to L4 in conditions supplemented with serum raises the possibility that the serum contains a component that activates *Dim*DAF-12 to start the molting process [46, 47]

2 Project Overview

2.1 Rationale and Hypothesis

Dirofilaria immitis is a parasite globally affecting pets, potentially leading to severe cardiac pathology. The emergence of resistance to MLs, the primary drugs used for prevention, necessitates exploring alternative approaches. While circulating microfilariae of *D. immitis* remain inactive in the bloodstream, they undergo rapid development and molting upon ingestion by their arthropod vector. This suggests that the cues initiating the transition in the vector are closely related to those initiating microfilarial molting.

D. immitis belongs to Clade III of filarial nematodes and exhibits distinct characteristics compared to Clade IV/V nematodes in terms of life stages and developmental regulation. Unlike nematodes with free-living stages that rely on the DAF-12 pathway, *D. immitis* lacks a free-living stage and employs both the DAF-12 pathway and an ecdysteroid signalling pathway, similar to insects. Previous studies have implicated the DAF-12 pathway in regulating development from iL3 to L4 in *D. immitis*. Additionally, research has demonstrated that stimulation of microfilarial development occurs through 20-E and results in the upregulation of key genes in the ecdysteroid signalling pathway.

Our current hypothesis posits that the ecdysteroid and DAF-12 signalling pathways may be involved in *D. immitis* microfilariae development inside the host. However, further investigation is necessary to identify the specific factors involved and their optimal concentrations. Mammalian serum inhibits microfilarial development, but the components responsible for this inhibition and the potential for artificially blocking *D. immitis* microfilarial development remain unknown.

A more comprehensive understanding of these processes and the factors involved could potentially lead to interventions that disrupt the parasite's life cycle and effectively prevent the development of mature heartworm infections.

2.2 Specific aims

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

I. Titrate the effects of FBS and charcoal-stripped FBS-supplemented media on *D. immitis* microfilariae.
II. Titrating the effects of 20-E and DA on microfilarial development.
III. Determining component(s) released from mosquito cells and FBS that influence microfilarial development via untargeted metabolomics.

To carry out the objectives, *D. immitis* microfilariae will be cultured in the presence of 20-E or Δ4-DA in mosquito cell conditioned media supplemented with FBS or CS-FBS for signs of morphological development. Metaboanalyst 5.0 will be used to analyse UPLC-FTMS results of different culture media sample-control pairs to determine components secreted by mosquito cells and those present in FBS which may affect the development of microfilariae.

3 Methodology

3.1 *Dirofilaria immitis* microfilariae

The Missouri (MO) strain of *D. immitis* microfilariae or early L1 stage larvae were provided by the University of Georgia's NIH Filariasis Research Reagent Resource Center (FR3).

3.2 *D. immitis* microfilariae extraction from dog blood

EDTA vacutainer tubes were used to collect dog blood samples containing *D. immitis* microfilariae. Tubes were shipped overnight after being put in a thermos to keep the temperature stable. The blood was processed immediately upon receipt. The sample was subjected to a two-fold dilution in a biological safety cabinet with 0.22 µM filtered sodium bicarbonate solution (2 g L-1) (Sterlitech; PCT3025100). The membrane was then washed with 3-5 mL of sodium bicarbonate. To allow microfilariae to migrate off the filter and into the phosphate-buffered solution (PBS), the filter was submerged in 2-3 mL of PBS (Gibco; 20012). Following a light PBS rinse, the filtrate containing microfilariae was collected into 15 mL centrifuge tubes (Corning; 430791).

To retrieve microfilariae that might have gotten past the filter, the flowthrough from filtering was collected, and the procedure was performed thrice. PBS containing live microfilariae was centrifuged at 1500 rpm for 5-6 min. The pellet was resuspended using fresh PBS after the supernatant was discarded. The centrifuged supernatant containing microfilariae was washed

with PBS 7-8 more times to acquire clean parasites, with the final two washes using 1:1 mix of DMEM (Gibco; 12430) and Ham's F12 (Gibco; 11765).

Microfilariae were counted on a microscope slide (Fisher Scientific) by placing 10 µL of microfilariae suspension between the slide and coverslip. To calculate the total number of microfilariae in the solution, the dilution factor was studies after being quantified.

3.3 *D. immitis* microfilariae *in-vitro* co-culturing

Using a 24-well cell culture plate (Corning; 3524), extracted *D. immitis* microfilariae were cultured in 1 mL culture media at a density of 500 microfilariae/well. Additionally, 0.25 µg/mL amphotericin B (Gibco; 15290), 0.01 mg/mL gentamicin (Sigma; G1397) 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco; 15140) was added to the culture media DMEM/Ham's F-12 and heated to 26°C before use. Prior to microfilariae co-culturing, mosquito (cell-line 4a-3B) cells were used to condition the culture media. The parasites were maintained at 26°C, 5% CO_2 for 22 days in a controlled atmosphere with or without FBS (Gibco; A31607-01), CS-FBS (Gibco; 12676029), DA (Cayman Chemical; 14100), or 20-E (Cayman Chemical; 16145).

To investigate the effects of CS-FBS compared to regular FBS, FBS or CS-FBS were added to wells To test the effects of DA, dimethyl sulfoxide (DMSO) (Sigma; D4540) was used to dissolve Δ4 DA to make a stock solution of 1 mM, which was then utilised at a final concentration of 10 µM and added to wells. For treatment with 20-E, DMSO was used to dissolve 20-E to make a stock solution of 10 mM and used at 1 µM, 2 µM, and 4 µM mixed with 0.004% HP-β-CD ((2-hydroxypropyl)-β-cyclodextrin) + 001% DMSO.

Sixty percent of the culture media containing the microfilariae was changed daily or once every three days.

3.4 4a-3B (*Anopheles gambiae*) mosquito culture media

An. Gambiae cells (Cell line 4a-3B from Dr G.K. Christophides, Imperial College, London) were routinely cultured in Schneider's insect media supplemented with 100 U/mL penicillin, and 100 g/mL streptomycin and 10% (v/v) heat-inactivated FBS, at 26°C with 5% CO_2. 48hrs prior to co-culturing with *D. immitis* microfilariae, in a T-25 cell culture flask (Corning; 430639), mosquito cells were planted at a density of $5x10^4$ cells / cm^2 in Schneider's insect media. After 24 hours,mosquito cells were washed with PBS and incubated in the DMEM/Ham's F12 media for 24 hours. The mosquito cell-conditioned media was collected and centrifuged for 5-6 minutes at 1300 rpm to eliminate any cells. The supernatant was utilised for further *D. immitis* co-culturing.

3.5 Morphological studies

In a 24-well cell culture plate, extracted live microfilariae were cultured with 5% CO_2 at 26°C (Thermo Scientific, Forma Series II Water Jacketed CO_2 Incubator) and imaged using a microscope (Fisher Scientific Invitrogen™ EVOS™ XL Core Imaging System) at 20X magnification.

3.6 Untargeted Metabolomics

3.6.1 Samples for metabolite extraction

Six culture media samples were prepared; non-conditioned media (NCM), non-conditioned media supplemented with FBS (NCMFBS), non-conditioned media supplemented with charcoal-stripped FBS (NCMFBSCS), mosquito-conditioned media (MSCM), mosquito-conditioned media supplemented with FBS (MSCMFBS) and mosquito conditioned media supplemented with charcoal-stripped FBS (MSCMFBSCS) where a mixture of DMEM and Ham's F12 was used in a ratio of 1:1 and supplemented with 0.25 µg/mL amphotericin B, 0.01 mg/mL gentamicin 100 U/mL, penicillin, 100 g/mL and streptomycin, with or without mosquito cell conditioning or 2.5% (v/v) CS-FBS or 10% (v/v) regular FBS.

3.6.2 Purification of conditioned media

The prepared culture media were passed through a 0.22 µM sterile filter (Fisher Brand; 09-720-004). The tubes were placed on ice. The filtered media was passed through a 3 KD Molecular weight cut-off filter (Pierce ™ Thermo scientific; PI88525). It was centrifuged at 3000 rpm at 4°C in batches until the proteins and small molecules were separated. The filter membranes were washed with 200-300 µL sterile PBS (x2). The proteins (roughly 500µL) (on ice) were pipetted out and stored at -20ºC. The flowthrough containing metabolites of interest from the same day was pooled together (on ice) and stored at -20ºC until use.

The tubes were thawed to proceed with the experiment. The flowthrough was acidified by adding a final concentration of 0.1% Formic acid, i.e. 100 µL per 100 mL culture media while

maintaining a pH between 2-4 using a pH paper (Fisher Brand; 13-640-508). The amount of formic acid/pH was made consistent among all batches.

3.6.3 Metabolite extraction by Solid phase extraction (SPE)

The C18 (Strata®; C18-E) columns were mounted and attached to the tubes that lead to the falcon tubes in ice. The columns were activated/preconditioned with 10 mL methanol. The flowthrough was collected separately. This was followed by column re-equilibrium by passing 10 mL double-distilled water (ddH_2O)+ 0.1% formic acid through the column. The flowthrough was collected.

While passing the sample through the column, a yellowish band appeared, which happens when a certain amount of sample has passed through the C-18 column. 30 mL ddH_2O+ 0.1% formic acid wash was passed through the column to remove all unbound particles, and the polar flowthrough was collected.

For eluting metabolites of interest, three acetonitrile solutions of 10 mL total volume were prepared at 25%, 50% and 100% concentrations, which contained acetonitrile, ddH_2O and 10 µL formic acid and briefly vortexed to mix. Ten eppendorf tubes were prepared for each test tube containing acetonitrile, and 1 mL eluted media was collected per eppendorf tube and stored at 4°C. The eluted samples were dried using SpeedVac vacuum concentrators (Thermo Scientific Savant SPD1010 SpeecVac Concentrators) and sent for UPLC-FTMS analysis to the Segal Cancer Proteomics Center (SCPC) in Jewish General hospital.

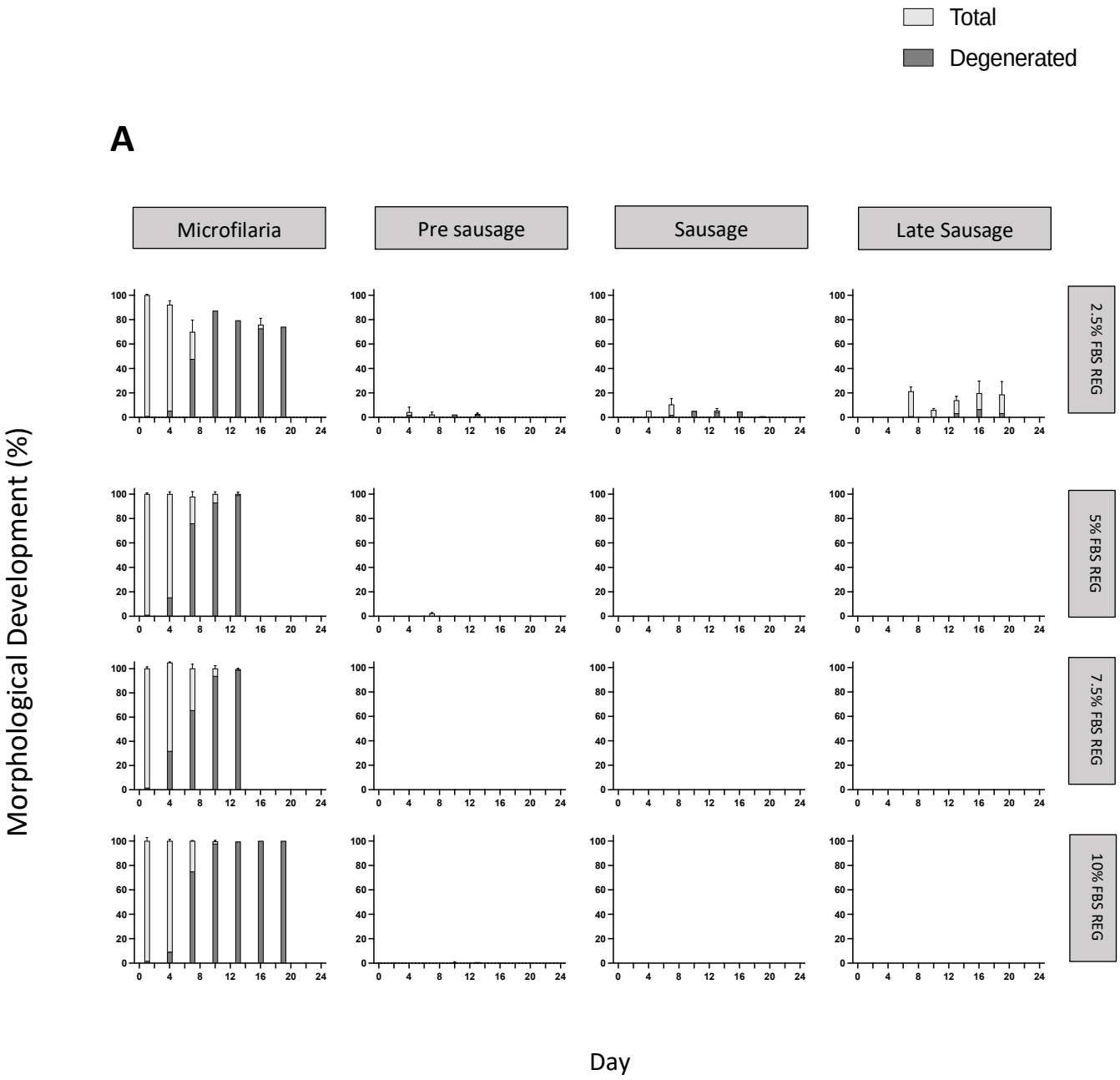
4 Results

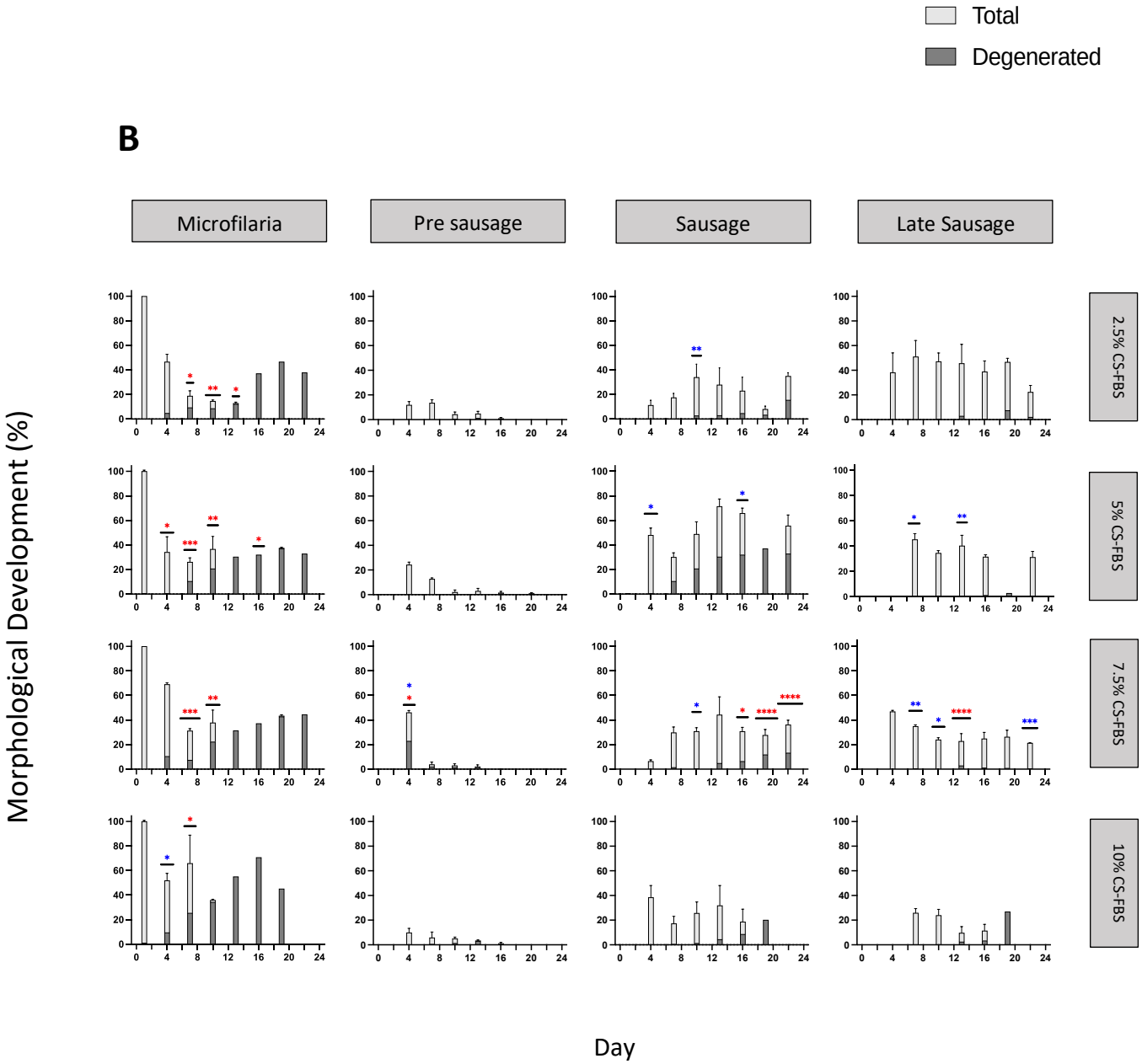
4.1 *D. immitis* microfilariae co-culturing

4.1.1 Charcoal-stripped FBS promotes microfilariae development.

The effect of varying concentrations of FBS and CS-FBS on the development of microfilariae was investigated by adding 2.5%, 5%, 7.5%, and 10% (v/v) FBS or CS-FBS to the culture media and incubating the *D. immitis* microfilariae at 5% CO_2, 26°C for 22 days. The number of parasites at microfilaria, pre-sausage, sausage and late-sausage stages were counted. With increasing concentrations of FBS, microfilariae development significantly reduced, and microfilariae appeared slower and degenerated, with no development into pre- or late-sausage stages **(see Figure 4A).**

However, when CS-FBS was added to the culture media, rather than complete FBS. The microfilariae developed into pre-sausage, sausage, and late-sausage stages, in lower concentrations of CS-FBS **(see Figure 4B)**. Therefore, 2.5% (v/v) CS-FBS was used for further co-culturing studies.


Total
Degenerated
A
Microfilaria
Pre sausage
Sausage
Late Sausage
2.5% FBS REG
5% FBS REG
7.5% FBS REG
10% FBS REG
Morphological Development (%)
Day


Total
Degenerated
B
Microfilaria
Pre sausage
Sausage
Late Sausage
2.5% CS-FBS
5% CS-FBS
7.5% CS-FBS
10% CS-FBS
Morphological Development (%)
Day

C

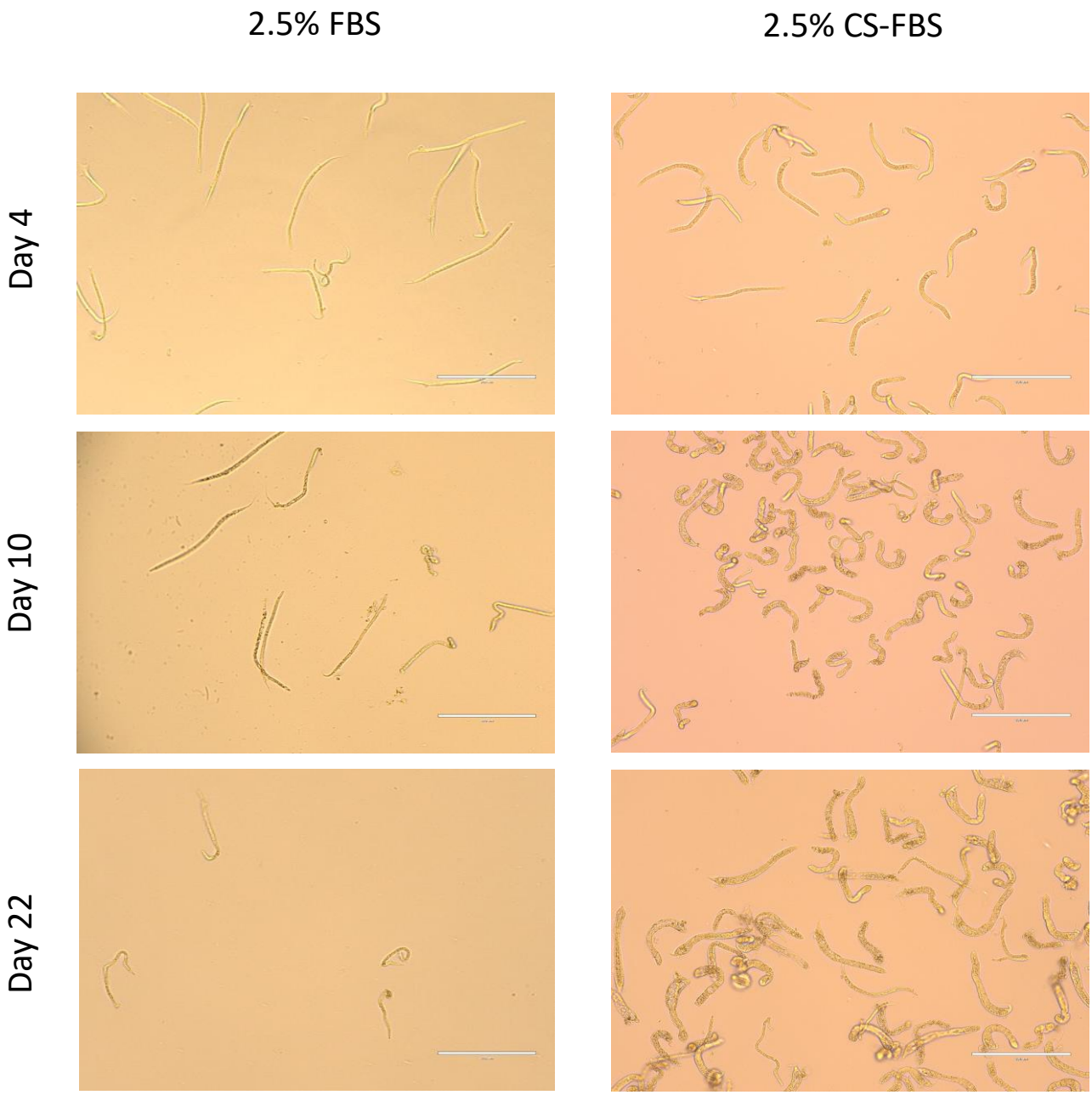
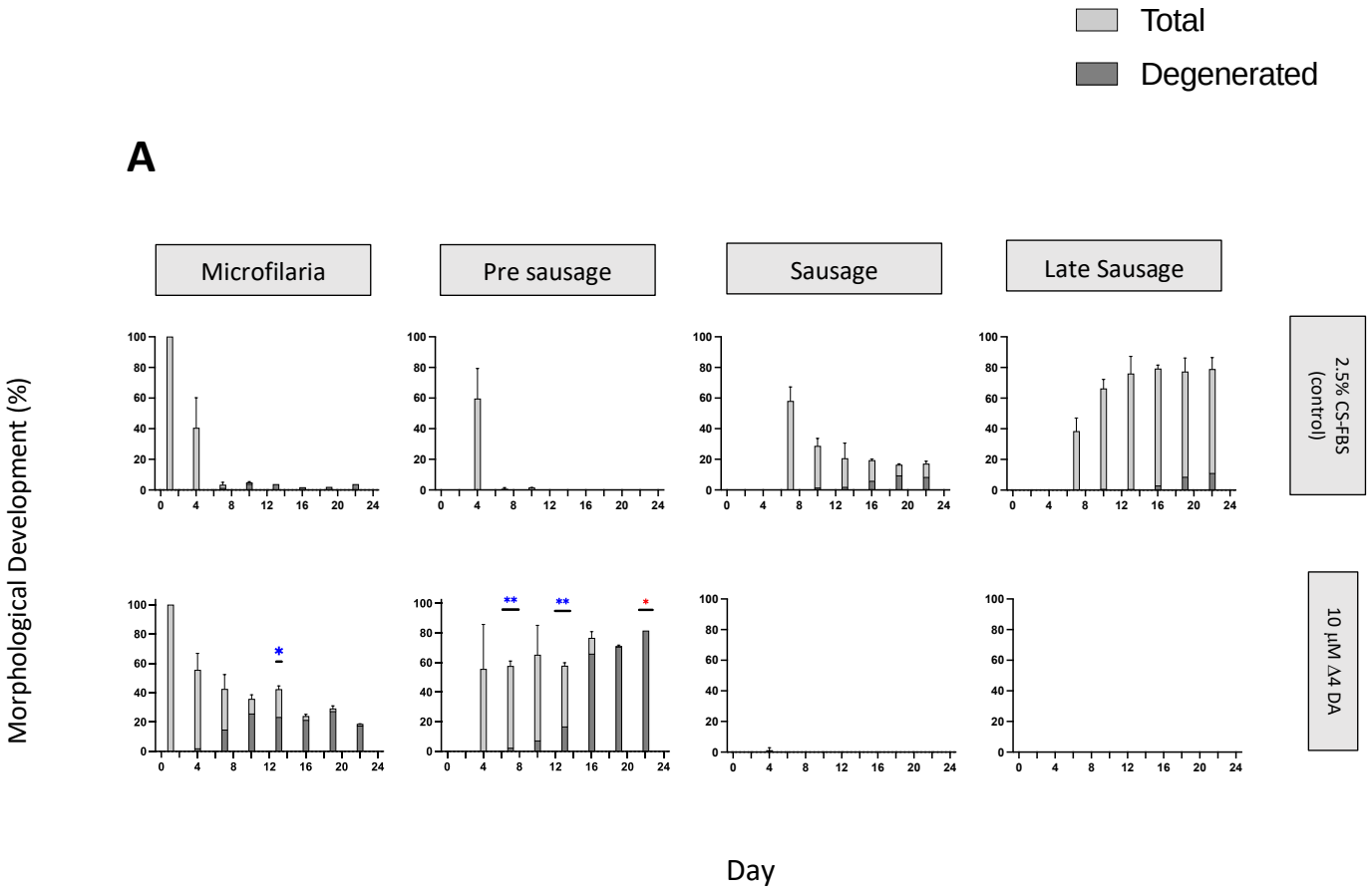


Figure 4. **Development of *D. immitis* microfilariae co-cultured in cell-conditioned media in the presence of FBS (regular) or Charcoal-stripped FBS (CS-FBS).** Results depict the percentages of larvae through various L1 stages of the parasite, namely the microfilaria, pre-sausage, sausage, and late-sausage stages cultured (A) in the presence of regular FBS at concentrations of 2.5%, 5%, 7.5% and 10% (v/v). Three independent replicates were performed; every dog blood sample containing microfilariae of MO strain was received on a different day. (B) In the presence of CS-FBS at varying concentrations of 2.5%, 5%, 7.5% and 10% (v/v). Three dog blood samples containing microfilariae of MO strain were collected from a canine with three independent replicates performed; each blood sample was shipped on a different day. (C) Light microscopy of *D. immitis* microfilariae cultured with 2.5% FBS or CS-FBS in mosquito-conditioned media at day 4, day 10 and day 22. All data are shown as means ± standard deviations (SD) based on all of the replicates mentioned for every procedure. Asterisks indicate the statistical difference between parasites that have total healthy (*) and total degenerated (*) larvae when compared to the FBS (regular) group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p<0.0001$. Statistical analysis was carried out with a 95% confidence interval using 2-way ANOVA and Sidaks multiple comparison test (Graph Pad Software, Inc.).

4.1.2 10µM Δ4 Dafachronic acid (DA) is detrimental to the early development of *D. immitis* microfilariae *in vitro.*

By adding 10 µM Δ4 DA to the culture media mix every three days and incubating the microfilariae for 22 days at 5% CO2, 26°C, the effect of Δ4 DA on microfilarial development was investigated. The addition of Δ4 DA slowed microfilariae development. The microfilariae seemed degenerated, with no development to the -sausage or late sausage stages **(see Figure 5A).** In contrast, microfilariae incubated in DMEM/Ham's F12 supplemented with 2.5% (v/v) CS-FBS developed to the later sausage stages (See Fig 5A). A similar trend was observed when treatment was done every day (no individual replicates) (**see supplementary file Figure S1).**



B

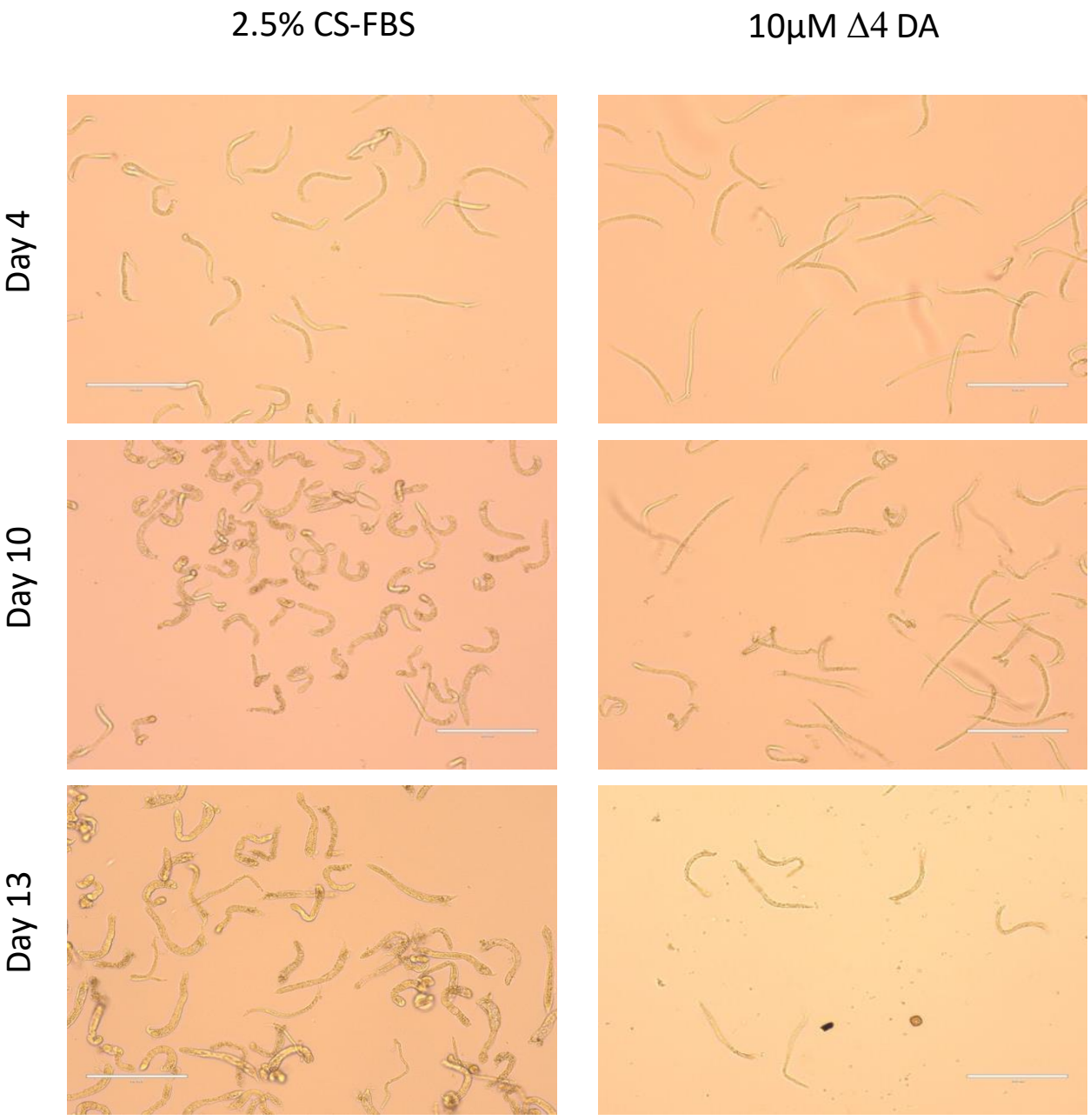

2.5% CS-FBS
10µM Δ4 DA
Day 4
Day 10
Day 13

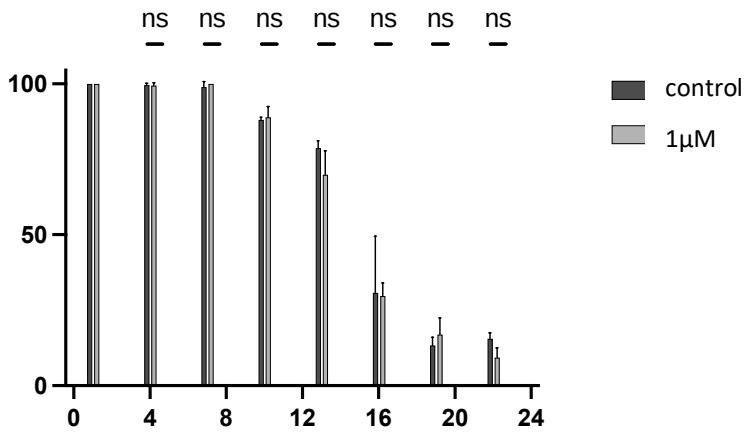
Figure 5. **Development of *D. immitis* microfilariae cultured in cell-conditioned media in the presence of Δ4 Dafachronic Acid or 2.5% (v/v) CS-FBS alone.** Results depict the percentages of larvae through various L1 stages of the parasite, namely the microfilaria, pre-sausage, sausage, and late sausage stages cultured (A) in the presence of 10 μM Δ4 DA in 2.5% (v/v) CS-FBS or 2.5% (v/v) CS-FBS alone. Three blood samples containing live microfilariae MO strain were collected from a canine, and three individual replicates were performed; each blood sample was shipped on a different day. Microfilariae were treated once every three days with Δ4 DA or 2.5% (v/v) CS-FBS alone. (B) Light microscopy of *D. immitis* microfilariae treated every three days with 10 μM Δ4 DA or 2.5% CS-FBS alone in conditioned media at days 4, day 10 and day 13. All data are shown as means ± standard deviations (SD) based on all of the replicates mentioned for every procedure. Asterisks indicate the statistical difference between DA-treated parasites that are total healthy (*) and total degenerated (*) larvae when compared to the 2.5% (v/v) CS-FBS group. *$p < 0.05$, **$p < 0.01$.Statistical analysis was carried out with a 95% confidence interval using 2-way ANOVA and Sidaks multiple comparison test (Graph Pad Software, Inc.).

4.1.3 20-Hydroxyecdysone (20-E) promotes microfilarial development.

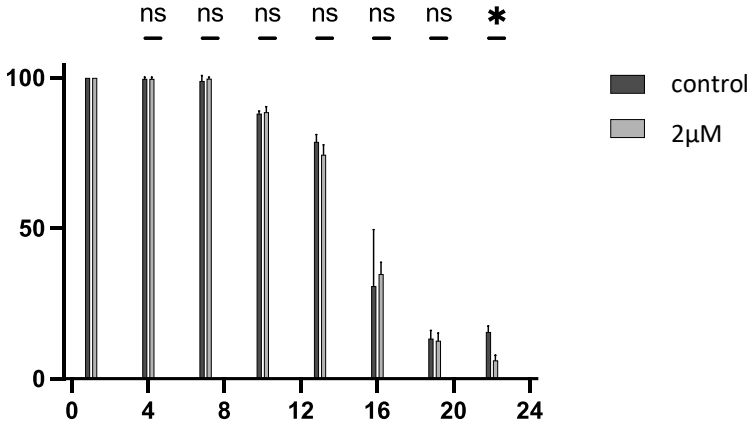
The effects of the ecdysteroid 20-E on the development of microfilariae were investigated by adding 20-E at 1 µM, 2 µM, and 4 µM dissolved in DMSO along with a hydrophobic compound carrier (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) to the culture media and incubating the microfilariae at 26°C, 5% CO_2 for 22 days; this was compared to DMSO + HP-β-CD control group. Again, the number of parasites at microfilaria pre-sausage and sausage and late-sausage stages were counted. The addition of 20-E to the culture media stimulated development of the microfilariae to later sausage stages; the percentage of total parasites that failed to molt into late-sausage stages was significantly lower at 4 µM 20-E by the end of the incubation period **(see Figure 6A).**

A

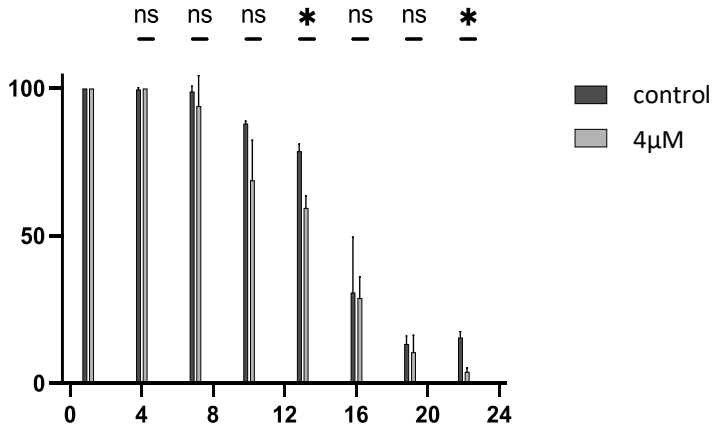
(i)


ns ns ns ns ns ns ns
100
50
0
0 4 8 12 16 20 24
control
1µM

(ii)


ns ns ns ns ns ns *
100
50
0
0 4 8 12 16 20 24
control
2µM

(iii)


ns ns ns * ns ns *
100
50
0
0 4 8 12 16 20 24
control
4µM

Day

B

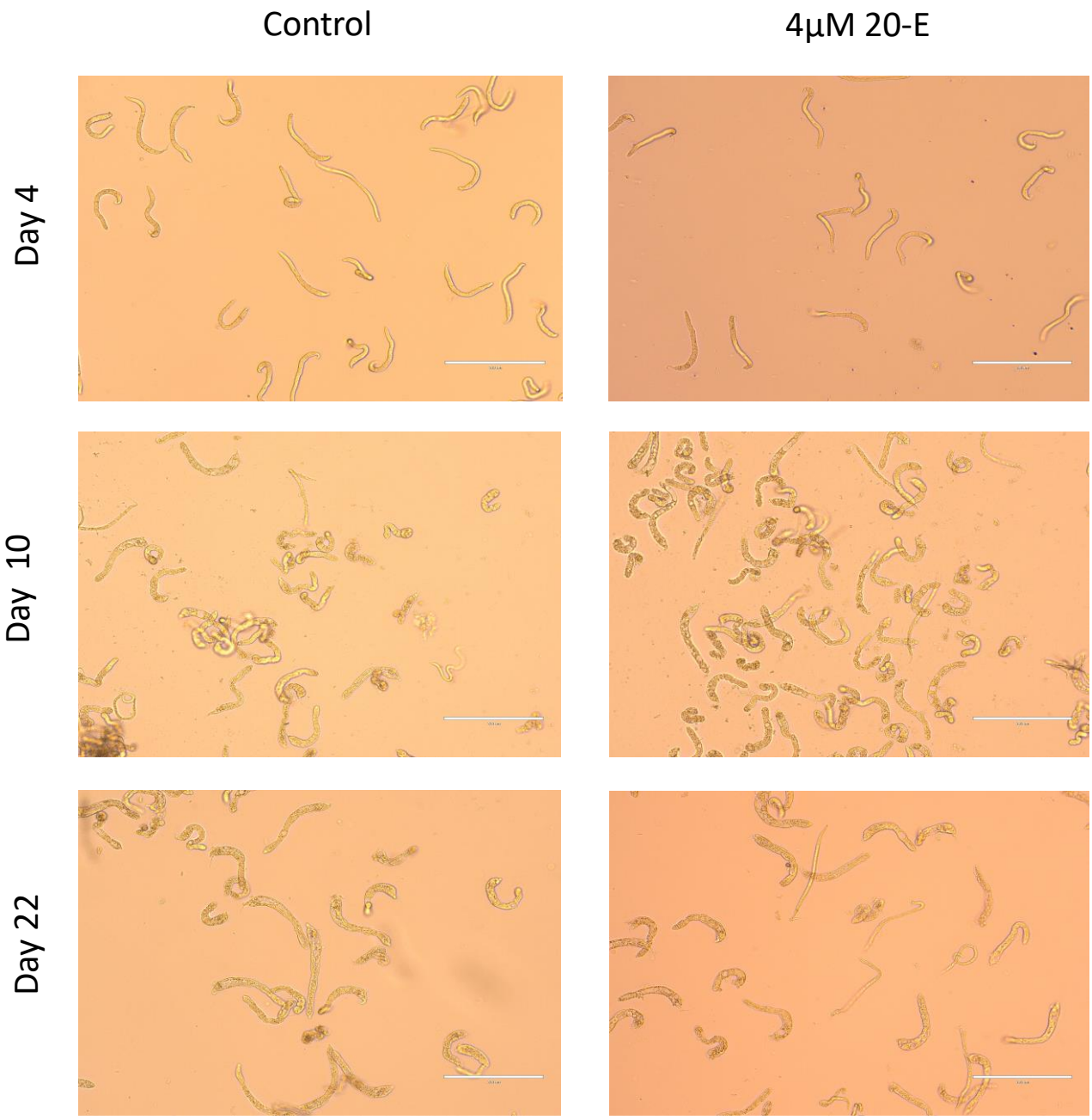


***Figure 6*. Development of *D. immitis* microfilariae cultured in cell-conditioned media in the presence of 20-Hydroxyecdysone + DMSO + HP-β-CD or DMSO + HP-β-CD.** Results show percent of total larvae (alive+degenerated) that failed to molt into late sausage stage (A) in the presence of 0.05% DMSO + 0.004% HP-β-CD (control) and (i) 1 µM 20-E+ 0.05% DMSO + 0.004% HP-β-CD, (ii) 2 µM 20-E +0.05% DMSO + 0.004% HP-β-CD or (iii) 4 µM 20-E + 0.05% DMSO + 0.004% HP-β-CD. Three blood samples containing live microfilariae MO strain were collected from a canine with three individual replicates performed; each blood sample was shipped on a different day, and microfilariae were treated every day with 20-E. (B) Light microscopy of *D. immitis* microfilariae treated daily 4 µM 20-E and 0.05% DMSO + 0.004% HP-β-CD (control) in mosquito-conditioned media at day 4, day 10 and day 22. All data are shown as means ± standard deviations (SD) based on all of the replicates mentioned for every procedure. Asterisks indicate the statistical difference between total parasites that failed to molt into the late sausage stage compared to the control group, 2.5% CS-FBS+ (0.05% DMSO + 0.004% HP-β-CD) ; *$p < 0.05$. Statistical analysis was carried out with a 95% confidence interval using 2-way ANOVA and Sidaks multiple comparison test (Graph Pad Software, Inc.).

4.2 Untargeted Metabolomics

4.2.1 Univariate analysis

Data were analysed using Metaboanalyst 5.0. Univariate analysis was carried out for study groups MSCM and MSCMFBS. Data were used for fold change (FC) analysis and t-test. FC was calculated through fold change analysis (unpaired), where FC is calculated as the ratios between two group means (M1/M2), and the p-value is obtained through t-test.

A volcano plot was generated with $\log_2$ (FC) on the x-axis and $-\log_{10}$ (p-value) on the y-axis. Based on the screening conditions, FC threshold of 2 and p-value <0.05 were used. The different screening results of the comparative analysis group are shown in **Figure 7**, which shows the differentially significant metabolites in the study groups.

The volcano plot for positive ion mode shows 88 significantly upregulated features/metabolites and 89 differentially downregulated significant features/metabolites in the MSCM group, and 1608 insignificantly different metabolites. The volcano plot for the negative ion mode shows 87 upregulated features/metabolites, 81 downregulated features/metabolites and 1703 insignificant ones **(see figure 7A & 7B).**

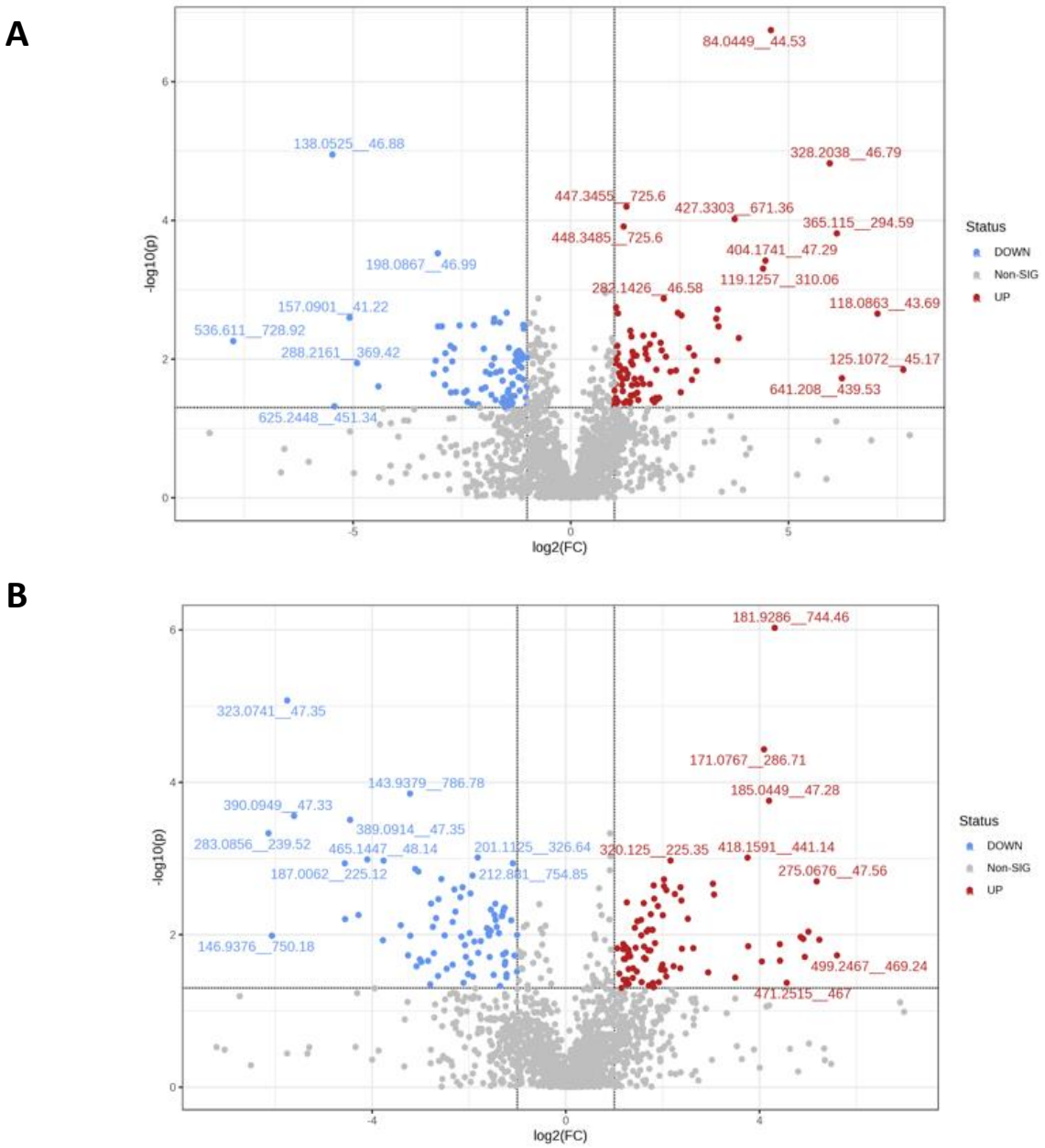


Figure 7. **The volcano plot** visually represents differentially expressed metabolites, highlighting significant features based on a FC threshold of 2 and a t-test threshold of 0.05. Significant features are denoted by red and blue circles above the threshold, while insignificant features are represented by grey dots. Fold changes and p-values are log-transformed, and the plot's positioning indicates the level of significance, with features further away from the (0,0) point considered more significant. Overall, the volcano plot offers a concise overview of the

differentially expressed metabolites and their significance. (A) is the volcano plot of differentially expressed features/metabolites in MSCM/MSCMFBS study groups in positive ion mode; (B) is the volcano plot of differentially expressed features/metabolites in MSCM/MSCMFBS study groups in negative ion mode.

4.2.2 Multivariate analysis

PLS (Partial Least Squares) is a supervised method that utilizes multivariate regression techniques to extract information from the original variables (X) in order to predict class membership (Y). Using a permutation test, the importance of class discrimination was assessed. Using the ideal number of components obtained by cross-validation based on the initial class assignment, a PLS-DA (Partial Least Squares Discriminant Analysis) model was built between the data (X) and the permuted class labels (Y) in each permutation. Metaboanalyst offers two types of test statistics for assessing class discrimination. The first is determined by the training's prediction accuracy, while the second is determined by the B/W ratio, which compares the total of squares within and between groups. The class discrimination cannot be regarded as statistically significant if the observed test statistic falls within the distribution obtained from the permuted class assignments.

PLS-DA, or Partial Least Squares Discriminant Analysis, employs two measures to assess the importance of variables. VIP, which stands for Variable Importance in Projection, is the first measure. To account for how much of the variation in the Y variable is explained by each dimension, it computes a weighted sum of squares of the PLS loadings. VIP scores are computed for each component, and when multiple components are used, the average of VIP

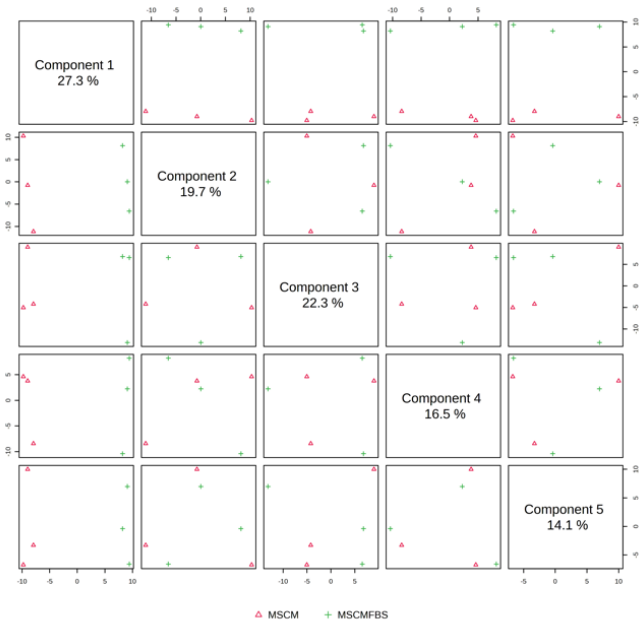
scores is used as a representative value. VIP score greater than one is generally used as a criterion for detecting the relative importance of an indicator.

PLS-DA was applied to identify changes in the metabolic profile of the MSCM group. The resulting pairwise PLS scores plot demonstrated that PC1 captured a higher proportion of variance, specifically 27.3% and 30.3%, while PC2 accounted for 19.7% and 16.7% variance in MSCM positive and negative ion modes, respectively. Additionally, the PLS-DA metabolic profiles of the MSCM and MSCMFBS study groups revealed statistically significant cluster separation in each scores plot in the negative and positive ion modes. These results show significant differences between the groups, as seen in **Figures 8 and 9**. Also, several metabolites with VIP score greater than one were observed, indicating the important features acting as a significant driving force for the separation/difference between MSCM and MSCMFBS study groups.

Q2 is a metric that assesses the predictive performance of a model through cross-validation. It measures the agreement between the projected data and the actual data by calculating the sum of squared errors. This value, known as the Predicted Residual Sum of Squares (PRESS), is normalized by the total sum of squares and subtracted from 1 to match the scale of R2. A high Q2 indicates good prediction quality, while a negative value suggests that the model is either non-predictive or overfitted.

The validation plots for the PLS-DA model showed that the permutation tests were valid with the R2 values being close to 1, and lower Q2 intercepts indicated the robustness of the models and showed a low risk of overfitting and reliability **(see Figure 11A & 11B**). These findings implied that the PLS-DA model could be utilized to identify the difference between pairwise groups

A



B

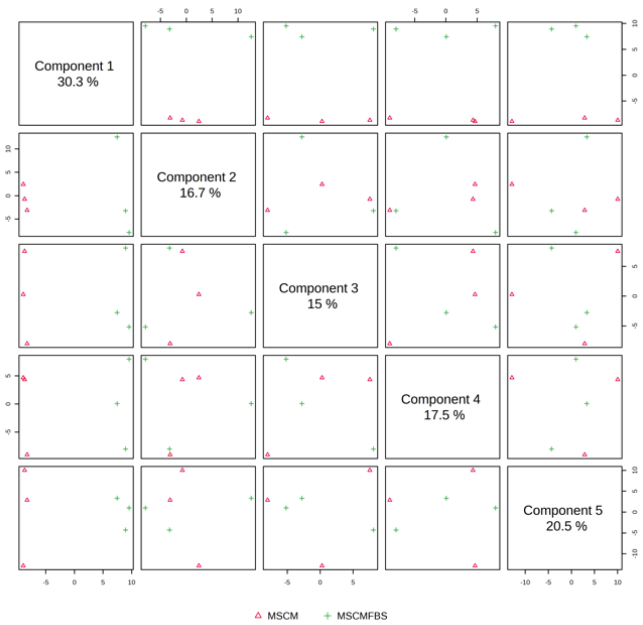
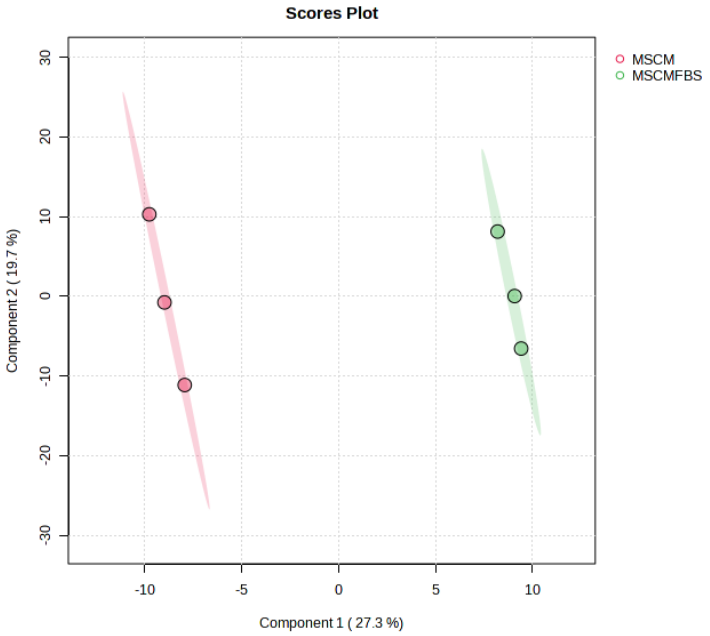


Figure 8. **The pairwise scores plot** shows the relationship between the selected components in both (A) positive (B) and negative ion modes. The diagonal cells in the plot represent the explained variance of each component.

A



B

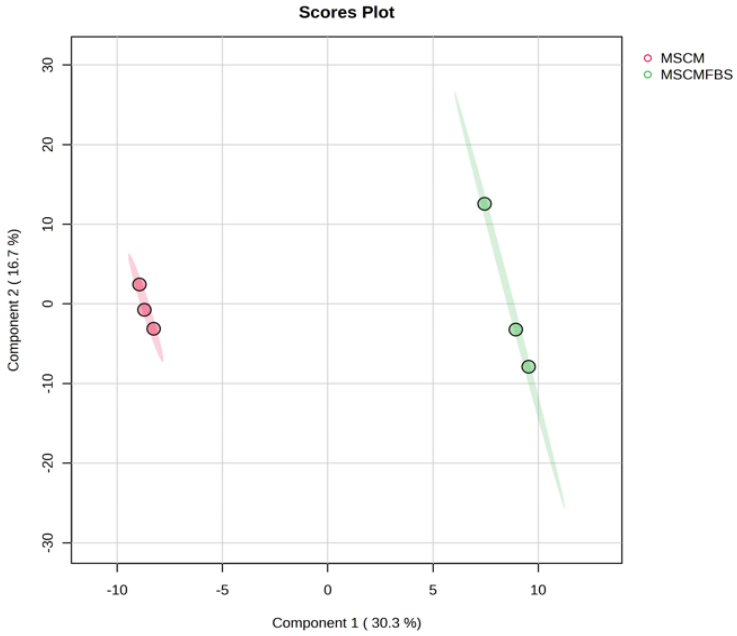
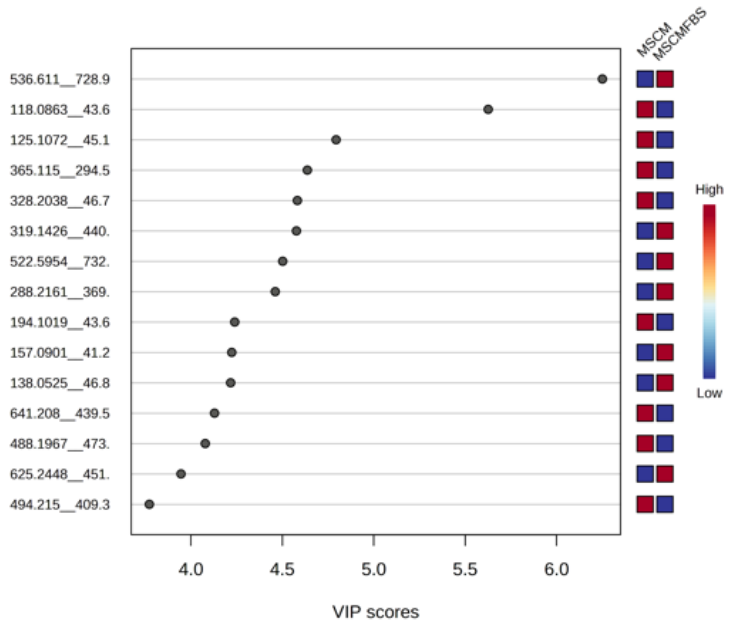


Figure 9. **Scores plot** displays the relationship between the selected principal components (PCs) in both (A) positive and (B) negative ion modes. The explained variances of each PC are indicated within brackets.

A



B

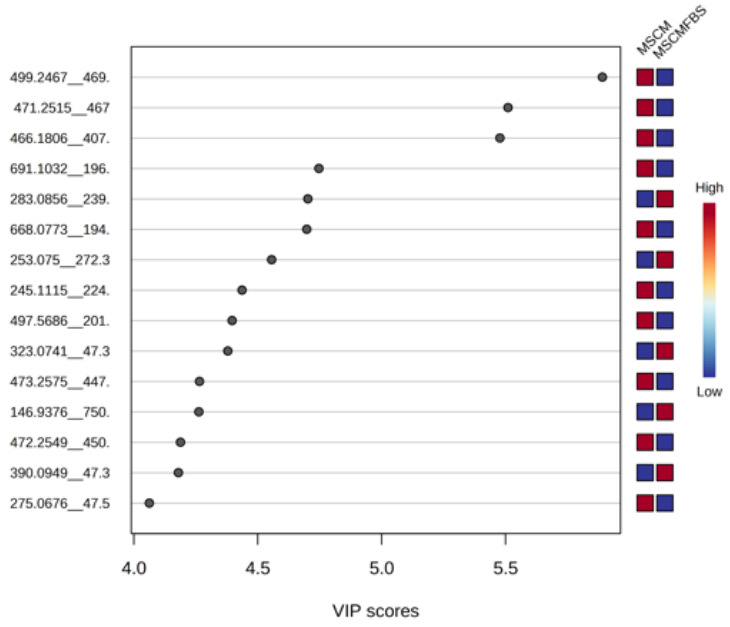
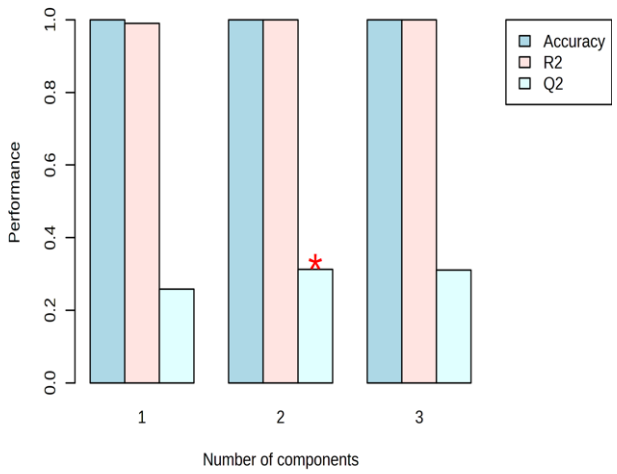


Figure 10. **VIP scores;** The PLS-DA analysis revealed important features in both (A) positive and (B) negative ion modes. The relative concentrations of the corresponding metabolites in each group under study are represented by colored boxes on the right.

A



B

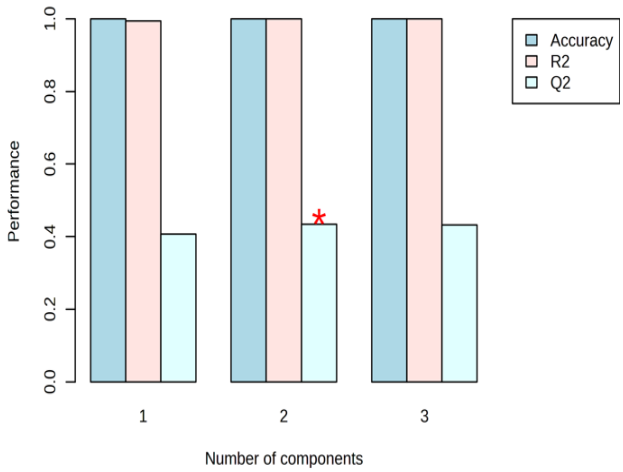


Figure 11. **Validation plots for PLS-DA** classification using a different number of components in (A) positive and (B) negative ion modes. The red asterisks indicate the best classifier.

4.2.3 Functional analysis

4.2.3.1 Pathway analysis

The differentially enriched pathways using KEGG (Kyoto Encyclopedia Genes and Genomes) and lipid database in MSCM/MSCMFBS study group in mixed ion mode are shown in **Figure 13A and 13B**, respectively. Scatter plots are generated using mummichog and GSEA algorithms on the x and y-axis for MSCM/MSCMFBS study groups. In the plots, the varying colours from yellow to red means the metabolites in our data have different levels of significance for enrichment analysis; the circles correspond to the p-values and enrichment factors.

The main lipids differentially enriched in the MSCM/MSCMFBS study group were sterols, flavonoids, glycerophosphoinositols, glycosphingolipids fatty esters, glycerophosphates aromatic polyketides, steroid Hydrocarbons, fatty alcohols, fatty acids, ceramides, phenylpropanoids,glycerophosphoglycerols,isoprenoids,octadecanoids,glycerophosphoethanolamines,steroid-conjugates,glycerophosphoserines, hydroxycinnamic acids, sphingoid bases, tannins, fatty amides, macrolides and analogues, quinones and hydroquinones, stilbenes, bile acids, fatty aldehydes, glycerophosphocholines, eicosanoids, and phosphosphingolipids (**see supplementary file Table S1).**

The main KEGG pathways which were differentially enriched in the MSCM/MSCMFBS groups were aminoacyl-tRNA biosynthesis, insect hormone biosynthesis, tyrosine metabolism, tryptophan metabolism, one carbon pool by folate, ubiquinone and other terpenoid-quinone, arginine and proline metabolism, arachidonic acid metabolism (ARA), and porphyrin and chlorophyll metabolism **(see supplementary file Table S2)**

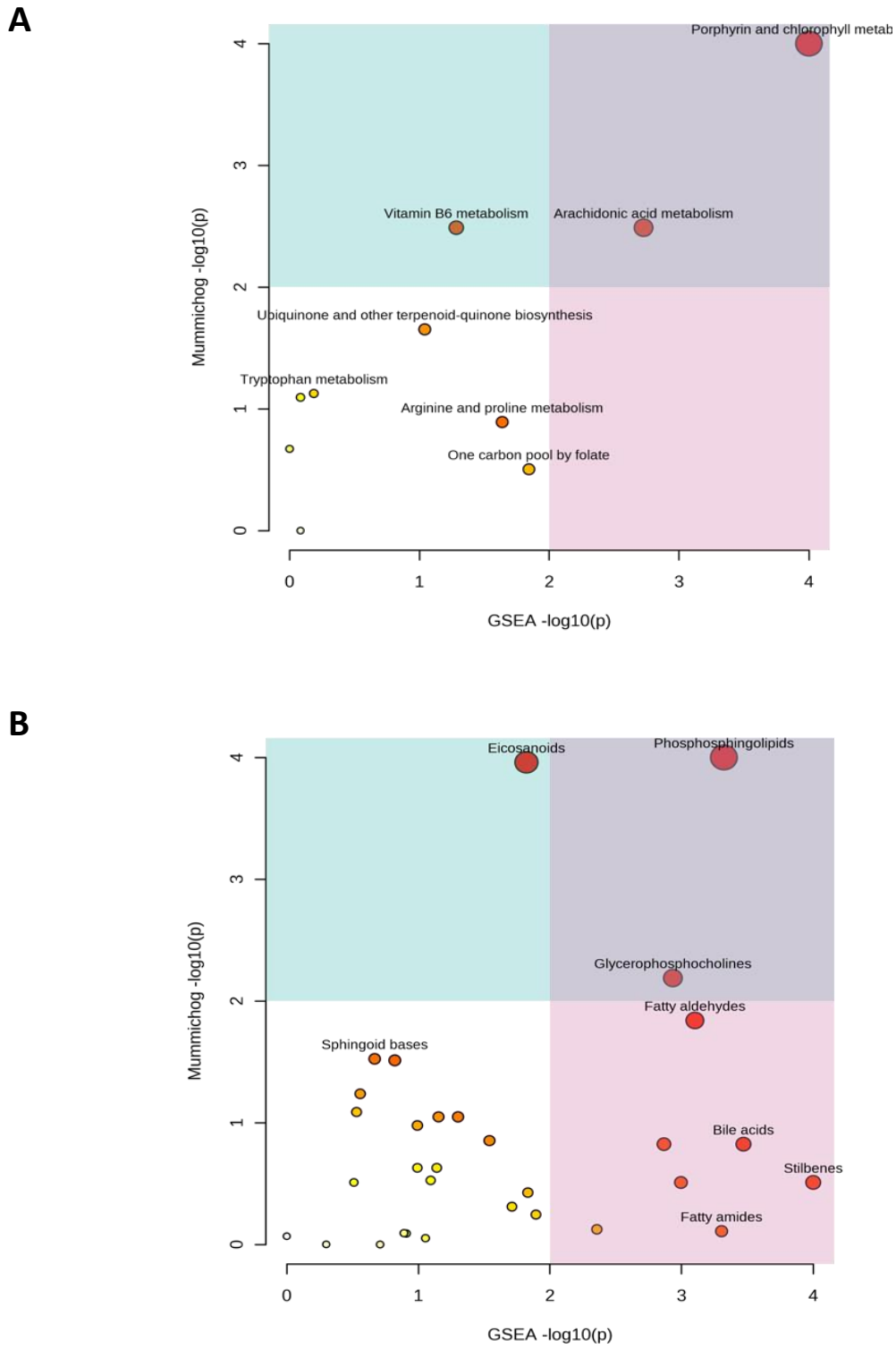


Figure 12. Enrichment analysis; Scatter plot; The significantly enriched metabolic pathways in mixed mode (A) KEGG and (B) Lipid database were predicted using the mummichog and

GSEA algorithms. The sizes of the data points correspond to their x values, and the colors represent their y values. Larger sizes indicate higher pathway enrichment, while darker colors indicate higher pathway impact values.

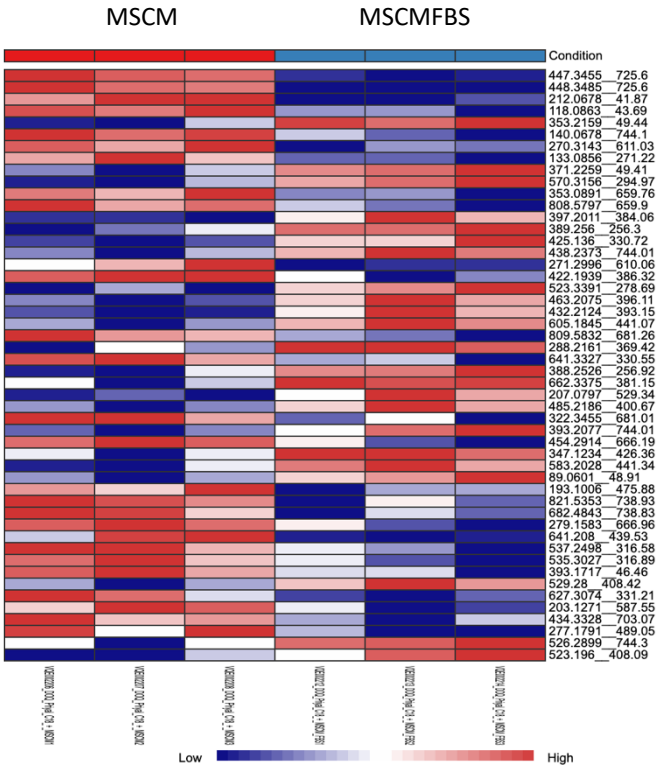
4.2.3.2 Cluster heatmap analysis Identification of significant metabolites

We utilized cluster heatmap analysis, a functional analysis method using KEGG and lipid databases, to further investigate and identify the metabolites within the studied pathways. The clustering was performed based on p-values.

Figure 14 displays the results. The top 10 metabolites and their association with KEGG and lipid database considered. The selection of these metabolites was based on their pathway impact and p-values ($p > 0.05$). The annotation of metabolites or pathways was done by clicking on any row on the heatmap corresponding to the metabolite mass. Both positive and negative ion modes were considered. The color-coding in the heatmap indicated the level of enrichment, with red representing significantly high enrichment in the specific study group and blue indicating low enrichment of the respective metabolite.

The metabolites and metabolites associated with pathways included sterols and secosterols like vitamin D3 analogs, FAs like 5-aminopentanoic acid, glycerophosphocholines like PE(39:2), fatty amines, insect hormone biosynthesis, and aminoacyl t-RNA biosynthesis, which were shown to be differentially enriched in MSCM study group. Whereas Fatty acids such as leucinic acid, Glycerophosphocholines such as PE(20:2/2:0), and other metabolites like tetracyclines, chalcones, flavonoids, fatty amides, porphyrin and chlorophyll metabolism, Arginine and proline metabolism were shown to be significantly differentially enriched in MSCMFBS group **(see supplementary file Table S3 & S4)**

A


MSCM
MSCMFBS
Condition
447.3455__725.6
448.3485__725.6
212.0678__41.87
118.0863__43.69
353.2159__49.44
140.0678__744.1
270.3143__611.03
133.0856__271.22
371.2259__49.41
570.3156__294.97
353.0891__659.76
808.5797__659.9
397.2011__384.06
389.256__256.3
425.136__330.72
438.2373__744.01
271.2996__610.06
422.1939__386.32
523.3391__278.69
463.2075__396.11
432.2124__393.15
605.1845__441.07
809.5832__681.26
288.2161__369.42
641.3327__330.55
388.2526__256.92
662.3375__381.15
207.0797__529.34
485.2186__400.67
322.3455__681.01
393.2077__744.01
454.2914__666.19
347.1234__426.36
583.2028__441.34
89.0601__48.91
193.1006__475.88
821.5353__738.93
682.4843__738.83
279.1583__666.96
641.208__439.53
537.2498__316.58
535.3027__316.89
393.1717__46.46
529.28__408.42
627.3074__331.21
203.1271__587.55
434.3328__703.07
277.1791__489.05
526.2899__744.3
523.196__408.09
Low
High

B

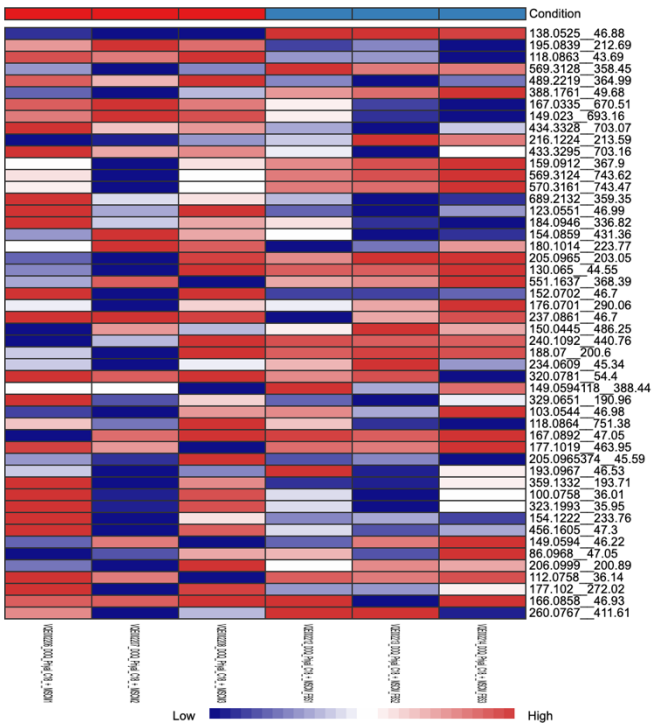

MSCM
MSCMFBS
Condition
138.0525__46.88
195.0839__212.69
118.0863__43.69
569.3128__358.45
489.2219__364.99
388.1761__49.68
167.0335__670.51
149.023__693.16
434.3328__703.07
216.1224__213.59
433.3295__703.16
159.0912__367.9
569.3124__743.62
570.3161__743.47
689.2132__359.35
123.0551__46.99
184.0946__336.82
154.0859__431.36
180.1014__223.77
205.0965__203.05
130.065__44.55
551.1637__368.39
152.0702__46.7
176.0701__290.06
237.0861__46.7
150.0445__486.25
240.1092__440.76
188.07__200.6
234.0609__45.34
320.0781__54.4
149.0594118__388.44
329.0651__190.96
103.0544__46.98
118.0864__751.38
167.0892__47.05
177.1019__463.95
205.0965374__45.59
193.0967__46.53
359.1332__193.71
100.0758__36.01
323.1993__35.95
154.1222__233.76
456.1605__47.3
149.0594__46.22
86.0968__47.05
206.0999__200.89
112.0758__36.14
177.102__272.02
166.0858__46.93
260.0767__411.61
Low
High

Figure 13. Cluster Heatmap; metabolite identification. From the cluster heatmap of the MSCM/MSCMFBS study group in positive ion mode (A) Lipid (B) KEGG database. Peak clustering method by p-value. Differentially enriched metabolites correspond to red color while low levels of enrichment correspond to blue color.

5 Discussion

There is limited literature regarding the *in vitro* development of *D. immitis* microfilariae. Therefore, the primary focus of our current project was to gain insight into the regulatory mechanisms that govern the development of these microfilariae, particularly by investigating the internal cues within the host organisms. The significance of this study lies in its potential to enhance our understanding of the *D. immitis* life cycle and to identify innovative methods for interrupting this cycle, thereby combating the alarming spread of this potentially fatal disease.

By employing a combination of DMEM and Ham's F-12, the parasites were given access to ample quantities of glucose, amino acids, and vitamins, including diamines [85], polyunsaturated fatty acid [86], organosulfur compound, purine, and pyrimidine [87], establishing a favourable milieu for their initial growth and development. To further simulate the environment of the mosquito host, the culture medium was supplemented with media conditioned by culture of *An. gambiae* (4a-3B) cell line known to closely resemble the mosquito environment. Previous research [48] indicated that this cell line significantly enhanced the growth and development of *D. immitis* microfilariae *in vitro*. Notably, it greatly increased the number of parasites in the late-sausage stage in the culture medium, and this study observed a similar trend.

The combination of DMEM/F12 culture media is highly effective for *in vitro* culturing of *D. immitis* microfilariae. However, since this culture mix lacks certain essential growth hormones, it is necessary to supplement it with FBS. A recent study observed that sera from mammals selectively activated *Dim* and *Bma*DAF-12 in iL3 larvae, indicating the presence of DA in low concentrations that activate DAF-12 and promote parasite growth and development [46].

Interestingly, in our study, the presence of FBS led to the degeneration of microfilariae, contrary to expectations. Moreover, when FBS was introduced to the culture medium at increasing concentrations, it progressively hindered the development of the parasites, with higher concentrations leading to more significant degeneration. Other researchers observed similar inhibition of development of microfilariae when cultured in Ham's F-12 medium supplemented with 10% serum [88].

This phenomenon could potentially be attributed to the similarity between FBS in the culture medium and the host environment of mammals. It is well-known that microfilariae circulate in the blood of dogs for extended periods, up to 2.5 years, in a state of arrested development, which prevents development. However, the reasons behind the degeneration of microfilariae in the presence of FBS and infected dog blood serum remained unclear.

The observed inhibition of microfilariae development in the presence of serum in the culture medium suggests the presence of an inhibitory component, possibly a lipid derived from cholesterol, that may interfere with the activity of nuclear hormone receptors (NHRs) or by some other mechanism affecting the growth and development of the parasite.

To further investigate this phenomenon, our study incorporated CS-FBS into the culture medium. Charcoal-stripping FBS is a technique that involves the removal of lipids, steroids, and other growth factors and is commonly utilized in hormone studies [89-91]. The purpose of this approach was to eliminate the inhibitory factors present in FBS and observe the impact on the development of microfilariae *in vitro*.

CS-FBS is an essential reagent in the examination of steroid hormones. It undergoes a process where it is absorbed with activated carbon, effectively removing non-polar substances such as

lipophilic materials (including certain growth factors, hormones, cytokines, and viruses) regardless of their molecular weight. However, this process has minimal effect on salts, glucose, amino acids, and other essential components present in the serum [92].

By incorporating CS-FBS in our study, we aimed to discern the specific factors within FBS that hinder the growth of microfilariae. This approach, along with untargeted metabolomics of different culture media, allowed us to investigate potential inhibitory substances, providing valuable insights into the intricate interplay between these factors and the development of the microfilariae [89, 93]. A direct comparison between CS-FBS and FBS-supplemented culture media revealed a distinct pattern: parasites exhibited robust development in CS-FBS, particularly when the concentration of CS-FBS was reduced.

By demonstrating the superior performance of CS-FBS over FBS-supplemented media and the correlation with lipid-related factors, our findings shed light on the underlying mechanisms influencing the growth and viability of microfilariae. This highlights the importance of lipid components and their potential contribution to the regulation of microfilariae development. Further exploration and characterization of these lipid-related factors are necessary to gain a comprehensive understanding of their specific roles and mechanisms of action in the context of microfilariae growth and degeneration.

The combination of DMEM/F12 conditioned with mosquito cells and supplemented with CS-FBS was chosen to be optimal for culturing *D. immitis in vitro*. What was left to further evaluate were the factors present in FBS and those secreted by mosquito cells which lead to the inhibition and stimulation of microfilariae growth *in vitro*. To determine the component(s) responsible for the initiation and inhibition of the growth of the microfilariae, the next approach

was to fractionate the metabolites of the mosquito cell-conditioned/non-conditioned media using UPLC-FTMS.

Previous literature [69] indicated the significant impact of Δ4-DA on molting of iL3 larvae in *D. immitis*. However, contrary to those findings, our study revealed negative effects when microfilariae were treated with 10 µM of Δ4-DA. Surprisingly, no significant morphological changes were observed in the parasites. In fact, compared to the control group (no DA treatment), microfilariae were unable to progress beyond the pre-sausage stages of development. In a recent study, researchers investigated DAF-12 in different nematodes, including filarial worms (*Brugia malayi* and *D. immitis*) and non-filarial nematodes (*H. contortus* and *C. elegans*). Hormone-depleted sera did not activate DAF-12 in filarial worms, but complete sera from a variety of mammals selectively activated *Dim* and *Bma*DAF-12, as observed in delayed larval development. Restoring *Dim*DAF-12 activation with Δ4-DA in charcoal-stripped mouse serum indicated mammalian serum DA's role, suggesting that filarial nematodes detect low Δ4-DA levels in their mammalian host serum [46].

Based on the literature, it was expected that *D. immitis* microfilariae would survive and develop with Δ4-DA treatment, but the possible explanation for this unexpected outcome suggests that the response of *D. immitis* microfilariae to Δ4-DA may be complex and influenced by various factors. It is evident that the effect of DA on molting is not straightforward and may depend on specific concentrations or additional regulatory mechanisms that were not fully elucidated in this study.

One possible reason for Δ4-DA having a detrimental effect on microfilariae is that 10 µM was a very high concentration. Excessive concentrations of hormones or ligands can have various effects on nuclear receptor regulation. One such scenario can lead to the downregulation of

nuclear receptors. This occurs when the high concentration of ligands saturates the receptors, triggering a negative feedback mechanism that reduces the expression or activity of the receptors. This downregulation can limit the responsiveness of the cells to the ligands and regulate their signalling pathways, potentially leading to altered gene expression patterns and disrupted cellular processes [94]. Therefore, future research is needed to carefully assess and titrate the effects of Δ4-DA on *D. immitis* microfilariae. Exploring the growth of microfilariae in media without mosquito cell conditioning, solely supplemented with CS-FBS, and the subsequent effects of adding Δ4-DA could provide valuable insights.

The addition of 20-E at 4 µM improved development of microfilariae, progressing them to later sausage stages. This effect was evident by a significant decrease in the percentage of parasites failing to molt into the advanced stages compared to the control group and other treatment concentrations of 20-E, suggesting a positive role of 20-E in microfilariae development.

Notably, earlier studies indicated that co-culturing microfilariae in mosquito-conditioned media led to the upregulation of genes involved in the ecdysone signalling pathway, such as *dim*-ecr, *dim*-rxr-1, *dim*-nhr-6, and *dim*-nhr-7 in L1 stage larvae. These findings suggest the presence of hormone ligands secreted by mosquito cells, possibly 20-E or some other component(s) that potentially activate the ecdysone signalling pathway or other pathways responsible for the development of L1 parasites [48].

To further investigate this phenomenon, it may be beneficial to design an experiment in which the culture media is devoid of these hormones originating from mosquito cells. By supplementing exogenous hormones and observing the effects on the worms, we can assess if they produce similar outcomes. It is worth noting that microfilariae are reliant on dietary

cholesterol as they cannot synthesize their own fatty acids, which adds another layer of complexity to their growth and development.

This study utilized UPLC-FTMS in combination with Metaboanalyst 5.0 [95] to investigate the metabolomic profiles of various culture media. Univariate and multivariate statistical analyses were performed to identify differentially expressed metabolites. Specifically, the study group MSCM/MSCMFBS was examined, and significant differences in metabolite abundance were observed. In addition to identifying differentially expressed metabolites, pathway analysis was conducted to understand the functional relevance of these metabolites. This analysis revealed the involvement of specific metabolic pathways and associated lipids relevant to the growth, development, or degeneration of microfilariae under *in-vitro* conditions.

In the MSCM study group, various analogs of vitamin D3 were significantly differentially expressed; these analogs included 1α,25-dihydroxy-11β-methoxyvitamin D3, 1α,25-dihydroxy-26,27-dimethyl-22-oxavitamin D3, and 24,24-difluoro-25-hydroxy-26,27-dimethylvitamin D3. The active form of vitamin D, known as calcitriol, activates the vitamin D receptor (VDR) in mammals. The VDR, belonging to the nuclear receptor family of transcription factors, forms a heterodimer complex with the RXR upon binding of calcitriol. This complex plays a crucial role in regulating several biological functions [96].

Even though calcitriol or 1,25-dihydroxy vitamin D3 is the active form of vitamin D, their analogs can be potent activators of the VDR, with some exhibiting even higher biological activity than vitamin D3. In a 1990 study, a group found 24,24-difluoro-25-hydroxy-26,27-dimethylvitamin D3 to be a highly potent vitamin D analog with bioactivity similar to that of

25-hydroxyvitamin D3 *in-vivo* [97]. The identification of these differentially expressed vitamin D3 analogs suggests their potential significance in VDR-mediated processes.

In a previous study, researchers explored the relationship between vitamin D3 and longevity using *C. elegans*. The study showed that vitamin D3 supplementation increased the lifespan of *C. elegans* in a DAF-12-dependent manner. The study aimed to investigate the impact of vitamin D on aging and age-related diseases by measuring the lifespan and health span of three *C. elegans* strains: wildtype (N2), nhr-8 mutant worms (lacking a functional vitamin D receptor ortholog), and gnals2 worms (representing Alzheimer's disease). The results showed that treatment with vitamin D3 had significant effects on the lifespan of the worms. It extended the lifespan of N2 worms and rescued the decreased lifespans of nhr-8 worms compared to N2 [98, 99]. In summary, the study highlighted the positive effects of vitamin D3 on the lifespan and health of *C. elegans*. The differential gene expression patterns provided insights into the potential mechanisms underlying these effects, particularly related to the innate immune response and xenobiotic metabolism.

An interesting observation is that the VDR shares a relationship with the ecdysone receptor in *Drosophila* and the DAF-12 NR in *C. elegans* [100], where these receptors, as discussed before, play a crucial role in regulating life history decisions in nematodes and arthropods. Remarkably, the synthesis of DAs, ecdysteroids, and vitamin D3 all rely on a common initial precursor called 7-dehydroxycholestrol (7-DHC). Suggesting that 7-DHC-dependent hormone synthesis could represent a specialized hormonal signalling pathway specific to the environmental conditions in different organisms. The chemistry of these hormones and the sequence of NRs likely evolved in response to each organism's natural environment, highlighting the adaptive nature of hormonal signalling across the animal kingdom [101].

The conserved nature of these receptors and the dependence of their ligands on a common precursor suggest a potential interplay between the factors present in the MSCM/MSCMFBS and the activation or inhibition of these receptors. This interplay may contribute to the regulation of the parasite's growth and development. The presence of these vitamin D3 analogs can be understood now to have a positive effect on *D. immitis* microfilariae in MSCM study group. However, further research is needed to culture microfilariae with these analogs and examine their specific effects on the developmental regulation of microfilariae. This investigation will provide deeper insights into the potential benefits and mechanisms of action associated with vitamin D3 analogs in the context of microfilariae development.

The study group of MSCM/MSCMFBS showed the expression of glycerophospholipids, glycerophosphocholines, glycophosphatidylinositol and FAs.

These components play a significant role as substrates for the production of eicosanoids. Arachidonic acid (ARA) is a polyunsaturated FA consisting of 20 carbons. Intracellularly, ARA is mainly esterified into glycerolipids, including phospholipids and neutral lipids, with a smaller fraction existing as free FAs [102].

Following its release by phospholipase A_2 (PLA2), free ARA can undergo oxygenation through different enzymatic pathways depending on the cell type. These pathways include the cyclooxygenase (COX) pathway, resulting in the synthesis of prostaglandins and thromboxane; the lipoxygenase (LOX) pathways, leading to the production of hydroxy eicosatetraenoic acids (HETEs), leukotrienes (LTA, LTE), and lipoxins; and the cytochrome P450 pathway, involving monooxygenase enzymes that generate hydroxy and epoxy derivatives [89]. These eicosanoids are local lipid mediators, distinct from stable circulating peptides. They are

promptly released after synthesis, exerting potent effects on neighboring cells. Eicosanoids rapidly break down into inactive metabolites, limiting systemic impact. Notable actions include inhibiting platelet aggregation (prostacyclin, PGE, PGD) and down-regulating T cell responses (PGE), potentially serving an immunoregulatory function [103, 104].

Filarial parasites rely on polyunsaturated FAs obtained from the host since they cannot produce ARA themselves. In the initial study on ARA metabolism in a nematode organism, it was demonstrated that microfilariae of *B. malayi* efficiently absorb and incorporate exogenous ARA into phospholipids [105]. Subsequent studies indicate that microfilariae have the ability to metabolize ARA into eicosanoids. Following short-term incubation with exogenous ARA, microfilariae of *B. malayi* synthesize and release prostaglandins, primarily prostacyclin and PGE2. In long-term incubations, these prostaglandins are formed using endogenous stores of ARA [106]. Additionally, although limited studies have been conducted on adult filariae due to the scarcity of parasite samples, adult *Brugia* worms also incorporate exogenous ARA [107] and release PGE2 [108].

These findings suggest that *D. immitis* microfilariae may possess a similar ability to utilize host-derived polyunsaturated FAs to generate specific eicosanoids. These synthesized eicosanoids may then be released into the local environment, as expected for molecules involved in mediating interactions with neighboring cells. The production of specific eicosanoids with selective effects on host cells may play a crucial role in the survival and pathogenicity of *D. immitis* microfilariae in mammalian hosts.

Furthermore, in the MSCM study group, the significant differential expression of 3β,5β-Ketotriol was observed. This metabolite is known to be involved in insect hormone

biosynthesis and is derived from 7-DHC, which is a derivative of cholesterol. It is possible that 3β,5β-Ketotriol plays a role in the production of 20-E or is directly involved in activating the ecdysone receptor, thereby facilitating the molting and development of the microfilariae in an *in-vitro* setting.

The MSCMFBS study group showed a significant differential expression of tetracyclines, flavonoids, chalcones, D-Proline associated with arginine and proline metabolism and protoporphyrinogen IX (PIX) associated with porphyrin metabolism. Tetracyclines are known for their anti-filarial activities, as demonstrated by a previous study conducted *in vitro* with microfilariae showing that increasing concentrations of tetracycline (20-100 μg/ml) led to a significant loss of microfilarial motility, indicating its potent anti-filarial effect [109]. Moreover, a dose-dependent association was observed between agents with high anti-filarial activity and oxidative parameters. Additionally, various flavonoids and their analogs have been shown to possess anti-leishmanial, anti-protozoal, and anti-trypanosomal activities [110-112]. Interestingly, D-proline, which was found to be significantly enriched in the MSCMFBS study group, has implications in a metabolic disorder known as hyperornithinemia with gyrate atrophy (hoga) in humans [113, 114]. It is important to note that D-proline is considered a potentially toxic compound.

PIX is formed through the oxidative decarboxylation of coproporphyrinogen. Under specific conditions, PIX can exhibit characteristics of phototoxin, neurotoxin, and metabotoxin. Phototoxicity refers to cellular damage caused by exposure to light, while neurotoxicity results in harm to nerve cells and tissues. Metabotoxins can lead to adverse health effects when present at high levels for extended periods of time [115-118]. In a previous study, PIX showed promising potential as a treatment for leishmaniasis by inhibiting the growth of *Leishmania* promastigotes compared to DMSO controls. The effectiveness of PIX varied depending on the

concentration and light exposure [119]. Additionally, another study highlighted the antileishmanial activity of certain chalcones [120].

The presence of several metabolites identified in the MSCMFBS study group has demonstrated inhibitory effects on other parasites, indicating a possible collective action in impeding the development of microfilariae *in vitro*. This collective action may explain why the microfilariae were unable to survive beyond day 13 when cultured in FBS-supplemented culture media. These findings highlight the possible detrimental impact of these metabolites on microfilariae survival and growth in FBS-supplemented culture media.

Further investigations are needed to elucidate and validate the specific contributions of these metabolites to microfilariae development. This will help us understand the precise mechanisms by which these factors interact with the receptors and influence the stimulation or inhibition of the growth and development of the parasite. Conducting follow-up experiments can help us gain a deeper understanding of the functional implications of these pathways and lipids, potentially uncovering novel targets for intervention and control of microfilariae development.

6 Conclusion

The emergence of drug resistance has become a significant challenge in the ongoing efforts to prevent heartworm infection. In order to overcome this obstacle, gaining a deeper understanding of the microfilariae stage of *Dirofilaria immitis* could potentially open up new avenues to disrupt the parasite's life cycle. Therefore, the objective of this study was to investigate the developmental regulation of *D. immitis* microfilariae and examine the influence of the mosquito host environment and ecdysteroids on larval growth. By exploring these factors, we aimed to uncover crucial insights that could inform the development of novel strategies for combating heartworm infection.

This study focused on investigating the developmental regulation of *Dirofilaria immitis* microfilariae. To facilitate their *in-vitro* cultivation, a culture media mixture of DMEM and Ham's F-12 in a 1:1 ratio was utilized. The media was additionally conditioned with mosquito *Anopheles gambiae* cells. Interestingly, the presence of FBS in the media was found to hinder parasite growth and motility. However, when the FBS was charcoal-stripped, an opposite effect was observed, resulting in enhanced development and motility of the microfilariae in the *in-vitro* setting.

Previous research has demonstrated that Δ4-DA has biological effects on iL3-stage larvae, promoting their transition to the L4 stage. However, in the present study, Δ4-DA was found to have negative effects on the development of microfilariae. Conversely, treating the microfilariae with 20-E led to a significant reduction in the number of microfilariae failing to molt into later sausage stages.

By employing untargeted metabolomics, this study yielded valuable insights into the potential mechanisms and biological pathways that influence the growth and development of *D. immitis*

microfilariae. The examination of metabolomic profiles in different culture media provided a deeper understanding of their impact on microfilariae growth. The identification of differentially expressed metabolites, and their associated pathways enhances our comprehension of the underlying mechanisms governing microfilariae development. These findings contribute to future research endeavours aimed at targeting the life cycle of parasitic organisms and devising innovative strategies to combat parasitic diseases. Notably, this study revealed that hormones derived from 7-DHC and their associated NRs represent a conserved pathway for the developmental regulation of *D. immitis*. Additionally, the metabolites identified in FBS-supplemented media likely work together to inhibit the growth of *D. immitis* microfilariae *in vitro*. Furthermore, the study indicates the involvement of ARA in the innate immunity of *D. immitis*, thereby stimulating its development *in vitro.*

However, these findings also raise numerous unanswered questions. For future investigations, it is important to determine: (1) whether the identified metabolites exert a direct effect on the development of *D. immitis* microfilariae *in vitro*, (2) the potential regulation of genes involved in the Ecdysone, DAF-12, and VDR signalling pathways by these components, and (3) how to better optimize the *in vitro* culture conditions for microfilarial development. These areas of focus will contribute to further advancing our understanding of *D. immitis* development and aid in finding novel interventions to prevent this parasitic disease and the development of more effective interventions against this parasitic disease. Knowledge of regulation of the development of microfilariae might be exploited to disrupt the life cycle, for example, by causing precious and lethal development of the microfilariae in the mammalian host or by blocking the microfilarial development in the mosquito.

7 References

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Supplementary File:

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Figure S1. **Co-culturing *D.immitis* with 10 μM Δ4 Dafachronic Acid or 2.5% CS-FBS treated every day.**

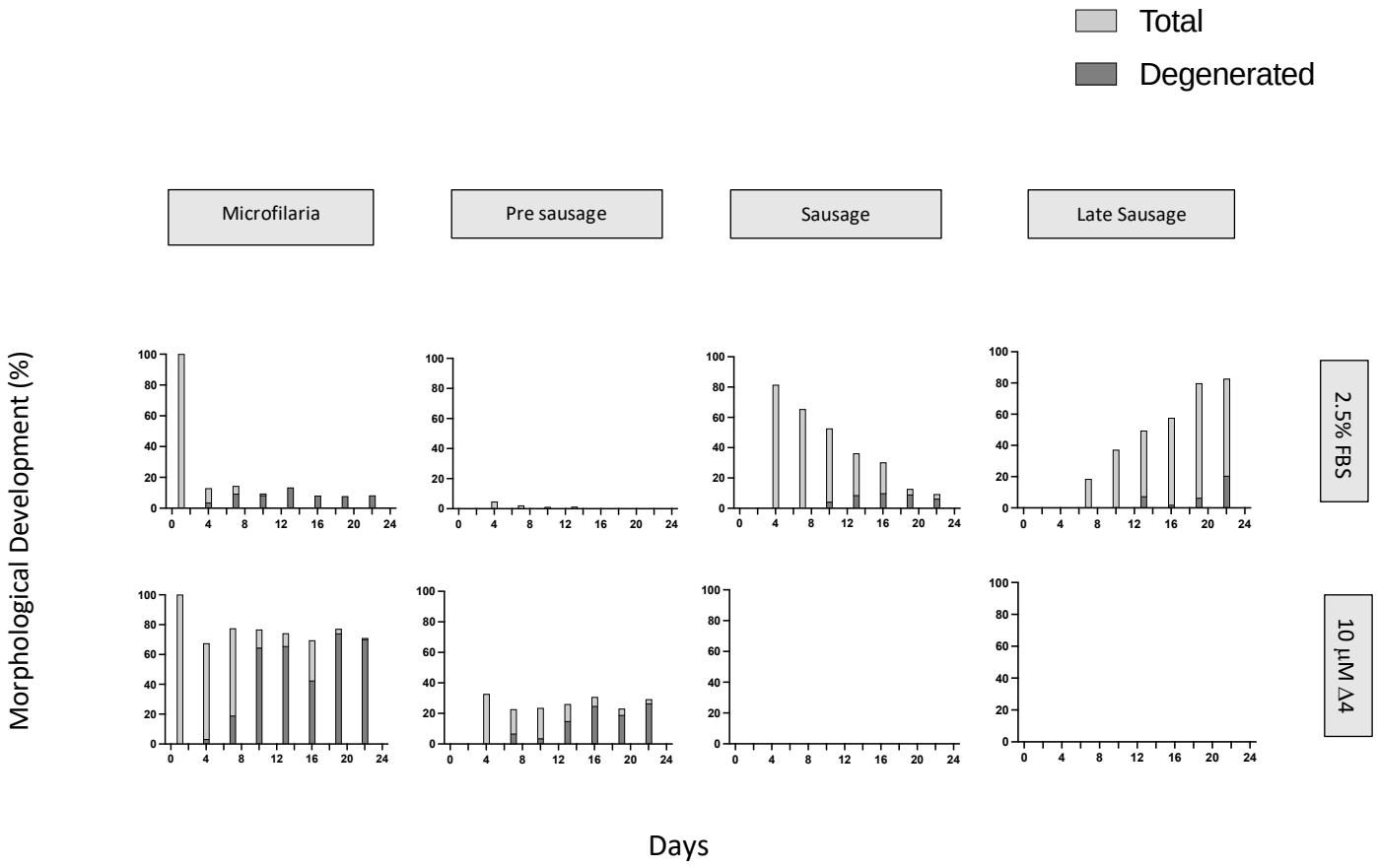

Table S1: Lipid database, pathway hits in MSCM/MSCMFBS in mixed ion mode.

Pathway hits	Total_Size	Hits	Sig_Hits	Mummichog_Pvals	GSEA_Pvals	Combined_Pvals
Phosphosphingolipids	4581	106	32	0.00448	0.07143	0.00289
Eicosanoids	297	52	44	0.00473	0.2353	0.00868
Glycerophosphocholines	3120	74	12	0.05178	0.09722	0.03167
Fatty aldehydes	29	4	3	0.0829	0.08511	0.04201
Bile acids	125	37	17	0.3269	0.06349	0.1012
Stilbenes	11	2	1	0.4998	0.04167	0.1015
Quinones and hydroquinones	35	6	2	0.3269	0.1026	0.1474
Macrolides and analogues	43	5	1	0.4998	0.09259	0.1885
Fatty amides	141	16	1	0.8584	0.07246	0.235
Tannins	9	1	1	0.129	0.5208	0.2486
Sphingoid bases	78	11	3	0.1269	0.5882	0.2683
Hydroxycinnamic acids	11	2	1	0.2415	0.3556	0.2967
Glycerophosphoserines	3031	106	23	0.3143	0.2941	0.3126
Steroid conjugates	26	3	2	0.2415	0.4	0.3224
Glycerophosphoethanolamines	3153	111	14	0.187	0.6418	0.3745
Octadecanoids	131	77	23	0.2657	0.4545	0.376
Isoprenoids	423	61	7	0.8409	0.1538	0.3938
Glycerophosphoglycerols	3058	112	26	0.5594	0.2333	0.3963
Phenylpropanoids	36	14	3	0.2288	0.6562	0.4348
Ceramides	5424	75	2	0.7139	0.2222	0.4507
Fatty acids	799	144	17	0.654	0.2568	0.4676
Fatty alcohols	50	8	1	0.4252	0.4043	0.4746
Hydrocarbons	67	6	2	0.4252	0.4545	0.5109
Steroids	173	27	4	0.4885	0.4194	0.5297
Aromatic polyketides	13	5	1	0.4998	0.6667	0.6994
Glycerophosphates	2996	93	7	0.9277	0.4328	0.7679
Fatty esters	569	39	6	0.8802	0.4857	0.7908
Glycosphingolipids	13440	180	24	0.8771	0.4928	0.7948
Glycerophosphoinositols	3219	104	9	0.9948	0.5692	0.8883
Flavonoids	287	49	5	0.9934	0.7879	0.9745
Sterols	188	16	1	0.9075	1	0.9956

***Table S2*: KEGG database ,pathway hits in MSCM/MSCMFBS in mixed ion mode.**

Pathway hits	Total_Size	Hits	Sig_Hits	Mummichog_Pvals	GSEA_Pvals	Combined_Pvals
Porphyrin and chlorophyll metabolism	21	2	1	0.03876	0.01667	0.00539
Arachidonic acid metabolism	12	3	3	0.1224	0.05769	0.04205
Vitamin B6 metabolism	8	1	1	0.1224	0.2353	0.131
Arginine and proline metabolism	30	5	1	0.4121	0.1667	0.2527
Ubiquinone and other terpenoid-quinone biosynthesis	9	2	1	0.231	0.2982	0.2532
One carbon pool by folate	8	5	1	0.5534	0.1364	0.2705
Tryptophan metabolism	30	10	3	0.3447	0.6857	0.5773
Tyrosine metabolism	33	9	2	0.3532	0.7576	0.6203
Insect hormone biosynthesis	21	5	1	0.4872	0.8226	0.7672
Aminoacyl-tRNA biosynthesis	22	8	1	0.8118	0.7576	0.914

***Table S3* : Compound identification, lipid database, in positive and negative ion mode**

Query.Mass	Matched.Compound	Matched.Form	Retention.Time	Mass.Diff	Empirical.Compound
447.3455	1alpha,25-Dihydroxy-11beta-methoxyvitamin D3	M+H[1+]	725.6	0.00137646677001158	EC00034
447.3455	1alpha,25-Dihydroxy-26,27-dimethyl-22-oxavitamin D3	M+H[1+]	725.6	0.00137646677001158	EC00034
447.3455	24,24-Difluoro-25-hydroxy-26,27-dimethylvitamin D3	M-H2O+H[1+]	725.6	0.00222353322999425	EC00052
448.3485	Stylisterol B	M(C13)+H[1+]	725.6	0.00178646676999961	EC00034
448.3485	1alpha,25-Dihydroxy-11beta-methoxyvitamin D3	M(C13)+H[1+]	725.6	0.00177646677002485	EC00034
448.3485	1alpha,25-Dihydroxy-26,27-dimethyl-22-oxavitamin D3	M(C13)+H[1+]	725.6	0.00177646677002485	EC00034
118.0863	5-Aminopentanoic acid	M+H[1+]	43.69	4.493222999713E-05	EC00054
118.0863	2S-Amino-pentanoic acid	M+H[1+]	43.69	2.35332300064783E-05	EC00054
133.0856	3-hydroxyhexanoic acid	M+H[1+]	271.22	0.000320712770019327	EC00039
133.0856	3-Hydroxyhexanoic acid	M+H[1+]	271.22	0.000276466770003481	EC00039
133.0856	5-Hydroxyhexanoic acid	M+H[1+]	271.22	0.000320716770005447	EC00039
133.0856	6-Hydroxyhexanoic acid	M+H[1+]	271.22	0.000320716770005447	EC00039
133.0856	D-Leucic acid	M+H[1+]	271.22	0.000320716770005447	EC00039
133.0856	Hydroxyisocaproic acid	M+H[1+]	271.22	0.000320716770005447	EC00039
133.0856	Leucinic acid	M+H[1+]	271.22	0.000320716770005447	EC00039
133.0856	2-Ethyl-2-Hydroxybutyric acid	M+H[1+]	271.22	0.000320716770005447	EC00039
133.0856	2-Hydroxy-3-methylpentanoic acid	M+H[1+]	271.22	0.000320716770005447	EC00039
133.0856	2-Hydroxycaproic acid	M+H[1+]	271.22	0.000320716770005447	EC00039
570.3156	PC(15:1/4:1)	M+Na[1+]	294.97	0.00108346676995552	EC000359
570.3156	PC(17:2/2:0)	M+Na[1+]	294.97	0.00108346676995552	EC000359
570.3156	PC(19:2)	M+Na[1+]	294.97	0.00108346676995552	EC000359
570.3156	PE(18:1/4:1)	M+Na[1+]	294.97	0.00108346676995552	EC000359
570.3156	PE(18:2/4:0)	M+Na[1+]	294.97	0.00108346676995552	EC000359
570.3156	PE(20:2/2:0)	M+Na[1+]	294.97	0.00108346676995552	EC000359
808.5797	PC(14:0_22:2)	M+Na[1+]	659.9	0.00303346676992078	EC000357
808.5797	PC(14:1_22:1)	M+Na[1+]	659.9	0.00303346676992078	EC000357
808.5797	PC(16:0_20:2)	M+Na[1+]	659.9	0.00303346676992078	EC000357
808.5797	PC(16:1_20:1)	M+Na[1+]	659.9	0.00303346676992078	EC000357
425.136	12-Dehydrotetracycline	M-H2O+H[1+]	330.72	0.00170753323004647	EC00025
425.136	Methacycline	M-H2O+H[1+]	330.72	0.00170784323000817	EC00025
389.256	N-Linoleoyl taurine	M(C13)+H[1+]	256.3	0.00102353322995441	EC000315
270.3143	Octadecylamine	M+H[1+]	611.03	0.00122671976998845	EC000327

275.0676	3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid	M+Cl[-]	47.56	0.00107362199997851	EC00043
347.2438	Sativic acid	M-H[-]	359.86	0.000123533229952955	EC000246
668.1936	Nevadensin 5-gentiobioside	M(C13)-H[-]	73.09	0.00217646676992445	EC000142
668.1936	Jaceidin 7-neohesperidoside	M(C13)-H[-]	73.09	0.00217646676992445	EC000142

***Table S4*: Compound identification, metabolites identified in the KEGG database, in positive and negative ion mode**

Query.Mass	Matched.Compound	Matched.Form	Retention.Time	Mass.Diff	Empirical.Compound
138.0525	C00148	M+Na[1+]	46.88	0.000105003769988343	EC0007
138.0525	C00763	M+Na[1+]	46.88	0.000105003769988343	EC0007
569.3128	C01079	M+H[1+]	358.45	0.000567745230000583	EC00040
569.3124	C01079	M+H[1+]	743.62	0.000167745229987304	EC00039
570.3161	C01079	M(C13)+H[1+]	743.47	0.000467745229911998	EC00039
434.3328	C16494	M(C13)+H[1+]	703.07	0.00183646676998706	EC00054
433.3295	C16494	M+H[1+]	703.16	0.00173646677001216	EC00054
388.1761	C00445	M-HCOONa+H[1+]	49.68	0.000892094229982376	EC00021
118.0863	C00183	M+H[1+]	43.69	4.493222999713E-05	EC00011
118.0863	C00719	M+H[1+]	43.69	4.493222999713E-05	EC00011
195.0839	C16595	M(C13)+H[1+]	212.69	0.000669689770006698	EC00058
195.0839	C16587	M(C13)+H[1+]	212.69	0.000669689770006698	EC00058