Detection of microfilariae (Nematoda: Filarioidea) in mosquito vectors trapped at bird and wildlife facilities in Durban, South Africa

By

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DECLARATION

I declare that this dissertation was compiled by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.

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Dr Louwrens Pieter Snyman _____ Date: _____

DEDICATION

TO MY MUM,

whose love and sacrifice lead to all that I am today.

TO MY HUSBAND,

my best friend and soul mate.

AND TO MY DAD

always on my mind, forever in my heart.

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LIST OF ABBREVIATIONS

AgriSETA	Agricultural Sector Education and Training Authority
BLAST	Basic Local Alignment Search Tool - NCBI
COI	Cytochrome Oxidase I
CROW	Centre for the Rehabilitation of Wildlife
DNA	Deoxyribonucleic acid
ITS2	Internal transcribed spacer 2
MAFFT	Multiple alignment using fast Fourier transform
MEGA7	Molecular Evolutionary Genetics Analysis version 7.0
PCR	Polymerase chain reaction
RAxML	Randomized Axelerated Maximum Likelihood
sp or spp.	Species
UBRP or UBP	Umgeni River Bird Park
WHO	World Health Organization
%	Percentage
°C	degrees Celsius

ABSTRACT

Detection of microfilariae (Nematoda: Filarioidea) in mosquito vectors trapped at animal shelters in Durban, South Africa

Mosquitoes belonging to the genera, *Aedes, Anopheles, Coquillettidia, Culex* and *Mansonia* are vectors of microfilariae (Superfamily: Filarioidea), the specialised larvae of filarial nematodes that infect the lymphatic system, tissues and body cavities of vertebrates. It was previously established that the microfilariae of *Dirofilaria repens* and *Acanthocheilonema reconditum* circulate within the domestic animals in South Africa, however, there is a lack of information relating to the presence and prevalence of microfilariae harboured in mosquito vectors in the region. The objectives of this study were to: i. capture mosquitoes from two animal-dense localities, namely, Centre for the Rehabilitation of Wildlife (CROW) and the Umgeni River Bird Park located in eThekwini (Durban) Metropolitan Municipality, KwaZulu-Natal (KZN) Province in South Africa and identify them using morphological means; ii. determine if microfilariae are present in the mosquito species captured from the two animal-dense sites iii. determine the prevalence of microfilariae in potential mosquito vectors by means of dissection and microscopy and iv. infer molecular identifications of microfilariae in potential mosquito vectors collected from areas with high animal densities in Durban, KZN.

To adress the objectives of the study, mosquitoes were captured from the Centre for the Rehabilitation of Wildlife (CROW) and the Umgeni River Bird Park (URBP) in

Durban per month from April 2021 to March 2022. All collected mosquitoes were identified morphologically using standard taxonomic keys.

The prevalence of microfilariae in wild-caught mosquitoes, was determined by microscopically dissecting the mosquitos' Malpighian tubules, thoracic muscles, head and mouthparts in search of microfilariae. Of the total number of mosquitoes collected (n= 2886), 1490 moquitoes were manually dissected. More specifically, a minimum of 59 mosquitoes per month at each of the two study localities was calculated to be dissected with 95% confidence level and a 5% margin of error, using Raosoft® (Sample Size Calculator; Raosoft inc.). The sample size was based on estimated 4% prevalence of microfilariae obtained from a study in West Africa (Abogye-Antwi et al., 2015).

The remaining 1396 mosquitoes that were collected from the two sites, were pooled into conspecific groups for further molecular screening. Polymerase chain reaction (PCR) was used to detect Filarioidea DNA from the mosquitoes using primer pairs designed to amplify the internal transcribed spacer-2 (ITS2) and the filarial mitochondrial DNA cytochrome oxidase subunit I (COI) gene regions. The amplicons for the COI and ITS2 genes were cloned to determine if multiple infections were present. In addition, to infer potential identity, a BLASTn search and phylogenetic inference using a maximum likelihood approach was employed.

A total of 1490 female mosquitoes were dissected during the study period, with 765 mosquitoes collected at from CROW and 725 collected at URBP. Across both sites, twelve mosquito species were caught and divided into pools including, *Culex pipiens* (s.l) (54.1%), *Mansonia africana* (32.0%), *Coquillettidia microannulata* (5.6%), *Aedes aegypti* (5.2%), *Anopheles* species (1.2%), with the remaining 1.9% (n=27) comprising

seven other species including *Eretmapodites quinquevitattus, Culex neavei, Cx. subdentatus, Cx. cinerrellus, Cx. duttoni, Cx. simposoni* and *Cx. univitattus*. The most abundant mosquito species were *Culex pipiens* (s.l) (54.1%) and *Mansonia africana* (32.0%), both of which have a high transmission potential for microfilariae. No microfilariae were detected in the dissected mosquitoes. This result demonstrated that prevalence of microfilariae circulating in the most common mosquitoes in Durban was lower than 4%, as used to calculate the sample size of mosquiotes dissected in the study.

Amplification of the COI and ITS2 genes of microfilarial DNA determined that one *Cx. pipiens* pool was found to be positive with Onchocercidae sp, thus supporting *Cx. pipiens* as a possible vector in the region. Phylogenetic analysis did not yield a genus or species level identification as variation in COI and ITS2 topologies, because molecular libraries available for the Onchocercidae were incomplete, and could not support a definitive identification. The ITS2 provided the most acceptable topology (81% bootstrap support; 99.05% identity), inferring that the positive Onchocercidae sp. is closely related to *Mansonella*.

It is recommended that, to determine true prevalence of microfilariae, the two common mosquito species in Durban, *Culex pipiens* and *Mansonia africana*, should be targeted for surveillance. Additionally, sampling should be intensified between November and February (wet season) in which a larger sample size should be collected due to the very low prevalence in the eThekwini Metropolitan Municipality, KZN, South Africa.

CHAPTER 1: General Introduction

1.1 Background

Filarial helminths are thread-like endoparasitic roundworms in the superfamily Filarioidea (Spirurida: Spirurina). The members of this taxonomic group have medical and veterinary importance because they infect most vertebrates, which serve as definitive (final) hosts (Anderson 2000). In addition, the worms have an indirect life cycle, with haematophagous arthropods serving as biological vectors and intermediate hosts (Anderson, 2000; Otranto et al., 2015).

The elongated and narrow morphology of the worms facilitates passage in both the blood capillaries of the definitive host as well as the arthropod vector's gastrointestinal tract (Anderson, 2000; Cancrini & Gabrielli, 2007). Each filarial worm species has a specific predilection site within the vertebrate definitive hosts, such as subcutaneous connective tissue (e.g., *Dirofilaria repens*), lymphatic system (e.g., *Wuchereria bancrofti*) or the heart and lungs (e.g., *Dirofilaria immitis*) (Anderson, 2000; Siers et al., 2010). Within the confines of the predilection sites, the worms develop into sexually mature adults and are seemingly protected. They can remain undetected for several years, often without prompting an immune response from the host (Orihel & Eberhard, 1998; Bravo-Barriga et al., 2016; Capelli et al., 2018). Female filariae produce larvated eggs which hatch inside the uterus releasing thousands of highly specialised larvae (microfilariae) into the host tissues, that circulate in blood awaiting transmission by vectors (Anderson, 2000; Genchi et al., 2007; Schwan, 2009).

The transmission of filariasis is dependent on haematophagous arthropod vectors known to feed on the blood of vertebrates, primarily mosquitoes from the genera, *Aedes*, *Anopheles*, *Armigeres*, *Coquillettidia, Culex*, *Mansonia*, *Ochlerotatus* and

Psorophora (Ahid et al., 2000; Anderson, 2000; Siers et al., 2010). As biological vectors, mosquitoes have particular significance in the development of the microfilariae into the infective third (L3) stage (Otranto et al., 2015).

When a mosquito takes a blood meal of an infected definitive host, they ingest the newly released microfilariae (Capelli et al., 2018). In the mosquito, the microfilariae move into the Malpighian tubules from the gut, wherein they moult into second stage larvae (L2) and then infective L3 (Capeilli et al., 2015). In preparation for transmission to a new host, the mouthparts of the mosquitoes become laden with infective L3, some of which exude from the tip of the proboscis (Anyanwu et al., 2000). During a mosquito's blood meal, the microfilariae enter the vertebrate host through the incision in the dermis (Ahid et al., 2000; Sehgal et al., 2005).

Presence of readily available vector-competent mosquitoes is one of the main factors influencing transmission of microfilariae (Anyanwu et al., 2000). Mosquitoes that display vector competence need to be both susceptible to microfilariae invasion and able to sustain the development of the microfilariae to the infective stages. Without the presence of susceptible mosquitoes in the natural environment, infective microfilariae would not have a means to infect definitive hosts to perpetuate their life cycle. Therefore, the study of microfilariae in their mosquito vectors adds knowledge to host-parasite relationships and is vital in assisting in control efforts in areas where filarial infection is widespread. Due to the significance of mosquitoes in transmitting microfilariae, fundamental knowledge of the mosquito species responsible for the transmission of microfilariae is essential to understanding the epidemiology and biology of the filarial nematodes.

1.2 Justification

There is high transmission potential for mammalian and avian filariasis in subtropical regions, due to suitable climatic conditions, including high temperatures and rainfall, which creates favourable breeding, mating and resting sites for mosquitoes. Two species, *Dirofilaria repens* and *Acanthocheilonema reconditum* are confirmed as endemic in companion animals in South Africa, with the highest prevalence occurring in Kwazulu-Natal Province (Schwan, 2009). Despite this, mosquito vectors of filarial nematodes are given little to no attention in South Africa. The Durban Metropolitan Municipality in the KwaZulu-Natal Province of South Africa provides an ideal natural study site, characterized by its warm and humid climate in which mosquitoes thrive. Though the pathogenesis and prevalence of filarial nematodes in companion animals has been investigated in the Durban area, vector studies have not been carried out despite the importance of vectors in disease transmission (Schwan, 2009). Though the transmission and pathogenicity of the major diseases-causing mosquito-borne filarial nematodes has gained attention, there are uncertainties about the mosquito species that act as vectors of these nematodes in subtropical Africa.

1.3 Aim and Objectives

The overall aim of this study was to determine the presence of filarial nematode larvae (microfilariae) in mosquitoes found in Durban, Kwazulu-Natal Province, South Africa.

The specific objectives were:

i. To identify the mosquito species that are potential vectors of microfilariae in Durban, Kwazulu-Natal.

ii. To determine the occurrence and prevalence of microfilariae in mosquito populations in Durban, Kwazulu-Natal.

1.4 Hypotheses

H_{0i}: There are no microfilariae present in mosquito populations in Durban, KwaZulu-Natal Province, South Africa.

H_{0ii}: There are no mosquito species that have the potential to transmit microfilariae in Durban, KwaZulu-Natal Province, South Africa

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CHAPTER 2: Literature review

A review of mosquito-borne microfilariae (Nematoda: Filarioidea) from the Afrotropical region, with emphasis on the vectors.

2.1 Introduction

Mosquito-borne filarial nematodes have received worldwide attention due to their importance in veterinary and human medicine as well as conservation (Edgerton et al. 2020). A range of filarial helminths found in subtropical Africa are transmitted from one vertebrate host to another by mosquitoes (Anyanwu et al., 2000; Cancrini et al., 2007). This chapter reviews the current state of knowledge of the epidemiology and distribution of filarial helminths affecting mammals and birds in subtropical Africa and the role of mosquitoes in transmission of their larvae (microfilariae). The methods used to detect filarial helminths in potential vectors are also discussed.

2.2 Microfilariae in mosquitoes within subtropical Africa

2.2.1 *Wuchereria bancrofti*

In much of the subtropical Africa, except for the southern-most countries, including South Africa and its neighbours, *Wuchereria bancrofti* is endemic (Cano et al., 2014). *Wuchereria bancrofti*, causes Bancroftian human lymphatic disease (elephantiasis) which damages the lymphatic system, resulting in build-up of fluid and swelling of the extremities of the body including, the arms, legs and genitalia (Hoerauf et l., 2011). This species is the source of 90% of infections. A further two species, *Brugia malayi* and *Brugia timori* are responsible for the remaining infections in tropical and

subtropical regions in Asia, South America and Indonesia but are not prevalent in the Afrotropics (Manyi et al., 2014). *Wuchereria bancrofti* causes debilitating disease for 406 million people in Africa (Cano et al., 2014). Additionally, it is one of the neglected tropical diseases (NTD) prioritised by the World Health Organisation (WHO) in which the elimination programme strategy involves mass chemotherapy with anthelminthic prophylaxis and vector control.

Two mosquito genera, *Culex and Anopheles* have been implicated in transmission of *W. bancrofti* in the Afrotropics. On the eastern Kenyan coast, in the 1960s, nearly 40 000 mosquitoes encompassing thirteen mosquito species were examined for *W. bancrofti*. Microfilariae were reported to be present in *Culex pipiens f. quinquefasciatus* (0.28%), *Anopheles gambiae (*0.09%) and *An. funestus (*0.07%) following dissections (Nelson et al., 1962). More recently, the three mosquito species were yet again found to harbour microfilariae following dissections and molecular screening of 1632 mosquitoes from six villages in Kenya. This finding confirmed the status of *Cx. pipiens f. quinquefasciatus*, *An. gambiae* and *An. funestus* as vectors of *W. bancrofti* in eastern Africa, even reporting similar infection rates of 1.7-1.8%, 0.3-0.4% and 0.1%, respectively (Kinyatta et al., 2018). The infectivity rates for *W. bancrofti* in the three mosquito species were calculated by assessing the percentage of infective L3 in the mosquito salivary glands and was found to be 1.5% (Kinyatta et al., 2018).

Nelson and colleagues posited the importance of *Cx. pipiens f. quinquefasciatus* in transmission in any area that it is prevalent provided climatic conditions are suitable and breeding sites are plentiful (Nelson et al., 1962). *Culex pipiens f. quinquefasciatus* is also capable of transmitting *W. bancrofti* in areas where the filaroid is found in low prevalence, suggesting that it is the most susceptible vector (Nelson et al., 1962). It

should however be noted that, all three species are effective vectors depending on their abundance in a given area (Nelson et al., 1962).

Similarly, in West Africa, *Cx. pipiens f. quinquefasciatus* is a major vector of *W. bancrofti*, while *Anopheles gambiae, An. funestus* and some *Aedes* spp. are considered potential vectors (Manyi et al., 2014). In Makurdi, Nigeria, 4320 *Culex* and *Anopheles* mosquitoes were dissected and examined for microfilariae and important vectors were found to be *Cx. quinquefasciatus* (7.5%), *Anopheles funestus* (1.4%) and *A. gambiae* (0.9%) (Manyi et al., 2014).

In a comparable study conducted in four local communities in Andoni, northern Nigeria over four months, *Cx. pipiens f. quinquefasciatus* (4.5-24.0%), *An. gambiae* (2.5-23%) and *Aedes* sp. (0.0-0.1%) were found to be infected by microfilariae (Dimpka et al., 2019). *Aedes* mosquitoes are less of a concern in the region due to the low prevalence and perhaps the lack of suitable breeding sites preferred by *Aedes* such as man-made clogged drainage and sewage pipes, often absent in rural communities (Dimpka et al., 2019). In areas where vectors (e.g., *Cx. quiquefasciatus*) are in high abundance and available perennially, there is continuous transmission of the parasite resulting in a high incidence in man (Dimpka et al., 2019).

2.2.2 *Brugia patei*

Brugia patei (previously *Wuchereria patei*) was found for the first time in 1957 by Nelson and Heisch (1957) and was so named after the origin of its discovery in Pate Island of Kenya in East Africa and to date its endemisity is limited to northern coast of Kenya (Heisch et al., 1962). The species was found to infect mainly domestic cats but

also dogs, with reservoirs occurring in wild genets and bush babies (Nelson & Heisch, 1957; Heisch et al., 1959; Nelson et al., 1962). In the vertebrate host, the adult worms were found to be located in the lymphatics of the hind legs or abdomen and the microfilariae were found in the ascitic fluid of the abdomen (Heisch, et al., 1959).

In a laboratory setting, following infection of wild mosquitoes, *Mansonia africana* and *Ma. uniformis* by feeding on *B. patei*-infected cats, the microfilariae were observed in the mosquitoes and successfully reached infectivity in at least six days, confirming the vector status of the two *Mansonia* species (Nelson et al., 1962; Laurence & Pester, 1961). Further experimental observations were performed by Heisch et al., (1959) on wild-caught *Aedes pembaensis* originating from the Faza region of Pate Island and it was found that the infected mosquitoes allowed for the development of infective larvae in eight days concluding that, *Ae. pembaensis* was a susceptible vector of *B. patei*. It was also noted that in areas where *Mansonia* was not present, *Aedes pembaensis* was the vector (Nelson et al., 1962). A further study observed development of *B. patei* microfilariae in *Anopheles gambiae*, a common vector of *Wuchereria bancrofti* in Africa and found that 90% larvae died in the stomach upon ingestion, however, some microfilariae showed signs of infectivity, developing and congregating in the mouth parts of the remaining mosquitoes (Laurence & Pester, 1961). This might demonstrate the adaptable nature of *B. patei* microfilariae in terms of the vector species in which the parasite can successfully complete its larval life cycle and further suggests that mosquitoes that are pre-disposed to microfilariae infection of one species, could perhaps accommodate the development of new species of microfilariae.

2.2.3 *Dirofilaria immitis* and *Dirofilaria repens*

From a veterinary perspective, *Dirofilaria* spp. has gained significance due to its incidence and prevalence in canids and felids (Simón et al., 2012). The two main species are *Dirofilaria* (*Nochtiella*) *repens,* commonly known as Old World *Dirofilaria* due to its prevalence in Europe, Asia and Africa, and *D. (Dirofilaria) immitis* which has worldwide distribution (Cancrini et al., 2006). *Dirofilaria immitis* is highly pathogenic causing cardiopulmonary dirofilariasis (heartworm), while *Dirofilaria repens* (subcutaneous dirofilariasis) is largely non-pathogenic (Capelli et al., 2018; Simón et al., 2012). Reports of infections in domestic canines and felines are found throughout the world while infections in wild animals such as red foxes, wolves, coyote, and otters are evidenced in necropsy reports, albeit outside of Africa (Ćirović et al., 2014; Magi et al., 2008; Penezić et al., 2018; Snyder et al 1989).

Both *D. immitis* and *D. repens* were found in humans through incidental reports and are, therefore, considered emerging zoonoses in areas of endemism, including subtropical Africa (Capelli et al., 2018). To date, *D. immitis* and *D. repens* are the most well-studied filarial parasites, presumably due to the pathogenicity in domestic companion animals as well as their zoonotic potential (Otranto et al., 2015).

In Africa, *D. immitis* was reported in domestic canines in Algeria, Canary Islands, Kenya, Malawi, Mozambique, Tunisia, South Africa and Zimbabwe (Rjeibi et al., 2017; Montoya et al., 2006; Schwan, 2009; Schwan & Durand, 2002; Tahir et al., 2017; Verster et al., 1991) and occurrence of *D. repens* was reported in domestic canines in Botswana, Egypt, Mozambique, Namibia, South Africa, Tunisia and Zambia (Abdel-Rahman et al., 2008; Ntesang, 2016; Rjeibi et al., 2017; Schwan et al., 2000; Schwan, 2009; Siwila, et al., 2015).

The most extensive work on microfilariae within a South African context focused on the seroprevalence of filariae in domestic carnivores in four South African provinces of Gauteng, KwaZulu-Natal (KZN), Mpumalanga and North-West (Schwan, 2009; Voigts 2019). *Dirofilaria repens* was prevalent in domestic canines in KwaZulu-Natal (12.47%) and Mpumalanga (1.5%) and domestic felines (10.98%) in KwaZulu-Natal, reaffirming endemicity in the country (Schwan 2009). Though mosquitoes play an important role in zoonotic transmission of *Dirofilaria* species, the species of mosquitoes responsible are not well investigated in South Africa. Urban areas with a high domestic canine population potentially provide important reservoirs for dirofilariosis as they harbour large dog populations, however, the different mosquito vectors for dirofilariosis in such areas have not been investigated.

A range of about 60 mosquito species of the genera, *Aedes, Anopheles, Culex, Mansonia, Psorophora* are potential intermediate hosts and vectors of *Dirofilaria* (Schwan, 2009; Schwan & Durand, 2002). In the Afrotropics, experimental investigations demonstrated that *Aedes aegypti* and *Ae. pembaensis* are competent vectors for both *Dirofilaria* species, while *Culex quinquefasciatus* (reported as *Cx. pipiens f. quinquefasciatus*) is susceptible to *D. immitis* and *Mansonia uniformis* and *Mansonia africana* displayed susceptibility to *D. repens* (Schwan, 2009).

The rate of microfilariae development in susceptible mosquito vectors varies according to vector species and temperature. Experimental infections investigated the role of mosquito species in *D. repens* transmission in Nigeria. The study determined that development of infective L3 larvae at 22.5-24.5°C occurred in *Aedes aegypti* following feeding on blood from a microfilaremic dog (Anyanwu et al., 2000). The growth of the sausage (L2) stage occurred in 5-7 days, the elongated (L3) stage larvae were in the

Malphigian tubules at day 10 and by day 14, infective larvae were observed in the head and proboscis (Anyanwu et al., 2000). Additionally, a 2°C drop in temperature caused a 4-day delay in development, supporting the dependence of microfilariae maturation and consequently transmission on climate and seasonal changes.

2.2.4 *Dirofilaria corynodes*

Dirofilaria corynodes is a mammalian filarial nematode that is prevalent in Nigeria and Kenya. Patas monkeys (*Erythrocebus patas*), African green monkeys (*Cercopithecus aethiops*) and possibly other species of monkeys are the definitive hosts while species of the genus *Aedes* has shown evidence of vector susceptibility for *D. corynodes* (Heisch et al., 1959; Orihel, 1969).

In coastal Kenya, 9966 *Ae. pembaensis* were dissected in search of microfilariae and 0.16% (n=16) were found to be infected with the parasitic worm (Heisch et al., 1959). In this same study site, peripheral blood smears from Patas monkeys were taken and 10 individuals were found to be positive confirming both the definitive host and the vector of *D. corynodes* (Heisch et al., 1959).

Some years later, *Aedes* was experimentally demonstrated to be a susceptible vector of *D. corynodes*. *Aedes aegypti* mosquitoes were fed on anesthetised microfilaremic patas monkeys and then housed in cages with controlled temperature (~28°C) and relative humidity (60-70%) to replicate ideal climatic conditions for microfilariae development (Orihel, 1969). The development of *D. corynodes* microfilariae, unlike other filarial worm species, which develop in the Malphigian tubules, occurs in the fat body cells of the *Aedes* species. In the first 24 hours following infection through a blood

meal, the microfilariae migrate from the stomach wall to the fat body (Orihel, 1969). On average, the microfilariae reach the infectivity stage at this site in 14 days however, subsequent blood meals that the infected mosquito ingests, accelerates microfilariae growth (Orihel, 1969; Travi et al., 1986).

2.2.5. *Acanthocheilonema* spp.

The two species of Acanthocheilonema that are prevalent in subtropical Africa are *Acanthocheilonema dracunculoides* (Cobbold, 1970) and *Acanthocheilonema reconditum* (Grassi, 1889), formerly named *Dipetalonema dracunculoides* and *D. reconditum* respectively. Both species are largely non-pathogenic and cause little damage due to the location of their respective predilection sites, the peritoneal cavity and subcutaneous tissues (Krücken et al., 2021). However, dermal lesions found in domestic dogs in Spain were caused by the parasite and provide evidence for occasional morbidity in infected animals (Schwan & Schröter, 2006; Costa, 2019).

Acanthocheilonema dracunculoides is enzootic in subtropical African countries including South Africa, Mozambique, Namibia, Kenya, Tanzania, Democratic Republic of Congo, Sudan, and Somalia (Nelson, 1963, Schwan, 2002, Schwan & Schröter, 2006). From an African perspective, the parasite was first encountered in South Africa in the aardwolf (*Proteles cristata*) during necropsy and has since been found and identified morphologically on a case-by-case basis in other canines including the domestic dog, brown hyaena (*Hyaena brunneal*) and fox (*Vulpes vulpes*) (Verster, 1991; Schwan, 2002; Schwan & Schröter, 2006; Krücken et al., 2021).

A study in Kenya by Lightner et al. (1983) also morphologically identified the microfilariae in the blood of spotted hyaena (*Crocuta crocuta*) as A. *dracunculoides*. However, a recent phylogenetic inference study analysing ITS2 rRNA gene sequence data concluded that the species found in the spotted hyaena, while morphologically indistinguishable, was most likely a species other than *A. dracunculoides* provisionally deemed *A. dracunculoides* ex. *Crocuta crocuta* (Krücken et al., 2021). Therefore, investigation is needed to provide further insight into the molecular identities of *Acanthocheilonema* spp.

Mosquitoes have yet to be implicated in the transmission of the two species of *Acanthocheilonema*. Thus far, the louse fly (*Hippobosca longipennis*) and the brown dog tick (*Rhipicephalus sanguineus*) have been confirmed as intermediate hosts and vectors of *A. dracunculoides* (Nelson, 1963, Schwan & Schröter, 2006). In terms of the epidemiology and identification of arthropod vector species, there are gaps in knowledge. The biological vectors of *Acanthocheilonema* spp. require further investigation as there is a wide range of haematophagous arthropods that could be possible vectors of filarial nematodes (Krücken et al., 2021).

In terms of the Afrotropics, the distribution of *A. reconditum* encompasses South Africa, Mozambique, Kenya, Uganda and Zambia (Schwan & Durand, 2002, Schwan, 2009). The species has been reported in domestic dogs, wild dogs and spotted and brown hyaenas (Verster, 1991, Schwan, 2009). There have been flea and lice species implicated, currently it is believed that the main biological vector of *A. reconditum* is the cat flea, *Ctenocephalides felis* even though infective larvae have also been found in lice, *Heterodoxus spiniger* and *Linognathus setosus* (lice), and fleas, *Ctenocephalides canis* (dog flea), *Echidnophaga pallinacea* (hen flea), *Pulex irritans*

(human flea) and *Pulex simulans* (Kamani et al., 2019, Napoli et al., 2014; Nelson, 1962, Verster, 1991).

Initially, it was hypothesised that A*. reconditum* larval stages developed near the body fat cells of fleas however, more recently, microscopic examination of microfilariae in *C. felis* demonstrated development in the subcuticular region within the abdomen (Napoli et al., 2014). The transmission of this species differs from those transmitted by way of a mosquito's bloodmeal. The large size of the infective larvae could suggest that transmission to the definitive hosts does not occur via the mouthparts but perhaps through ingestion of the infected flea by the host (Napoli et al., 2014).

Prevalence of *A. reconditum* in the vector has been investigated in Europe in which *C. felis* collected on dogs were analysed by way of dissection (Brianti et al., 2012). *Ctenocephalides felis* contained L1 and L3 larval stages with a prevalence of 5.1% following dissection and morphological identification (Nelson et al., 1962; Brianti et al., 2012).

Manoj et al. (2021) employed molecular analyses using two sets of COI primers to detect filaroid DNA in mosquitoes and the biting midge, *Culicoides* collected in Portugal. *Culex. quinquefasciatus* and *Ochlerotatus caspius* (= *Ae. caspius*) mosquitoes were shown to have a prevalence of 0.2% and 3% of *A. reconditum* respectively. However, infective larvae were not confirmed in the mouthparts and no other evidence in the literature indicates that either of the mosquito species vectors *A. reconditum*. The unanticipated occurrence is most likely attributed to consumption of microfilariaemic blood, which can remain in the mosquito two weeks after ingestion (Manoj et al., 2021). Thus, non-susceptible mosquitoes may provide information about

circulating microfilariae in the animal population upon which they feed rather than being a reflection of transmission (Manoj et al., 2021).

2.2.6 *Setaria* spp.

The species of the genus *Setaria* largely plague domesticated ungulate and wild game animals in several countries, worldwide i.e., *Setaria equina* (Kenya), *S. labiato-papillosa* (Kenya, Italy), *S. scalprum*, *S. saegeri, S. africana* and *S. thwaitei* (South Africa), *S. tundra* (Germany) (Cancrini et al., 1997; Czajka et al., 2012; Nelson et al., 1962; Watermeyer et. al., 2000; Watermeyer et. al., 2003; Watermeyer et. al., 2000). The literature agrees that *Aedes* spp. are the responsible vectors for the transmission of *Setaria*.

In Kenya, *Setaria* spp. were found in donkeys, cattle, bushbuck and bushpigs in a combined prevalence of 6.8% through the observation of Giemsa-stained peripheral blood smears and adults in the peritoneal cavity (Heisch et al., 1959). The donkeys harboured *S. equina* (9.09%), cattle harboured *S. labiatopapillosa* and bushbuck and bushpig were found to be infected with unknown *Setaria* species (Nelson et al., 1962). There were no signs of visible disease in natural animal hosts suggesting that it has low or no pathogenicity, however, in non-host animals, infections may give rise to destructive lesions in the brain and spinal column (Cancrini et al., 1997).

In the same study, field collections of mosquitoes demonstrated that microfilariae prevalence in *Ae. pembaensis* was 0.29%. Further artificial feeding experiments of donkey blood infected with S. *labiatopapillosa* found that development to infective larvae occurred in *Ae. pembaensis* and to a lesser extent in both *Ae. aegypti* and *Cx.*

pipiens f. quiquefasciatus (Heisch et al., 1959; Nelson et al., 1962). Furthermore, *Setaria* spp. were observed to develop in the thoracic muscles of the *Aedes* mosquitoes and L3 larvae were seen in all parts of the mosquito, including the legs, antennae and palps (Heisch et al., 1959). A study conducted by Cancrini et al. (1997) on a cattle-dense farm in Italy, following feeding on the blood of microfilaraemic cattle, found 69% developed microfilariae in *Ae. caspius,* 22.7% in *Ae. vexans*, 6.1% in *Ae. maculipennis* and 1.4% in *Ae. claviger*. This further solidifies the connection between the *Setaria* species and *Aedes* mosquitoes.

2.2.7 Avian microfilariae

There are 160 species of filarial nematodes within 16 currently accepted genera, worldwide, that have been associated with birds (Atkinson et al., 2008). Avian microfilariae are generally not specific and thus infect a broad variety of bird families. Adult worms have been observed in the heart, blood vessels, leg joints or the brain while microfilariae are usually found in the blood of birds but in some cases, may be found in the skin (Anderson, 2000).

The vectors of avian microfilariae are a diverse array of biting midges, black flies, lice and mosquitoes (Anderson, 2000). Three genera of microfilariae, *Aproctella*, *Cardiofilaria* and *Pelecitus* are confirmed to be vectored by mosquitoes (Atkinson et al., 2008). *Coquillettidia crassipes* is a confirmed vector of *Cardiofilaria nilesi* and *Pelecitus ceylonensis* in Sri Lanka and Malaysia, and *Armigeres* and *Culex* spp. were also found to harbour *C. nilesi* infective larvae in Malaysia (Anderson et al., 2000; Klinaewnarong et al., 1985). Given the ornithophilic feeding habits of *Culex pipiens*, it is also hypothesised that the species and its forms and hybrids act as vectors for avian

microfilariae (Czajka et al. 2012). Possible evidence is that unknown microfilariae of avian origin were found, through molecularly means, in *Cx. pipiens f. pipiens* in Spain, however, further investigation is needed to confirm the species as a vector for avian microfilariae (Bravo-Barriga et al., 2016).

Table 2.1: Prevalence of avian blood-borne microfilariae in sub-tropical Africa.

Wild bird species	% Infection	Locality	Reference
Various Avian orders represented	4	South Africa	Earle et al., 1991
Turdus pelios (African thrush)	0.35	Ethiopia	Ashford et al., 1976
Alethe castanea (fire-crested alathe); *A. diademata* (white-tailed alathe)	3.6	Cameroon, Equatoria Guinea, Ivory Coast	Sehgal, Jones & Smith, 2005; Kirkpatrick & Smith, 1988
Various Avian orders represented	2.6	Kenya, Tanzania and Zaire	Bennet & Herman, 1976
Various Avian orders represented	5.2	Ghana	Wink & Bennett, 1976
Various Avian orders represented	28.1	Chad	Williams, Bennett & Troncy, 1977
Various forest dwelling bird species	11.0	Madagascar	Savage et al., 2009
Various Avian orders represented	3.9	Uganda	Valkiunas et al., 2005

To date, little scientific attention has been dedicated to the filarial nematodes of Afrotropical birds, with few fragmented studies available in the literature (Table 2.1). Several authors conducted studies examining African birds for a collection of blood parasites in the 1970s and 2000s (Ashford et al., 1976; Bennet & Herman 1976; Earle et al., 1991; Kirkpatrick & Smith, 1988; Savage et al., 2009; Sehgal, et al., 2005; Valkiunas et al., 2005; Williams, et al., 1977; Wink & Bennett 1976). In the series of studies conducted in the 1970s, intravenous blood from wild birds was collected and stained with Giemsa's stain to detect microfilariae. The prevalence varied between 0.35-28.1% among the bird species sampled across the continent suggesting a low to medium prevalence (Table 2.1). In some cases, measurements, and descriptions of the cephalic ends of the filarial larvae were reported. It was also noted that there was difficulty in identifying microfilariae due to the fickle nature of the mediums used to diagnose the parasites (Bennet & Herman 1976).

In Cameroon, Côte d'Ivoire and Equatorial Guinea, between 1990 and 2001, around one thousand rainforest birds were bled from the brachial vein (Sehgal et al., 2005). Blood smears were made, and nematode DNA was amplified and sequenced and 35 birds, mainly Fire-crested Alethe (*Alethe diademata*) were positive for filarial DNA (3.6%) (Sehgal et al., 2005). It was noted that the true prevalence may be even higher, as blood from the brachial vein has lower microfilariae density than blood sampled from the pulmonary artery (Sehgal et al., 2005). The vectors were not alluded to and studies on the ecology of vector transmission are required to identify specific vectors for the avian filariae found during the study (Sehgal et al., 2005).

In a study conducted in Madagascar between 1994 and 2003, blood samples were collected from toe clippings of birds that were trapped and released as well as heart

punctures from birds taken as voucher specimens across elevation gradients and forest types (Savage et al., 2009). Thin blood films were examined microscopically for various haemoparasites and microfilariae were found to be the second most abundant group of parasites suggesting that susceptible vectors are present in the forest areas sampled (Savage et al., 2009).

The studies focussing on avian filariae reference the importance of microfilariae-carrying arthropods and propose that the presence of microfilariae suggests a presence of suitable vectors (Savage et al., 2009). However, the specific role of the vectors has not been explored and therefore, there is a lack of information in natural areas where filariae are known to be circulating in wild bird communities. It is also suggested that the avian-specific microfilariae found during the studies have co-evolved to fit into the available niches inhabited by wild bird communities and some level of endemism occurs, but further investigation is required to better understand the interactions between the filariae and their avian hosts (Savage et al., 2009).

2.3 Filarial zoonoses

The zoonotic potential of mosquito-borne filarial helminths, particularly species of the genera, *Dirofilaria* and *Brugia*, has been well documented. In Europe, zoonotic filariosis and human dirofilariasis caused by *D. repens* is ever-increasing (Otranto et al., 2015). Even though human dirofilariosis is present throughout Africa, reports are few and scattered and in the form of case studies (Argy et al., 2011; Genchi & Kramer, 2020). This is likely due to severe under-reporting of cases rather than a reflection of prevalence (Penezic et al., 2014).

In the Republic of South Africa, two autochthonous cases of *D. repens* zoonotic infections have been documented (Moodley et al., 2015). The cases occurred in Gauteng and Kwa-Zulu Natal in two middle-aged women, who presented with an ocular cystic lesion and a subcutaneous nodule, respectively (Moodley et al., 2015). The cases of *D. repens* in domestic animals suggests that occasional local human cases can be expected and though, it is likely that the occurrence of zoonoses are higher, cases most likely go undiagnosed or undetected (Moodley et al., 2015; Voigts, n.d.). In addition, *D. repens* is considered an emerging zoonotic disease in the Old World (McKay et al., 2013).

2.4 Importance of circadian periodicity of microfilariae in transmission

The most favourable site for microfilariae in the definitive hosts is within the lungs, however, the microfilariae need to be present in the peripheral blood, to enable uptake and transmission by mosquito vectors (Hawking, 1967). The microfilariae tend to follow the 24-hour circadian rhythm of the host so that they circulate in the capillaries of the lungs for the maximum amount of time possible and circulate in the periphery when necessary (Hawking, 1967). This interesting phenomenon is called circadian periodicity and it occurs in a number of filarial species (Lynch, 1919).

In circadian periodicity, the maximum proportion of microfilariae circulate in the peripheral blood of the definitive host at the height of activity of a competent mosquito vector, to favour uptake of microfilariae during the mosquito's blood meal (Hawking, 1967; Klein et al., 2019). Periodicity can occur at dusk, night, dawn or day depending on the species, though nocturnal periodicity is most common, as most susceptible

mosquitoes, *Culex, Anopheles* and *Mansonia* species are nocturnal or crepuscular (Klein et al., 2019).

In filarial species that display nocturnal periodicity, microfilariae accumulate in the cutaneous vessels of the host at night, when susceptible mosquito vectors are actively searching for hosts to feed (Klein et al., 2019). In contrast, during the day, the microfilariae accumulate in the vessels of the lungs (Hawking, 1967). Microfilariae of *D. immitis* are most numerous in the peripheral blood of a dog host between 18h00 and 21h00 which coincides with the feeding pattern of the mosquito species responsible for transmission (Hawking, 1967).

Heisch et al. (1959) also found strong evidence for nocturnal periodicity displayed in *Dirofilaria corynodes* found in the blood of grivet monkeys (*Cercopithecus aethiops*) on the Kenyan Coast. Three microfilariae per 20mm^3 blood were observed during the day and 900 mm^3 in blood found during night collections (Heish et al., 1959). The determination of periodicity of filariae adds important knowledge for timing transmission of the parasite (Lynch, 1919). This knowledge can also assist in determining the time period for mosquito collections when microfilariae are accumulated in the peripheral blood and consequently reflect true prevalence (Sehgal et al., 2005).

2.5 Vector importance in filarial transmission

The source of filarial infection is largely represented by mosquitoes living in the natural environment and the filarial life cycle continuity is dependent upon an abundance of competent vectors (Otranto et al., 2015; Otranto & Deplazes, 2019). There is limited knowledge of the ecology and epidemiology of filariae and mosquitoes (Otranto et al.,

2015). There are roughly 3500 mosquito species worldwide, approximately 200 in southern Africa and about 30 species in Durban located within KwaZulu-Natal Province of South Africa (unpublished data; Foster & Walker 2002). Worldwide, 60-70 mosquito species are considered potential competent vectors for members of the genus, *Dirofilaria* alone (Ludlam et al., 1970; Schwan & Durand 2002).

Filarial transmission is dependent on mosquito flight range, survival and ability to locate, feed and infect new hosts (Bocková et al., 2013). In addition, the ability of mosquitoes to ingest microfilariae and development of microfilariae in mosquitoes are important factors affecting transmission of this parasite (Bryan et al., 1990). In a study conducted by Gleave et al. (2016), the *Ae. aegypti* females that fed on blood with *Brugia malayi* infective L3 microfilariae were more likely to take a blood meal than non-infected mosquitoes thus suggesting that mosquito behaviour is altered by microfilariae infection. In addition, the biting, probing and feeding behaviour of female mosquitoes have adapted for transmission of microfilariae to varying degrees across species (Kinyatta, 2010).

Microfilariae density in an infective blood meal is compromised by the mosquito's immune response to the invasion of pathogens (Bryan et al.1990). Mosquitoes susceptible to invasion by microfilariae have evolved various lines of defence to ensure their survival while allowing for the development of some microfilariae for transmission to vertebrate hosts (McGreevy et al., 1978; Bryan et al., 1990; Ahid et al., 2000). The defence mechanisms destroy a large proportion of microfilariae therefore affects vector efficiency by reducing the number of microfilariae that develop into infective third stage larvae (McGreevey et al., 1978; Bryan et al., 1990; Ahid et al., 2000; Lai et al., 2000; McCrea et al., 2021).

Melanisation and encapsulation occur within the Malphigian tubules and thoracic muscles of susceptible mosquitoes. When microfilariae infiltrate the mosquito, they are recognised as foreign by the haemocyte cells responsible for immune response (Forton et al., 1985; Ahid et al., 2000). In response to the invasion, the incoming larvae are enclosed by melanin or a thick clear capsule, for eventual expulsion through the anus (Forton et al., 1985; Ahid et al., 2000). In a study conducted by Forton et al. (1985), *D. immitis* microfilariae isolated from dog blood was inserted into the thoraces of laboratory bred *Aedes trivittatus* to study the melanisation process. The mosquitoes were dissected and observed microscopically over the first 2 hours and then daily for five days showing that within 24 hours of infection, the microfilariae were completely encased by melanin and by 48 hours, the microfilariae were deceased (Forton et al., 1985).

Mosquitoes have also evolved teeth-like structures in the foregut of the gastrointestinal tract called cibarial and pharyngeal armatures as well as other papillae and spines, which vary from primitive to highly developed across species (McGreevy et al., 1978; Bryan et al., 1990). *Anopheles* spp. have been observed to have both cibarial and pharyngeal armatures, while *Culex* spp. have poorly developed cibarial armatures and *Aedes* spp. in turn, only have pharyngeal armatures (McGreevy et al., 1978). The cibarial and pharyngeal armatures provide varying degrees of protection, by causing cuticle damage, loss of motility and mortality to microfilariae, thus preventing a proportion of ingested microfilariae from advancing further into the mosquito's body (Bryan et al., 1974; Bryan et al., 1990).

In a study conducted by McGreevy et al. (1978), *An. farauti* and *Cx. pipiens* fed on cats infected with *Brugia pahangi*. The midguts and hindguts were dissected and

examined microscopically to assess the number of damaged and immobilised microfilariae. In *An. farauti* and *An. gambiae*, 36-96% damaged microfilariae were observed (McGreevy et al., 1978). In contrast, the under-developed cibarial armature in *Cx. pipiens* caused a mere 6% mortality of microfilariae (McGreevy et al., 1978). Bryan et al. (1974) observed that the percentage of damaged *B. pahangi* microfilariae was higher in *An. farauti* and *An. gambiae* which has both pharyngeal and cibarial armatures than *Ae. aegypti* and *Ae. togoi*, likely due to the lack of cibarial armatures in *Aedes.* In a similar study, natural vectors of *W. bancrofti,* namely *An. gambiae s.s., An. arabiensis, An. merus* and *An. funestus* fed on the blood of microfilaremic volunteers (Bryan et al., 1990). The *W. bancrofti* microfilariae found in the mosquito midguts were stained in Giemsa and observed microscopically and 26-67% of microfilariae were found to be damaged in the four *Anopheles* species (Bryan et al., 1990). These studies support that, well-developed pharyngeal armatures or a combination of pharyngeal and cibarial armatures are most successful at destroying invading microfilariae while still allowing some larvae to develop to the infective stage, thus enabling transmission. This suggests that microfilariae reduce the fitness of infected mosquitoes.

2.6 Influence of climate on microfilariae development in mosquitoes

The movement of microfilariae from vector to definitive host is affected by climatic conditions. High temperatures and rainfall perpetuate the mosquito populations, maintaining their abundance and thus facilitating filarial transmission. Filarial infection is predominant in tropical and subtropical regions which typically experience seasonally high temperatures and rainfall (Schwan 2009).

The development of microfilariae within mosquito vectors is temperature dependent (Pietikäinen et al., 2017). The microfilariae of *D. immitis*, for example, develop to the L3 stage at an average daily temperature of 14°C, but once temperature drops, development ceases until the threshold temperature is once again reached (Ledesma et al., 2015; Pietikäinen et al., 2017). Temperature also influences the developmental rate of microfilariae in mosquitoes (Bocková et al., 2013). In a study on donor-feeding trials of *D. repens* in six common mosquitoes of Nigeria, microfilariae growth was accelerated by 4 days when temperature increased by 2°C, from 22.5°C in summer to 24.5°C in winter (Anyanwu et al., 2000).

The presence of filarial nematodes in definitive host populations are dependent on the presence and abundance of mosquitoes in the environment. Mosquitoes are therefore proxies for filarial transmission and the mosquito presence is often used for modelling of parasite transmission (Lippi et al., 2019). In Europe and the Americas, models have been developed to determine seasonality of filarial transmission however no such models are available in Africa (Schwan 2009).

In recent times, climate change is initiating an atypical spread in the mosquito's previously known natural distribution (Lippi et al., 2019). This distribution shift is creating suitable conditions for larval development in temperate regions (Kronefeld et al., 2014). The wide host ranges of some infective mosquitoes also exacerbate the zoonotic potential through accidental infection (Cirovic et al 2014).

Climate change and habitat change has led to incidences of vector spread to areas not previously observed (McCall 2008). In addition, in 1990 to 2008, over 20% of emerging infectious diseases (EID) have been attributed to vector-borne diseases accompanied by an increase in EID events over time showing the influence of climate

change on vectors (Jones 2008). Therefore, it is vital that attention is given to wild mosquito populations as potential reservoirs for microfilariae infection (Klein et al., 2019).

2.7 Microfilariae detection in mosquitoes

The two methods employed to detect microfilariae in mosquito populations are dissection and polymerase chain reaction (PCR) (Goodman et al., 2003).

2.7.1 Dissection and microscopic examination

The method of dissection entails separation and examination of the mosquito body parts, using dissecting tools, to locate larval developmental stages within the thoracic muscles, Malphigian tubules, head, and mouthparts (Goodman et al., 2003). The microfilariae are visible under a stereo microscope, and in infective mosquitoes, separation of the mosquito's head seems to cause a disturbance amongst the larvae, which can sometimes be found streaming out from the proboscis of the mosquito (Anyanwu et al., 2000).

Dissection, as a method for monitoring filarial infection in mosquitoes, is considered the gold standard to which other methods have been compared, for decades (Watts et al., 1999; Goodman et al., 2003; Czajka et al. 2012). Mosquito dissection is so ubiquitous, that the World Health Organisation employs the method as a standard for monitoring the progress of lymphatic filariasis global elimination programmes (de Souza et al., 2012; Goodman et al., 2003). It is an effective way to monitor filarial nematode prevalence in vectors that does not require overly specialised equipment.

Despite its practicality, when filarial prevalence in mosquitoes is 1% or lower, and thus large numbers of mosquitoes need to be screened by highly trained technical staff, this method of detection can be quite costly (Goodman et al., 2003). Additionally, the method is also time-consuming, arduous and lacks sensitivity and specificity (Watts et al., 1999; Goodman et al., 2003; Dyab et al. 2015). In addition, the morphological similarity amongst the different filarial nematode species increases difficulty of identification using morphology and morphometrics, and the use of inexperienced dissection technicians can likely lead to misidentifications (Sehgal et al., 2005; Aonuma et al., 2009).

2.7.2 Molecular detection

Since the late 1990s, PCR techniques were used to detect human filariasis and canine dirofilariasis in wild-caught and laboratory bred mosquitoes (Goodman et al., 2003). Diagnosticians are constantly developing genus or species- specific PCR-based assays for use in detection programmes. In 2002, Mar and colleagues found that *D. immitis* and *A. reconditum* are very similar in morphology and that specific and sensitive PCR tests were needed for identifying the species (Mar et al., 2022). The same authors designed specific primers derived from ITS2 to allow for a differential diagnosis for *D. immitis* and *A. reconditum*.

In Africa, human lymphatic filariasis is widespread and a large proportion of the population in endemic areas are at risk of infection (Derua et. al., 2017). Therefore, much of the literature focuses on the presence of *Wuchereria bancrofti* in *Culex* and *Anopheles* mosquitoes. A multiplex PCR technique was developed to detect DNA of several filarial nematode species in mosquitoes in a single test (Dyab et al. 2015). A

hundred mosquito pools were collected from Assiut Governorate, Egypt and the multiplex PCR used, found pools positive for *W. bancrofti*, *D. immitis* and *D. repens* (Simon et al., 2012; Dyab et al. 2015). Thus, the robust and specific nature of PCR-based methods enables the detection of multiple filarial nematode species from a single sample.

2.8 Concluding remarks

There is a plethora of information on filariasis transmission and infection in domestic canids and felids due to their close relationship to humans. However, investigations on microfilariae in mosquitoes, especially in areas inhabited by wild fauna is lacking, from an Afrotropical perspective (Schwan 2009; Voigts 2018; Cirovic 2014).

Many vector studies in the Afrotropics conducted in areas with wild animals and birds are historic and were reliant on methods of detection that are vastly outdated, given the advances made in molecular analyses. In addition, studies of animal filariae have focussed largely on prevalence of filarial nematodes in host blood (microfilariae) or body cavities (adults) and few explore the crucial role that the culicid vectors play as intermediate hosts in the region.

Multi-faceted vector studies which encompass mosquito blood meal analysis, dissection, molecular analysis and behavioural studies on mosquitoes naturally or artificially infected with microfilariae should be considered if comprehensive knowledge on host-vector dynamics can be elucidated. The aim of the current study was, therefore, to evaluate the prevalence of microfilariae in mosquito vectors found in areas with high animal densities in Durban, South Africa.

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CHAPTER 3: Short communication: In search of vector-borne filariae: comments on presence and prevalence in mosquito vectors in Durban, South Africa

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This chapter is under review in the Journal of Veterinary Parasitology: Regional Reports and has been formatted to this journal's specifications.

Abstract

Mosquitoes belonging to the genera, *Aedes, Anopheles, Coquillettidia, Culex* and *Mansonia* are vectors of microfilariae (Superfamily: Filarioidea), infecting the lymphatic system, tissues and body cavities of vertebrates. To investigate the prevalence of microfilariae in mosquito vectors in Durban, KwaZulu-Natal Province, South Africa, 1490 mosquitoes collected over a period of 12 months from animal-dense sites in the eThekwini (Durban) Metropolitan Municipality were analysed through dissection and molecular screening. No microfilariae were detected. The prevalence of microfilariae in mosquitoes from the region should, based on sample size, be considered to be lower than 4%. The most common mosquito species were *Culex pipiens* (s.l) (54.1%) and *Mansonia africana* (32.0%), which have a high transmission potential for microfilariae. Further investigation is needed, and it is recommended that (i) the two common species of mosquitoes in Durban, *C. pipiens* and *Ma. africana*, be targeted,

(ii) sampling should be conducted between November and February because the highest number of the target mosquito species were collected during these months and (iii) a larger sample size of mosquitoes per month is required to establish presence or determine prevalence, due to the low prevalence in the region.

Key words: *Culex pipiens*, *Mansonia africana*, microfilariae, mosquito, prevalence,

Vector, Durban, South Africa

3.1 Introduction

Mosquitoes (Family: Culicidae) are major vectors of microfilariae (Superfamily: Filarioidea) in tropical and subtropical parts of the world (Dimpka et al., 2019). As intermediate hosts and vectors, mosquitoes are associated with development of microfilariae, the specialised first stage larvae and transmission of the infective third stage filarial helminth larvae to new hosts (Anderson, 2000). Several mosquito species belonging to such genera *as Aedes, Anopheles, Coquillettidia, Culex* and *Mansonia*, are known to have high transmission potential for microfilariae in the Republic of South Africa however, this has not been confirmed (Schwan, 2009).

In the tropics and subtropics of Africa, the major filarial helminths transmitted by mosquitoes are *Dirofilaria immitis, Dirofilaria repens, Wuchereria bancrofti*, *Brugia patei* and *Setaria* species (Nelson et al., 1962; Schwan 2009; Ughasi et al., 2012; Kinyatta et al. 2018). To understand the epidemiology and dynamics of microfilariae transmission, it is essential that the mosquitoes that play a role as potential vectors in the region, are identified (Bravo-Barriga et al. 2016). As a vector, the mosquito is an important component in surveillance for filarial infection and monitoring the infection

levels in mosquitoes provides information about the prevalence of known infections and the possible emergence of new infections (Goodman et al., 2003).

The presence of readily available natural mosquito vectors is one of the pre-requisites for the transmission of microfilariae, along with the presence of definitive hosts and suitable climatic conditions (Anyanwu et al., 2000). The mosquito species, *Culex pipiens* and *Mansonia africana* are important vectors for microfilariae and both species are widespread in the tropics and subtropics of Africa including Nigeria, Kenya, Ghana and South Africa (Anyanwu et al., 2000; Nelson et al., 1962; Ughasi et al., 2012). Both species have demonstrated their vector status for microfilarial transmission in filariasis-endemic regions, wherein either of the species is common. Both mosquito species are known natural vectors of *Dirofilaria immitis* in Kenya and natural infections of *D. immitis* were also reported in *Ma. africana* in Tanzania (Nelson et al., 1962; Schwan 2009). *Mansonia africana* is also reported to be infected with *Wuchereria bancrofti*, with a prevalence of 0.12% in Kenya and 7.6% in Ghana (Nelson et al., 1962; Ughasi et al., 2012). With regards to *Cx. pipiens*, in two reports in Kenya, it was determined that the wild-caught mosquitoes had infection rates of 0.78% and 1.7-1.8% for *W. bancrofti* (Nelson et al., 1962; Kinyatta et al. 2018). Under artificial/laboratory conditions, *Ma. africana* collected in Kenya displayed susceptibility to *Dirofilaria repens* and *Brugia patei* while *Cx. pipiens* f. *quinquefasciatus* demonstrated infectibility with *W. bancrofti*, (Nelson et al., 1962).

Outside of the Afrotropics, *Cx. pipiens* f. *quinquefasciatus* reportedly harbours *D. immitis* (1.4%), *Onchocerca lupi* (0.2%), *D. repens* (0.2%) and *Acanthocheilonema reconditum* (0.2%) in Portugal (Manoj et al., 2021). The *Culex* genus has a high prevalence in this region, with *Cx. pipiens* as the dominant species, maintaining

prevalence of 52%. Similarly in Italy, the prevalence of *D. immitis* and *D. repens* in *Cx. pipiens* was confirmed to be 0.38-0.74% and 0.74%, respectively and the species was established as the most efficient natural vector of cardiopulmonary dirofilariasis in the studied area (Cancrini et al., 2007; Capelli et al., 2013).

Though the risk of infection from microfilariae of *D. repens* and *A. reconditum* is present in South Africa, there is no information regarding the prevalence of microfilariae in mosquito vectors (Schwan 2009). The current study aimed at (i) determining the prevalence of microfilariae in mosquito populations in Durban, South Africa (ii) identifying mosquito species that are potential vectors of filarial infection and (iii) discussing the prevalence of mosquito-borne microfilariae in a South African context.

3.2 Materials and methods

3.2.1 Survey areas, sample size and mosquito collection

Female adult mosquitoes were collected from April 2021 to March 2022 at the Centre for the Rehabilitation of Wildlife (29.91954°S 30.93628°E) and the Umgeni River Bird Park (29.80858°S 31.01745°E) in Durban, eThekwini Metropolitan Municipality, KwaZulu-Natal Province, Republic of South Africa. The Umgeni bird park is located along the Umgeni River, 4km from the coast and spans ca. 3.5 ha of tropical landscape. The park maintains 800 birds belonging to over 200 indigenous and exotic species (Umgeni River Bird Park/eThekwini Municipality [n.d]). CROW (Yellowwood Park, South Africa, 29.91954°S 30.93628°E) was established in 1980 and is a fully accredited private Rescue-Rehabilitate-Release wildlife facility (CROW, 2021). The organisation cares for injured and orphaned wild fauna, the most common of which

are vervet monkeys, mongoose, antelope, genets, and a variety of birds. The 4-ha plot is surrounded by forest patches and contains enclosures that replicate the animals' natural habitats. Notably, the bird park consists of several artificial pools of stagnant water in the enclosures, that are polluted with organic avian waste.The wildlife rehabilitation centre contains diverse natural vegetation offering many favourable niches mosquito breeding in comparison to the bird park which was built over an old quarry and surrounded by cultivated tropical vegetation.

Each month, trapping was conducted over a maximum of three nights for each of the sites. Female mosquitoes were captured with the use of two types of specialised mosquito traps, namely, the Encephalitis Vector Surveillance (EVS) traps (BioQuip Products, CA, USA) and BG-2 Sentinel mosquito traps (Biogents, Regensburg, Germany), baited with dry ice. Two EVS traps and two BG-2 Sentinel traps were used for each trapping event. During each trapping event, a 1 kg block of dry ice was placed into each specialised mosquito trap, the traps were positioned either within or near the animal enclosures, turned on in the afternoon at around 16:00 h and left standing overnight until 7:00 h the next morning, based on the peak activity period of crepuscular and nocturnal mosquito species. The dry ice released gaseous carbon dioxide (CO2) through sublimation, thus attracting flying mosquitoes that were in search for a bloodmeal.

A calculated minimum sample size of 59 mosquitoes per study site was calculated with 95% confidence level and a 5% margin of error, using Raosoft® (Sample Size Calculator; Raosoft inc.). The sample size was based on estimated 4% prevalence of microfilariae obtained from a study in West Africa (Abogye-Antwi et al., 2015).

3.2.2 Morphological identification of mosquito species

Mosquitoes were examined under a stereo microscope (SMZ800N, Nikon Corporation, Japan) and identified using standard taxonomic keys published in *Mosquitoes of Southern Africa: Culicinae and Toxorynchitinae* (Jupp, 1996).

3.2.3. Microscopic dissection and examination of mosquitoes

Live female mosquitoes were identified and transferred into petri dishes over a cold pack causing them to become immobile. One mosquito was dissected at a time. The mosquito's legs and wings were removed, and the insect fixed on a slide containing a drop of 0.9% sodium chloride (NaCl) solution, providing a medium to observe larval movement (Silaghi et al., 2017). The head, thorax and abdomen were separated, and each segment was dissected using extra fine stainless-steel tweezers and dissecting probes under the stereo microscope and examined at various magnifications. The Malpighian tubules and thoracic muscles were examined for the presence of stage one (L1) and two (L2) larvae, and the head and proboscis examined for infective L3 larvae. The dissected mosquitoes of each month were pooled each month and stored at -80°C until needed for molecular screening.

3.2.4 Confirmation using PCR

To confirm the accuracy of the dissection results, the dissected mosquitoes were subjected to DNA extraction and polymerase chain reaction (PCR). To prepare the samples for PCR, genomic filarioid deoxyribonucleic acid (DNA) from the dissected mosquito samples was extracted using the PureLink™ Genomic DNA Mini Kit

(Invitrogen™, Thermo Fischer Scientific, USA) according to the manufacturer's instructions with few modifications. The DNA was eluted in 100 µl of PureLink™ Genomic Elution buffer and DNA products were stored at -20°C.

PCR was used to detect Filarioidea DNA from the mosquitoes using primer pairs designed to amplify the internal transcribed spacer-2 (ITS2) and the filarial mitochondrial DNA cytochrome oxidase subunit I (COI) genes, as described in Bravo-Barriga et al (2016) (Table 3.1).

Table 3.1: COI and ITS2 primers sets used to detect microfilariae in mosquitoes as described in Bravo-Barriga et al. 2016.

Primer	Gene	Sequence (5' to 3')
DIDR-F1	ITS2	AGT GCG AAT TGC AGA CGC ATT GAG
DIDR-R1	ITS2	AGC GGG TAA TCA CGA CTG AGT TGA
COIintF	COI	TGA TTG GTG GTT TTG GTA A
COIintR	COI	ATA AGT ACG AGT ATC AAT ATC

Twenty microlitres of PCR reaction was achieved by synthesising 0.5 µl of 20 µm forward and reverse primers, 10 µl of Phusion Flash High-Fidelity Master Mix (Invitrogen™, Thermo Fischer Scientific, USA) and 6.5 µl double distilled water with each 2.5 µl of DNA sample. The Veriti™ 96-Well Fast Thermal Cycle (Applied Biosystems™) was used to run the PCR cycles. The conditions for PCR amplification

were as follows: initial denaturation at 98 °C for 10 s, 35 cycles consisting of denaturation at 98 °C for 1 s, annealing at 52 °C for 5 s, extension at 72 °C for 15 s, and final extension at 70 °C for 1 min.

Five microlitres of PCR products were electrophoresed with 1 µl of Tritrack DNA loading dye (6X) (Invitrogen™ Thermo Fischer Scientific, USA) on 2% Agarose LE (Benchmark Scientific, USA) gels, stained with 5 µl ethidium bromide. A molecular weight marker (GeneRuler 100 bp DNA Ladder, Thermo Fischer Scientific) was used. Gels ran at C at 120 V on a PowerPac™ Universal power supply (Bio-Rad Laboratories, USA). The DNA amplicons were analysed using a Gel Doc XR+ System and Image Lab Software (Bio-Rad Laboratories, Inc, USA). The amplicons were excised from the ethidium bromide-stained agarose gels under an ultraviolet transilluminator.

3.2.5 Data analysis

Plots were made to visualise the dissection counts for each mosquito species over the study period using base R (R Core Team, 2020) in R-Studio (R-Studio Team, 2020).

3.3 Results

A total of 1490 female mosquitoes were collected and dissected during the study period, with 765 mosquitoes having been collected from the Centre for the Rehabilitation of Wildlife (CROW) and 725 from Umgeni River Bird Park (URBP) (Table 3.2). Across both sites, twelve mosquito species were dissected in search of microfilariae and larval stages during the study period including, 806 *Culex pipiens*

(s.l) (54.1%), 477 *Mansonia africana* (32.0%), 84 *Coquillettidia microannulata* (5.6%), 78 *Aedes aegypti* (5.2%), 18 unidentified *Anopheles* species (1.2%), with the remaining 1.9% (n=27) comprising seven species including *Eretmapodites quinquevitattus, Culex neavei, Cx. subdentatus, Cx. cinerrellus, Cx. duttoni, Cx. simpsoni and Cx. univitattus*. (Table 3.2).

Overall, the two species, *Ma. africana* (n=806; 54.1%) and *Cx. pipiens* (s.l.) (n=477; 32.0%) made up 86.1% of mosquitoes dissected from both sites. *Mansonia africana* and *Cx. pipiens* were consistently dissected every month during the study period at CROW and URBP, respectively (Figure 3.1). The number of mosquito species dissected from CROW (n=12) was double that from Umgeni River Bird Park (n=6), over the study period.

Table 3.2: The total numbers of each mosquito species that was collected and dissected from two sites in Durban, South Africa, the Centre for the Rehabilitation of Wildlife (CROW, n=765) and Umgeni River Bird Park (URBP, n=725) and at both sites (n=1490) in Durban, South Africa.

Site	CROW	URBP	Both Sites
Culex pipiens s.l.	117	689	806
Mansonia africana	474	3	477
Coquillettidia microannulata	83	1	84
Aedes aegypti	52	26	78
Anopheles sp.	18	0	18
Eretmapodites quinquevitattus	4	4	8
Culex neavei	7	2	9
Culex subdentatus	1	0	1
Culex cinerellus	1	0	1
Culex duttoni	5	0	5
Culex simpsoni	2	0	2
Culex univittatus	1	0	1
TOTAL	765	725	1490

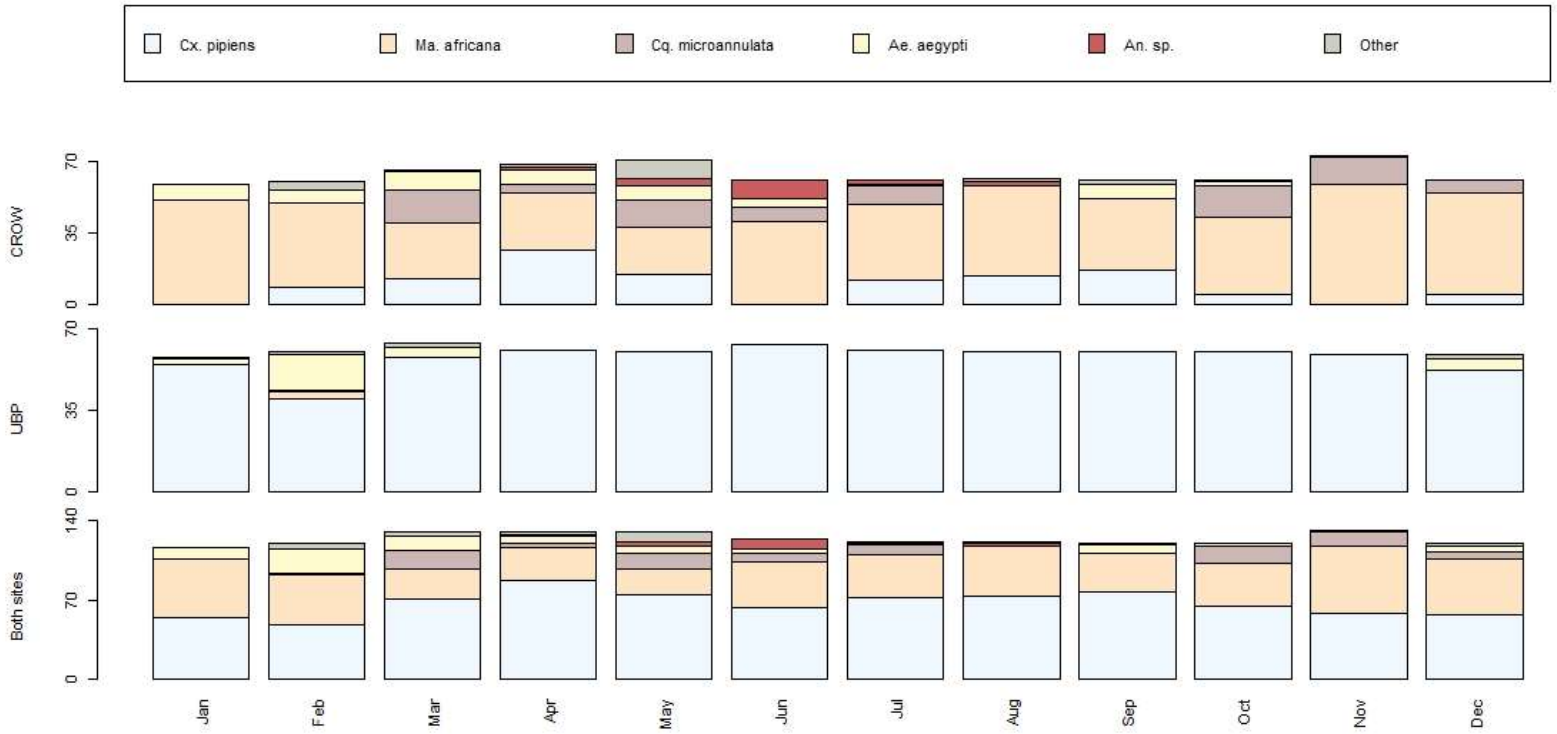

Figure 3.1: Bar graphs showing the numbers and composition of the five most common mosquito species collected each month at the Centre for the Rehabilitation of Wildlife (CROW) (top row), Umgeni River Bird Park (UBP) (middle row) and both sites (bottom row) combined, from April 2021 to March 2022.

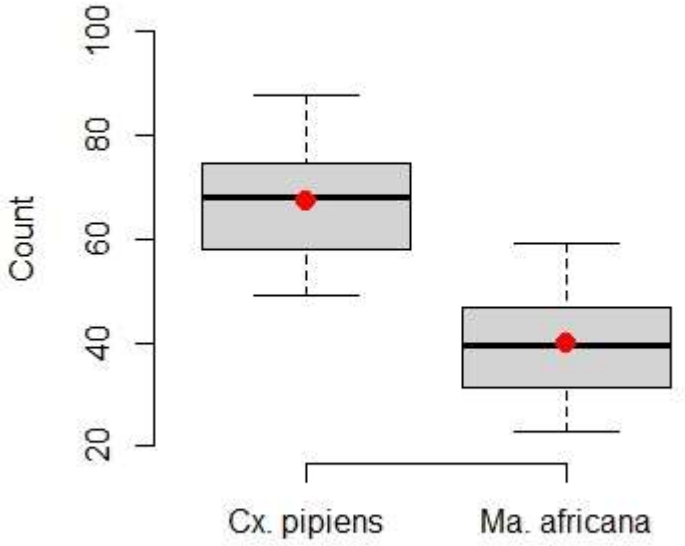

Figure 3.2: A box and whisker plots of the total numbers of *Culex pipiens* (s.l) and *Mansonia africana* mosquitoes that were dissected in the study. The red dot at the centre of each plot indicates a mean number of specimens collected for each of the two mosquito species (*Cx pipiens* = 67.17; *Ma. africana* = 39.75).

The *Cx. pipiens* (s.l.) (mean= 67.17) dissection counts across both sites were considerably higher than *Ma. africana* (mean= 39.75) (Figure 3.2). Overall, the number of *Cx. pipiens* (s.l.) dissections per month varied between 49 and 88 while that of *Ma. africana* was between 23 and 59. Between the months of April and November, *Cx pipiens* (s.l) was the only species dissected (n= 59-63) and for the remaining four months (December to March), the species made up 66.7-94.8% (n=43-63) of dissected mosquitoes while the remaining species dissected were *Ma. africana*, *Cq. microannulata*, *Ae. aegypti*, and other *Culex* spp. (Figure 3.1, 3.2). At CROW, *Ma. africana* was dissected throughout the year, with highest abundance (n=51-59) maintained in the summer (wet) season between November and January, while *Cx. pipiens* was collected in mid to high prevalence throughout the year. *Culex. pipiens* (s.l) was in high abundance all year round at Umgeni River Bird Park.

Mosquitoes dissected and evaluated for the presence of microfilariae showed no evidence of microfilarial activity in the midgut, Malphighian tubules, thorax or head and proboscis. Results of the molecularly analysed of the mosquito carcasses using PCR to detect the presence of filarial DNA using ITS2 and CO1 gene regions confirmed that no microfilariae were, in fact, present.

3.4 Discussion

The occurrence and prevalence of the microfilariae in various animals have been studied through a range of surveys conducted in the South African provinces of Gauteng, KwaZulu-Natal (KZN), Mpumalanga and North-West (Schwan, 2009; Voigts 2019). Using membrane filtration and acid phosphatase staining of blood samples, *Dirofilaria repens* (12.47 %) and *Acanthocheilonema reconditum* (1.2 %) were

prevalent in domestic canines and *D. repens* (10.98%) prevalent in domestic felines, reaffirming their endemism in KZN (Schwan 2009). The prevalence of *D. repens* and *A. reconditum* in the province is comparatively higher than Gauteng in which only 1.49% *D. repens* and no other species were found in domestic animals, thus establishing KZN as an important location for transmission of these filarial worms.

The current study aimed to investigate the presence and prevalence of potentially important mosquito vectors responsible for microfilariae transmission, to enhance the current knowledge of filarial disease in Durban, KZN. The expectation was that mosquitoes collected at animal-dense sites where a variety of exotic and indigenous birds such as African spoonbill, Grey crowned crane (indigenous) and Moluccan eclectus (exotic) or small to medium African mammals including vervet monkeys, mongoose, genets are found, would harbour endemic species, *D. repens*, *A. reconditum* and other filaroid species, however, it is likely that the prevalence of microfilarial infection is very low in the definitive host population. Though *A. reconditum* is known to be transmitted by lice and fleas, microfilariae have been detected using molecular techniques in *Culex pipiens f. quinquefasciatus* in a previous study, albeit at very low prevalence (0.2%) by accidental ingestion in blood meals (Manoj et al., 2012). Thus, non-susceptible mosquitoes provide information about circulating microfilariae in the infected animal population upon which they feed.

Due to the lack of existing work related to microfilariae incidence and prevalence in mosquito vectors in South Africa, the number of mosquitoes required for assessment was calculated using an estimated 4% prevalence from a study in West Africa (Abogye-Antwi et al., 2015), resulting in a minimum monthly sample size of 59 mosquitoes at each site. Based on the sample size, all the mosquitoes that were

analysed were found to be negative for microfilariae. Thus, prevalence of microfilariae in mosquitoes is below 4%, lower than originally presumed in the region.

Of the twelve endemic mosquito species investigated, *Cx. pipiens* had the highest abundance of 54.1% followed by *Ma. africana* (36.0%), confirming their endemism in Durban, South Africa. The two species reportedly have a high transmission potential for microfilariae, in particular, *D. repens* (Anyanwu et al., 2000; Manoj et al., 2021; Nelson et al., 1962; Schwan 2009). Thus, in the event of significant increase in prevalence or sudden onset of filarial infection recovered in domestic or wild animals in the region, it is recommended that these two species are screened as potential vectors.

The Durban metropolis has a vast number of ponds, river fringes, *s*torm drain catch basins and animal waste lagoons, which are preferable natural and artificial breeding sites for *Cx. pipiens* (Liu et al., 2019). The availability of these numerous breeding sites coupled with a generalist feeding nature and ability to shift between ornithophilic and mammalophilic preferences, identifies *Cx. pipiens* as a potential vector species in the region (Takken & Verhulst, 2012). As a confirmed vector of *D. repens* in other parts of the world such as Italy and Portugal, *Cx. pipiens* is an especially important potential vector species for subcutaneous dirofilariasis in Durban (Cancrini et al., 2012; Manoj et al., 2021).

Both the Centre for the Rehabilitation of Wildlife (CROW) and Umgeni River Bird Park contain a high faunal density as well as availability of preferable mosquito breeding sites and therefore were regarded as model sites for circulating microfilariae in the local mosquito populations living amongst the animals. The passive mosquito traps, baited with subliming carbon dioxide were positioned either within or near the animal

enclosures. Traps were operated between dusk and dawn, the peak period of most mosquito species which have crepuscular and nocturnal patterns of activity. These factors were considered to ensure the capture of an optimal number of relevant mosquitoes to investigate microfilariae prevalence.

The dominant species at each site differed due to variation in ecological factors (Kinyatta et al., 2018). Four traps were deployed in the same locations within each site every month. Notably, the bird park consists of several artificial pools of stagnant water in the enclosures, that are polluted with organic avian waste, which is a preferred breeding site of *Cx. pipiens*, thus the species constitutes 95.0% of the mosquitoes collected at this site (Liu et al., 2019). The wildlife rehabilitation centre contains diverse natural vegetation offering many favourable niches mosquito breeding in comparison to the bird park which was built over an old quarry and surrounded by cultivated tropical vegetation which attributed to the discrepancy in species number (Kinyatta et al., 2018). The high abundance of *Ma. africana* at CROW is ascribed to the presence of a vlei and the marshy forested areas at a neighbouring nature reserve, which are preferred breeding sites.

Previous studies, in subtropical Africa and further afield, focused on mosquito sampling in the wet season to coincide with the high abundance of mosquitoes which signals a much higher risk of microfilariae transmission (Capelli et al., 2013; Ughasi et al., 2012; Younes et al., 2021; Manoj et al., 2021). A study in Kenya revealed that 993 (60.8%) of the total mosquitoes collected over a 5-month long rainy season was *Cx. pipiens f. quinquefasciatus* and that 27 (1.7-1.8%) of these mosquitoes were found to be positive for microfilariae through dissection (Kinyatta et al., 2018). An estimated mean of 198.6 *Cx. pipiens f. quinquefaciatus*, were assessed for microfilariae per

month, nearly three times more than was investigated in the current study (mean= 67.17) (Figure 3.2). Given that no microfilariae were found using the proposed sample size and prevalence in mosquitoes is very low in the region, a larger sample size of between 200-300 is perhaps required to determine true prevalence (Kinyatta et al., 2018).

This preliminary study provided no positive result with regards to occurrence of microfilariae in dissected mosquitoes in Durban, South Africa. Further investigation is needed to determine true prevalence because the results demonstrate that the prevalence of microfilariae circulating in the most common mosquitoes in Durban is lower than 4%. It is recommended that (i) a larger sample size of mosquitoes per month should be screened to determine microfilariae prevalence due to the very low prevalence in the region and (ii) screening of captive birds and wild animals should be conducted in the Durban area, through microscopic or molecular examination of blood smears, to obtain more accurate data on the presence and prevalence of filarial infections.

3.5 Acknowledgements

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3.6 Author Contributions

N. Govender conducted the field collections and mosquito dissections. M. Troskie and N. Govender conducted the laboratory analyses. L.P. Snyman plotted the figures. N. Govender wrote the communication under the supervision of M.C. Marufu and L.P Snyman, who also conceptualised the project.

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3.8 Ethical approval

This study was approved by the Research Ethics (REC) and Animal Ethics (AEC) Committees (reference number: REC071-21), Faculty of Veterinary Sciences, University of Pretoria. In addition, a permit to conduct research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No 35 of 1984) was acquired from the Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development (DALRRD).

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CHAPTER 4: Presence of microfilariae (Family: Onchocercidae) in *Culex pipiens* s.l. in Durban Metropolitan Municipality, South Africa

Abstract

In South Africa, incidence and prevalence of microfilariae has been investigated in the domestic canines and felines, however, the role of mosquitoes in microfilariae transmission in the region remains obscure. The current study aimed to collect mosquitoes from two animal-dense sites in Durban (KwaZulu-Natal, South Africa) and molecularly screen for microfilariae. Mosquitoes were identified and pooled in conspecific groups. During PCR, two primer pairs, amplifying the COI and ITS2 regions were used for detecting microfilarial DNA in the mosquito pools. The amplicons for the COI and ITS2 genes were cloned to determine if multiple infections were present. To infer potential identity, a BLASTn search and phylogenetic inference using a maximum likelihood approach was employed. The most abundant mosquito species that were collected and screened were *Culex pipiens* (61.39%), *Mansonia africana* (21.13%) and *Aedes agypti* (8.02%). Both primer sets indicated that one pool of *Culex pipiens* was positive for microfilariae (Family: Onchocercidae). A species level identification could not be inferred from either the COI or ITS2 sequence obtained due to a lack of available reference sequences. Variation in phylogenetic topologies between COI and ITS data added complexity. The ITS2 phylogeny supported relatedness of the positive Onchocercidae sp. to *Mansonella* (81% bootstrap support; 99.05% identity). The results implicate *Cx. pipiens* as a possible vector.

Key words: COI, ITS2, mosquito-borne, Onchocercidae, phylogeny, vector

4.1 Introduction

There is a range of mosquito-borne filarial helminths that affect birds, reptiles, amphibians and mammals, including man (Anderson 2000). From an Afrotropical perspective, information on occurrence of filarial infections in their vertebrate hosts is predominantly published as incident reports (Sehgal et al., 2005). The main filariae species reported that are confirmed to be vectored by mosquitoes in the region are *Dirofilaria immitis, D. repens*, that infect domestic and wild canines and felines, *Wuchereria bancrofti*, that infects humans in most of the Afrotropics, except for southern-most countries including South Africa, and *D. corynodes* and *Brugia patei*, found in apes and genet cats respectively (Abdel-Rahman et. al., 2008; Cano et al., 2014; Heisch et al., 1959; Genchi & Kramer et al., 2020; Schwan, 2009).

With regard to avian microfilariae, general prevalence studies have been conducted in South Africa, Ethiopia, Cameroon, Equatorial Guinea, Ivory Coast, Kenya, Tanzania, Zaire, Ghana, Chad, Madagascar and Uganda, resulting in a low to moderate prevalence of 0.35-28.1% (Ashford et al., 1976; Bennet & Herman 1976; Earle et al., 1991; Kirkpatrick & Smith, 1988; Savage et al., 2009; Sehgal, et al., 2005; Valkiunas et al., 2005; Williams, et al., 1977; Wink & Bennett 1976). Nevertheless, the identification of avian helminths in the region is an enigma that requires further investigation.

Depending on the species, filarial nematodes have variable consequences for host fitness (Sehgal et al., 2005). Canine and feline heartworm disease (*Dirofilaria immitis*) has high pathogenicity, by presence of adult worms in the heart causing lesions in the pulmonary arteries and advancing to heart failure, if left untreated (Genchi et al., 2007). *Dirofilaria repens* (subcutaneous dirofilariasis), conversely, is less pathogenic

than *D. immitis*, in most cases producing painless hypodermal nodules however, there have been clinical reports of clinical variable dermatological irritations in dogs and cats (Genchi et al., 2007).

Thus far in South Africa, the incidence of microfilariae has been focused on domestic animals as well as incidental findings of zoonotic infection. Microfilariae presence or prevalence of wild mammals and birds have not been investigated. A few studies have investigated the occurrence of filariasis in domestic animals in South Africa and its neighbouring countries (Schwan & Durand; 2002; Schwan & Schrote 2006; Schwan 2009). These studies found that *D. immitis* (Mozambique), *D. repens* (Mozambique, South Africa), *Acanthochilonema reconditum* (Mozambique, South Africa) and *A. dracunculoides* (Mozambique, Namibia) are enzootic among dog and cat populations in the country, with specific infections of *D. repens* and *A. reconditum* reported in KwaZulu-Natal province, the prevalence of which was higher than the other three South African provinces that were surveyed. In addition, a case of human dirofilariasis was reported, in Kwazulu-Natal, South Africa (Moodley et. al., 2015), due to the close relationship between humans and their companion animals, thus characterising canine dirofilariasis as an emerging zoonosis (Dyab et al., 2015). These published reports provide substantial evidence that microfilariae are, in fact, circulating in definitive hosts in South Africa.

The role of mosquitoes, as intermediate hosts in the life cycle of filarial nematodes is essential. The mosquito's body provides a protected environment for larval development to the critical stage of infectivity. Mosquitoes act as agents for transmission for infective larvae (microfilariae). Identifying mosquito species involved in transmission of microfilariae and investigating their ecology and biology are

important for prevention and control of filariasis in birds, animals and humans (McKay et al., 2013; Noureldin et al., 2021). The mosquito species that have been reportedly involved in microfilariae transmission in subtropical Africa are *Culex pipiens sensu lato*, *Aedes aegypti, Mansonia africana* and *Ma. uniformis*, however, the presence and prevalence of microfilariae in mosquito vectors have not been specifically investigated in South Africa (Schwan 2009).

The use of dissection and microscopy for determining infection rate of microfilariae in mosquitoes has been the gold standard (Goodman et al., 2003). This method of detection is inexpensive and easy to conduct, requiring minimal equipment. However, when large quantities of mosquitoes are examined, it becomes time-consuming when dealing with low prevalence (Goodman et al., 2003). There is also difficulty in diagnosing microfilariae due to the vast morphological similarities (Sehgal et al., 2005).

Therefore, as a method of microfilariae detection to determine infection rate in mosquitoes, molecular detection provides specificity and sensitivity that is lacking in the method of dissection (Dyab et al., 2014). Over the last two decades, polymerase chain reaction (PCR) has been successfully used by several authors to test for microfilariae in mosquitoes and to distinguish between filarial nematodes in mosquitoes as well as blood samples (Bravo-Barriga et al., 2016; Kinyatta et al., 2018; Manoj et al., 2021; Rishniw et al., 2006). PCR is also used in molecular xenomonitoring for the detection of *W. bancrofti*, *D. immitis*, or *D. repens*, to assess the progress of filarial disease control programmes in areas of endemism (Dyab et al., 2014).

Investigations focussing on presence of microfilariae in mosquitoes have yet to be undertaken in South Africa. Studies of this nature provide much needed information on the epidemiology of filarial disease and the state of infection in the definitive host

population. Thus, the current study aimed at addressing this information paucity by (i) determining the mosquito species that harbour microfilariae in Durban, KwaZulu-Natal, South Africa by molecular means and (ii) inferring the potential identity of microfilariae found in mosquitoes by conduting a BLASTn search and phylogenetic inference using a maximum likelihood approach.

4.2 Materials and methods

4.2.1 Study locality

Mosquitoes were collected from two sites in the city of Durban, Republic of South Africa (29.9°S, 30.9°E) (Figure 4.1). The city is located in the only metropolitan municipality in KwaZulu-Natal province, eThekwini Municipal Area (EMA) which has a surface area of 2297 km^2 in size (eThekwini Municipality, [n.d]). Durban has a subtropical climate with the coldest, driest months occurring over June and July, and January and February are synonymous with warm, moist climate. The average maximum temperature ranges between 22.6-28.0°C and mean total precipitation ranges between 28.0-134.0mm (World Meterological Organisation, 2020).

The EMA contains a mosaic of urban, rural, and natural environments. According to Husqvarna Urban Green Space Index, Durban has 60% of green space within its perimeter and is classified as the greenest city in Africa and the second greenest city in the world (Husqvarna 2021). There are 34 proclaimed nature reserves maintained by either eThekwini municipality, the provincial conservation authority, Ezemvelo KZN Wildlife, or non-governmental organisations which is included in the 95 000ha system of natural areas under the Durban Metropolitan Open Space System (D'MOSS) (Roberts & O'Donoghue, 2013).

Table 4.1: Characteristics of collection sites, Umgeni River Bird Park and Centre for the Rehabilitation of Wildlife.

Umgeni River Bird Park	Centre for the Rehabilitation of Wildlife
3.5 ha	4 ha
Coastal/riverine	10 km Inland, Coastal forest
~800 indigenous and exotic birds	vervet monkeys, mongoose, geese, antelope, genets
bird population permanent	animals temporarily retained
several artificial water bodies	one vlei, other water bodies in neighbouring nature reserve
regular anti-helminthic treatment administered	new animals quarantined before treatment

The collection sites were the Umgeni River Bird Park (URBP) and the Centre for the Rehabilitation of Wildlife (CROW). The institutes house high densities of avian and mammalian fauna and contain water bodies, providing a breeding ground for mosquitoes (Table 4.1). The Umgeni River Bird Park (Durban North, South Africa, 29.80858°S 31.01745°E) is managed by eThekwini Municipality. The bird park is located along the Umgeni River, 4km from the coast and spans ca. 3.5 ha of tropical landscape. It was opened in 1984, with the aim of public education in diversity and bird conservation. The park maintains 800 birds belonging to over 200 indigenous and exotic species (Umgeni River Bird Park/eThekwini Municipality [n.d]). CROW (Yellowwood Park, South Africa, 29.91954°S 30.93628°E) was established in 1980 and is a fully accredited private Rescue-Rehabilitate-Release wildlife facility (CROW, 2021). The organisation cares for injured and orphaned wild fauna, the most common of which are vervet monkeys, mongoose, antelope, genets, and a variety of birds. The 4-ha plot is surrounded by forest patches and contains enclosures that replicate the animals' natural habitats.

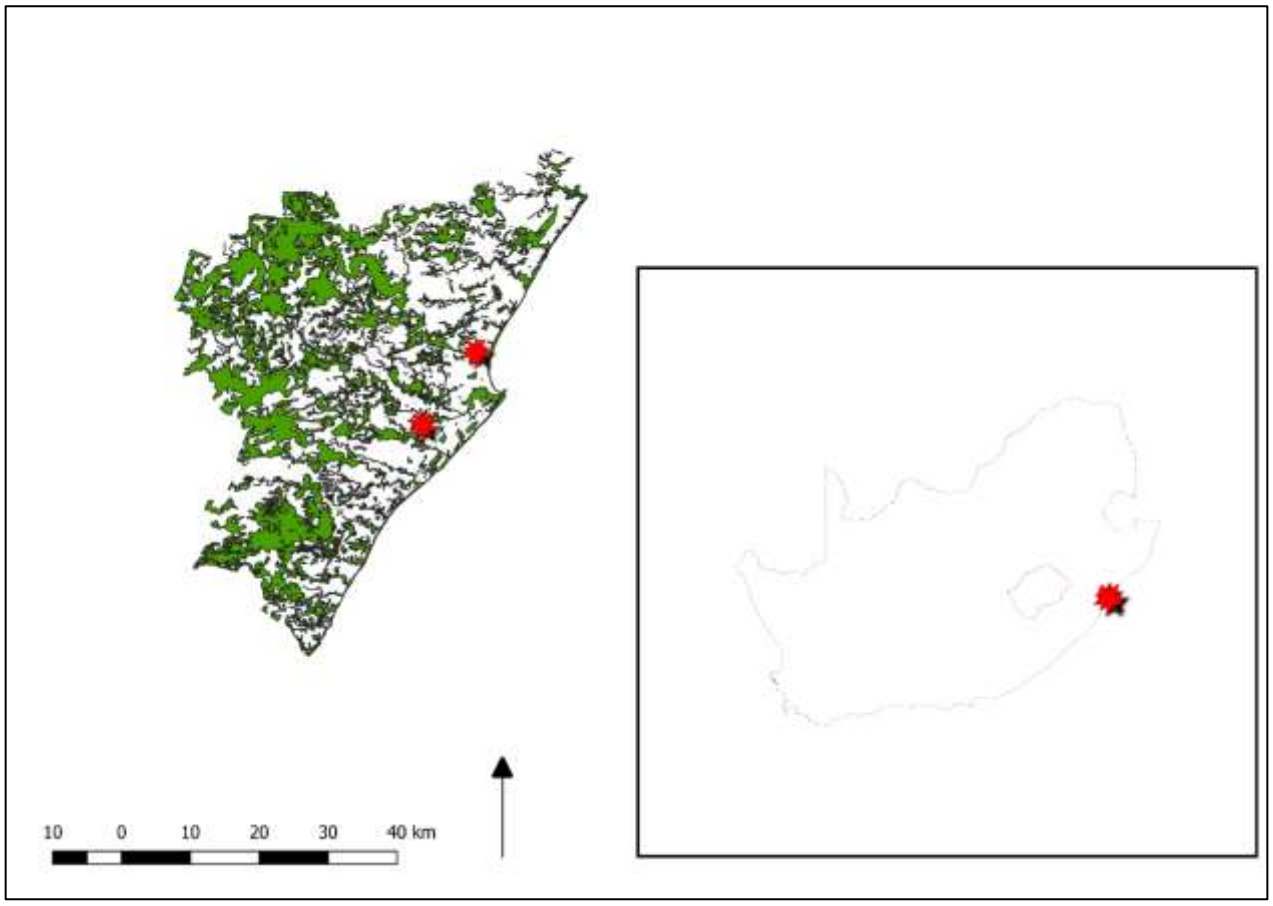

Figure 4.1: Republic of South Africa (inset) indicating location of Durban with green spaces (main) and two study sites (red stars).

4.2.2 Mosquito collections

This study was approved by the Research Ethics Committee and the Animal Ethics Committee (reference number: REC071-21) of the University of Pretoria. In addition, a permit to do research under Section 20 of the Animal Diseases Act, 1984 (Act No 35 of 1984) was acquired from the Directorate of Animal Health, Department of Agriculture, Land Reform and Rural Development (DALRRD).

Mosquitoes were collected monthly from April 2021 to March 2022. At each locality, four sites were selected. and two EVS-traps (BioQuip Products, CA, USA) and two

BG-2 Sentinel mosquito traps (Biogents, Regensburg, Germany) were set up at pre-selected sites for one to two nights per month (Figure 4.2). Traps were deployed at 16h00 and collected the next morning at 07h30.

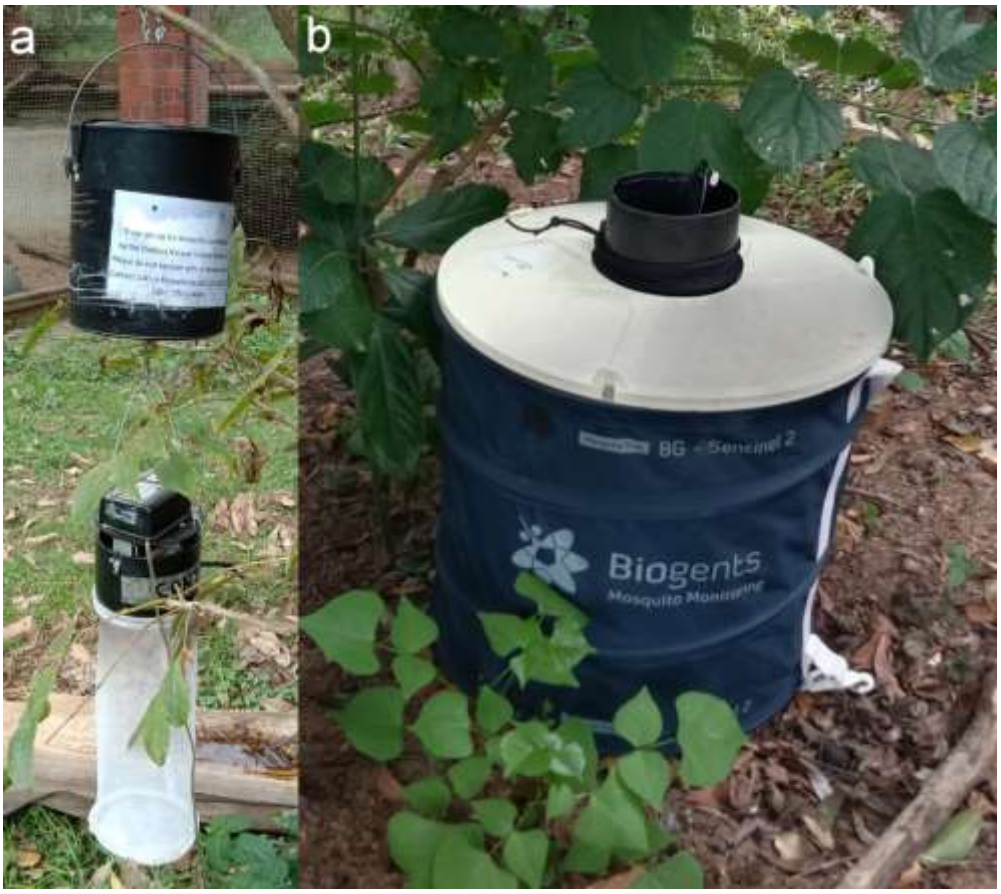

Figure 4.2: Encephalitis Vector Survey trap (a) and BG-2 Sentinel Mosquito Trap (b).

The EVS traps were placed up to 1.5 metres above the ground and the BG-traps were placed on the ground, baited with 1 kg dry ice as a source of CO_2. To ensure that the traps work independent to each other traps were placed at least 100m apart. Trap types were alternated each month amongst the sites, at each locality, to provide

opportunity for all possible mosquito species to capture as previous studies have suggested that collecting efficacy differs amongst trap designs (Lühken et al. 2014).

4.2.3 Morphological Identification

The trap collections were transferred from the field collection sites to Durban Natural Science Museum Research Centre's Invertebrate facility. Following each collection, the catches were sorted, and the female mosquitoes were identified microscopically using taxonomic keys published in *Mosquitoes of Southern Africa* (Jupp 1996) under a Nikon SMZ800N stereo microscope. For each trap catch, the taxonomic identification, number of individuals per species caught and abdominal status of females (blood fed, gravid or unfed) were recorded. The conspecific species were sorted into pools of one to ten mosquitoes.

4.2.4 Molecular analyses

4.2.4.1 DNA extraction

The pools of mosquitoes were placed into 2ml tubes with six 3 mm borosilicate solid-glass beads (Sigma-Aldrich, USA). The samples were homogenised using the Precellys® 24 lysis and homogeniser (Bertin Technologies, USA) using the following protocol, 5200rpm-2x18s-005. Genomic filarioid deoxyribonucleic acid (DNA) from the homogenised mosquito samples was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen™, Thermo Fischer Scientific, USA) according to the manufacturer's instructions with a few modifications. In each sample, 180 µl of PureLink™ Genomic Digestion Buffer and 40 µl of Proteinase K (20 mg/ml) was

added, briefly mixed by vortexing and incubated overnight at 56°C. After incubation, the samples were centrifuged at 14 000 rpm (maximum speed) for 3 minutes and the supernatant was transferred into 1.5 ml tubes.

Twenty microlitres of RNase A (20 mg/ml), mixed by vortexing and incubated for 2 minutes at room temperature. After incubation, 200 µl PureLink™ Genomic Lysis/Binding was added, vortexed and centrifuged thoroughly. Thereafter, 200 µl absolute (99%) ethanol was added, and the mixture was vortexed for 5 seconds. The entirety of the homogenous mixture was transferred into Purelink™ Spin Columns with Collection Tubes and centrifuged at 10 000 rpm for 1 minute. The flowthrough was discarded and thereafter, 400 µl of Genomic Wash (GW) Buffer 1 was added into the spin column and a new collection tube and centrifuged at 10 000 rpm for 1 minute and discarded the flowthrough again. The mixture was washed a second time using 400 µl of buffer GW2 and centrifuged at 14 000 rpm for 3 minutes. The spin columns were then placed in 1.5 ml Eppendorf tubes and 100 µl of PureLink™ Genomic Elution buffer was added and incubated at room temperature for 1 minute. To elute the DNA, the tubes were centrifuged at 14 000 rpm 1.5 minutes. The extracted DNA products were stored at -20 °C.

4.2.4.2 Conventional Polymerase Chain Reaction (PCR) and amplification

PCR was used to detect Filarioidea DNA from the mosquitoes using primer pairs designed to amplify the internal transcribed spacer-2 (ITS2) and the filarial mitochondrial DNA cytochrome oxidase subunit I (COI) genes, as described in Bravo-Barriga et al (2016) (Table 4.2).

Table 4.2: COI and ITS2 primers sets used to detect microfilariae in mosquitoes as described in Bravo-Barriga et al. 2016.

Primer	Gene	Sequence (5' to 3')
DIDR-F1	ITS2	AGT GCG AAT TGC AGA CGC ATT GAG
DIDR-R1	ITS2	AGC GGG TAA TCA CGA CTG AGT TGA
COIintF	COI	TGA TTG GTG GTT TTG GTA A
COIintR	COI	ATA AGT ACG AGT ATC AAT ATC

Twenty microlitres of PCR reaction was achieved by synthesising 0.5 µl of 20 µm forward and reverse primers, 10 µl of Phusion Flash High-Fidelity Master Mix (Invitrogen™, Thermo Fischer Scientific, USA) and 6.5 µl double distilled water with each 2.5 µl of DNA sample. The Veriti™ 96-Well Fast Thermal Cycle (Applied Biosystems™) was used to run the PCR cycles. The conditions for PCR amplification were as follows: initial denaturation at 98 °C for 10 s, 35 cycles consisting of denaturation at 98 °C for 1 s, annealing at 52 °C for 5 s, extension at 72 °C for 15 s, and final extension at 70 °C for 1 min.

Positive control: To validate the above protocol, two filarial-positive blood samples were obtained from the Helminthology Diagnostic Laboratory, Department of Veterinary Tropical Diseases (University of Pretoria) and were subjected to DNA isolation and PCR with both DIDR and COIint primers. The PCR products were sent for sequencing to confirm identification using BLASTn and phylogenetic inference.

4.2.5 Electrophoresis, gel extraction and PCR purification

Five microlitres of PCR products were electrophoresed with 1 µl of Tritrack DNA loading dye (6X) (Invitrogen™ Thermo Fischer Scientific, USA) on 2% Agarose LE (Benchmark Scientific, USA) gels, stained with 5 µl ethidium bromide. A molecular weight marker (GeneRuler 100 bp DNA Ladder, Thermo Fischer Scientific) was used. Gels ran at C at 120 V on a PowerPac™ Universal power supply (Bio-Rad Laboratories, USA). The DNA amplicons were analysed using a Gel Doc XR+ System and Image Lab Software (Bio-Rad Laboratories, Inc, USA). The amplicons were excised from the ethidium bromide-stained agarose gels under an ultraviolet transilluminator. The PCR products were purified from agarose gels using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen™, Thermo Fischer Scientific, USA) with a minor modification: elution in 15 µl E1 buffer to produce a concentrated purified product.

4.2.6 Cloning of positive samples

The Phusion Flash High-Fidelity PCR Master Mix amplification produces blunt ended amplicons for the COI and ITS2 genes which were cloned into pJET vector cells using the CloneJET PCR Cloning Kit (Invitrogen™, Thermo Fischer Scientific, USA) according to the manufacturer's instructions. The enzyme, T4 DNA ligase was used to ligate the purified DNA fragment to vector cells. The cells were transformed into C29921 5-alpha F'Iq Competent *E. coli* High Efficiency competent cells (New England BioLabs, USA). The transformed cells were plated onto imMediaTM AMP Agar media culture plates (Invitrogen, USA) and incubated overnight at 37°C.

Three to five white colonies per sample were selected. Each colony was screened for DNA inserts by using a colony PCR and placed into a 50 ml conical tube in 3ml of imMedia™ Amp liquid broth (Invitrogen, USA). The tubes were placed in a shaking incubator at 37°C to allow for overnight bacterial growth. Colony PCR was performed using the pJET1.2/blunt F primer (5'-dCGA CTC ACT ATA GGG AGA GCG GC-3') and pJET1.2/blunt R primer (5'-dAAGAACATCGATTTTCCATGGCAG-3') according to the manufacturer's instructions. The Invitrogen™ PureLink™ Quick Plasmid Miniprep Kit (Thermo Fischer Scientific, USA) was used to isolate recombinant plasmid DNA from the bacterial growths by following the manufacturer's instructions with a minor modification: recombinant plasmid DNA was eluted in 30µL buffer. The plasmid DNA was stored at 4°C.

4.2.7 Sequencing

The purified PCR products and recombinant plasmid DNA were sent to Inqaba Biotechnological Industries (Pretoria, South Africa) for sequencing. Sequencing was performed using BrilliantDye™ Terminator v3.1 kit (NimaGen, Netherlands). COI_F and COI_R and DiDF and DiDR primers were used for the purified DNA and pJET primers for the plasmid DNA. The sequences were obtained using an ABI 3500XL genetic analyser.

4.2.8 Sequence analysis and phylogeny

Similarity searches were conducted on microfilariae DNA sequences obtained from the PCR analyses using NCBI Nucleotide Basic Local Alignment Search Tool (BLASTn https://blast.ncbi.nlm.nih.gov/Blast.cgi).

In addition to BLASTn searches, three sequence matrices were prepared for analysis. Generated sequences were assembled using QIAGEN CLC Main Workbench and aligned to reference sequences using the online version of MAFFT (https://mafft.cbrc.jp/alignment/server/), with default parameters. The alignments were viewed, edited, and truncated using MEGA7 (Kumar et al., 2016). A maximum likelihood analysis was conducted in RAxML8 (Stamatakis et al., 2008) for each matrix, employing the GTRCAT approximation and calculating bootstrap support by invoking the autoMRE bootstopping criterion. The resulting topologies were viewed in FigTree4 (http://tree.bio.ed.ac.uk/software/figtree/) and graphically edited in Corel Paintshop Pro X8.

4.3 Results

A total of 1396 mosquitoes from 15 species were analysed by PCR for the presence of microfilariae. The most abundant species screened was *Culex pipiens* (61.39%) followed by *Mansonia africana* (21.13%) and *Aedes aegypti* (8.02%), while the remaining twelve species were in abundances lower than 3% (0.07-2.94%) (Table 4.3). With regards to site-specific mosquito abundances, *Culex pipiens* was most abundant at Umgeni River Bird Park (n=746) while *Mansonia africana* was found most abundantly at the Centre for the Rehabilitation of Wildlife (n=295).

Table 4.3: Numbers and percentages of mosquitoes per species, at each site that were molecularly screened over the course of the study period. CROW: 535; URBP: 861; $\%_{total}$: Percentage of total mosquitoes screened.

Species	Number	CROW	URBP	$\%_{total}$
Aedes aegypti	112	45	67	8,02
Aedes albocephalus	4	0	4	0,29
Aedes dentatus	3	3	0	0,21
Aedes hirsutus	3	0	3	0,21
Anopheles coustani	2	2	0	0,14
Anopheles sp.	22	22	0	1,58
Coquilettidea microannulata	41	41	0	2,94
Culex duttoni	2	2	0	0,14
Culex neavei	24	8	16	1,72
Culex nebulosus	4	4	0	0,29
Culex pipiens	857	111	746	61,39
Culex simpsoni	3	0	3	0,21
Culex zombaensis	1	0	1	0,07
Eretmapodites quinquevittatus	23	2	21	1,65
Mansonia africana	295	295	0	21,13

The mosquitoes pooled into 204 conspecific groups of one to ten individuals were screened to detect microfilariae using two microfilariae-specific primer sets, COI and ITS2 (Table 4.2), resulting in one pool testing positive for microfilariae DNA, following amplification. The positive pool contained two *Culex pipiens* mosquitoes which were collected from Umgeni River Bird Park in November 2021 (early summer- wet season).

The positive pool was cloned and resulted in four sequences, three amplifying COI and one amplifying the ITS2 gene regions. Similarity searches conducted on the sequences acquired from both COI and ITS2 genes were, unfortunately not possible beyond the familial level of Onchocercidae.

However, the analysis did not definitively correspond to any sequences deposited in Genbank (https://www.ncbi.nlm.nih.gov/genbank/). Only 91.64% identity with *Chandlerella quiscali*, could be acquired with the COI sequences (622 bp) (Table 4.4). Conversely, the ITS2 sequence (464 bp) matched *Mansonella perstans* with 99.05% identity but only 22% query coverage and consequently the identification was indeterminant, according to the results from the Nucleotide BLAST.

Table 4.4: BLASTn results for the clones, UB106_A3, UB106A4, UB106A5 (COI) and UB106_A4 (ITS2) of the positive mosquito pool.

Name	BLAST_ID	Region	Alignment Length (bp)	Max Score	Total Score	Query Cover	E value	% identity	Accession
UBP106_A3	*Chandlerella quiscali*	COI	622	861	861	100%	0.0	91.64%	HM773029.1
UBP106_A4	unclassified *Filaria*	COI	604	830	830	86%	0.0	95.39%	MH379965.1
UBP106_A5	*Chandlerella quiscali*	COI	622	861	861	100%	0.0	91.64%	HM773029.1
UBP106_A4	*Mansonella perstans*	ITS2	464	187	187	22%	1,00E-42	99.05%	MN432520.1

The maximum likelihood topology recovered from analysing the large COI dataset is presented in Figure 4.3. The barcoding portion of COI performed reasonably well for species delimitation, generating trees mostly conforming to the current taxonomy. A sequence designated to *Dipetalonema gracile* (AM749279) that had perfect support as the sister to *Mansonella atelensis* (AM749278), rendering the *Mansonella* genus paraphyletic. Similarly, *Acanthocheilonema viteae* rendered *Setaria* paraphyletic. It should be noted that the grouping lacked support. *Splendidofilaria* was also recovered as a paraphyletic genus with a sequence only designated as belonging to a *Splendidofilaria* sp. (JQ867052) being rendered the sister group to a monophyletic *Eufilaria* clade (bootstrap = 66) and a monophyletic *Splendidofilaria* + undetermined Filaroidea species (bootstrap = 95) closely related to *Eufilaria*. The three clone sequences formed a perfectly supported monophyletic unit (bootstrap = 100) that was recovered as the sister clade to *Chanderella quiscali*, albeit with weak support (bootstrap = 62).

The resulting trees generated from the taxon-comparative COI-ITS2 datasets displayed different topologies and showed ambiguity in the proposed identification of the positive pool. The topology recovered from the ITS2 analysis conformed into well-supported clades conforming to taxonomic designations, generally with strong bootstrap support (Figure 4.4). The cladogram from the COI analysis, however, did not support *Dirofilaria* as a monophyletic genus, instead placing *D. repens* as an outlier to a clade comprising *Wuchereria bancrofti* + *Brugia* and the sequence from this study (Figure 4.4). It is worth noting that no branch support for the placement of *D. repens* or the test sequence was recovered (bootstrap >50). The test sequence was

however placed as sister (bootstrap = 87) to a well-supported *Mansonella* clade (bootstrap = 100) in the topology generated by the ITS2 sequence analysis. The test sequence lacks species or genus level resolution due to incomplete taxon representation in the molecular libraries, as well as conflicting phylogenetic placement. Therefore, identification cannot be inferred from phylogenetic placement. It can only be concluded that species present in the *Cx. pipiens* pool was within the family Onchocercidae and is either closely related to *Mansonella* or *Chandlerella quiscali*.

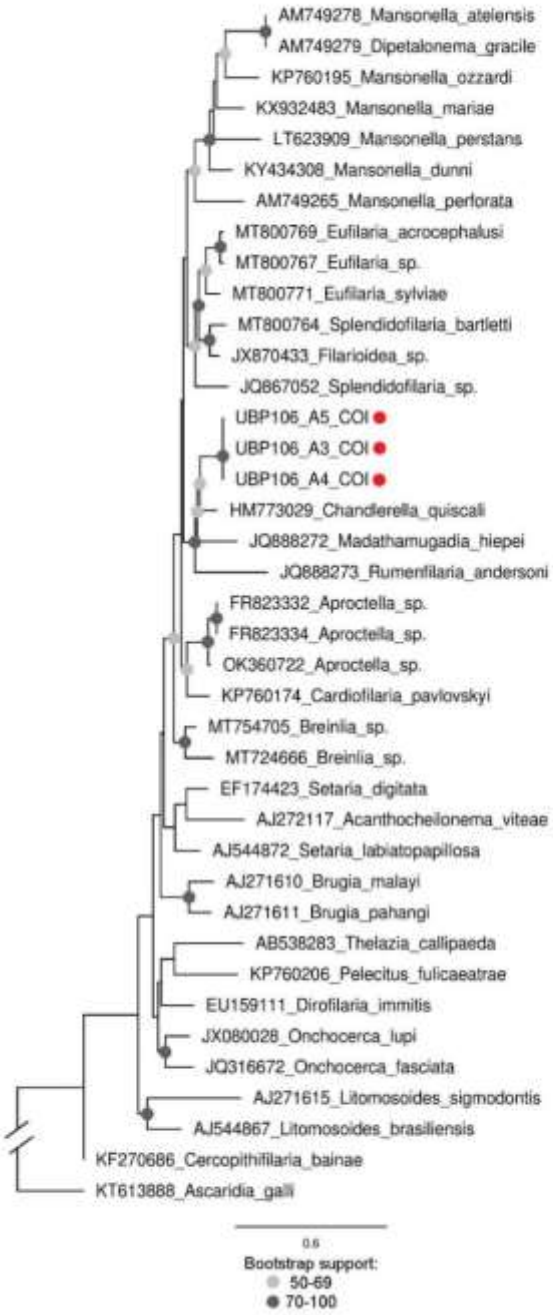

Figure 4.3: Maximum likelihood phylogenetic analysis of COI sequences for three clones (UBP106_A3 COI, UBP106_A4 COI, UBP106_A5 COI). The sequence data obtained in this work is marked with a red dot. The size bar indicates the number of nucleotide substitutions per site.

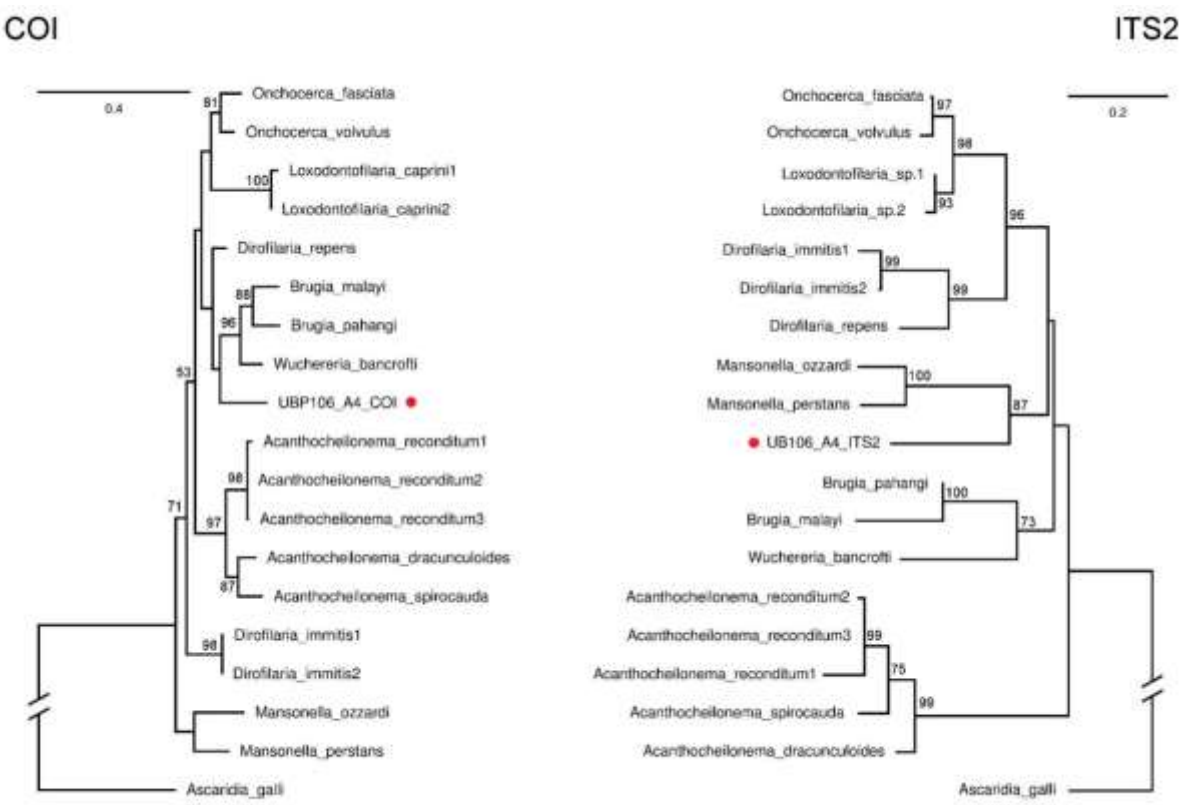

Figure 4.4: Conflicting phylogenetic topologies recovered from maximum likelihood analysis of COI and ITS2 sequences. The red dots indicate the sequence generated from *Culex pipiens* mosquitoes. Bootstraps calculated from 1000 replicates present as nodal support. (UBP106_A4). The size bar indicates the number of nucleotide substitutions per site.

4.4 Discussion

The positive mosquito pool detected in the present study, comprised *Culex pipiens* and therefore it can be proposed that this species most likely has vector potential for microfilariae in Durban. This finding supports conclusions made about the significant transmission potential of the mosquito species in the previous chapter (Chapter 3).

The microfilariae found in *Cx. pipiens* were detected in early summer (wet season). This finding coincides with the knowledge that there is higher microfilariae activity and infectivity rates in mosquitoes in the wet season. Mosquito surveillance studies have shown higher abundances in summer months (Kasili et al., 2009). Temperature is known to be a factor affecting both mosquito and microfilariae development. While designing *D. immitis*-specific diagnostic tools, Oh et al. (2016) found a direct correlation between ambient temperature and *D. immitis* larval development within mosquitoes. This suggests that temperature is a limiting factor for larval development.

Since whole mosquitoes were macerated and analysed in the study, it cannot be confirmed at this stage, that *Cx. pipiens* is responsible for transmission of infective microfilariae. To confirm vector competence, development of infective stages and migration to the head and proboscis needs to be determined (Schwan, 2009). This can be done by analysing the mosquito head, thorax and abdomen regions separately because, in general, presence of microfilariae in the abdomen indicates development of L2 and L3 larvae and presence in the head-thoracic muscles shows migration of infective (L3) microfilariae into the mouthparts in preparation for host infection (Bravo-Barriga et al., 2016).

In the current study, both Nucleotide BLAST results and phylogenetic topologies constructed using COI and ITS2 markers produced conflicting results which does not

provide conclusive evidence of a species-level or genus-level identification for the mosquito pool found to be positive for microfilariae.

The phylogeny of the large COI dataset, in general conformed to the currently accepted taxonomy however, a few deviations were observed. *Dipetalonema gracile* was the misplacement as a sister to *Mansonella atelensis*. The *Dipetalonema* and *Mansonella* do not share similar morphological or biological traits and in current taxonomy, they are not regarded as sister taxa. *Dipetalonema* rather forms a clade as a sister to *Acanthocheilonema*, *Monanaema*, *Cruorifilaria*, *Cercopithifilaria*, *Yatesia* and *Litonsoides* whereas *Mansonella* is currently grouped with *Wuchereria*, *Brugia* and *Breinlia* (Lefoulon et al., 2015). Though the misplacement of *Dipetalonema gracile* in the current study could, however, be due to erroneous designation or identification to the sequence. Additionally, the genus, *Splendidofilaria bartletti* is placed alongside the monophyletic *Eufilaria* clade, deviating from the current taxonomy which groups the genera as two separate but closely related clades (Binkienė et al., 2021).

In the taxon-comparative COI-ITS2 datasets, the COI topology isolated the canine filariae, *Dirofilaria repens* (Dirofilariinae) from the *Dirofilaria* clade whereas the ITS2 topology produced a representation of *Dirofilaria* that corresponds to the current taxonomy by grouping *D. repens* and *D. immitis* sequences in a single clade, with extremely high support. Therefore, the proposed grouping of *Dirofilaria* as an outlier to *Wuchereria* in the COI topology is not accepted. *Dirofilaria* is one of the only two genera that has a very early first larval moult in the animal host (2-3 days) whereas the other taxa that have been studied have a late first moult (6-10 days) suggesting that this group evolved separately from the rest of the Onchoceridae (Lefoulon et al., 2015).

Outside of the well-studied species, there is a huge diversity of other filarial nematodes that lack molecular characterisation that results in unresolved phylogenetic relationships within Onchocercidae (Whitton et al., 2004). In recent study by Lefoulon et al. (2015), phylogenies were proposed based on sampling of a wide diversity of genera within the family. The phylogenies varied with regard to conformity to the current classification of the filarial nematode family. Most notably, three closely related subfamilies, Onchocercinae, Splendidofilariinae and Dirofilarinae did not display monophyly, as expected and it was concluded that the presence of shared morphological, biological and molecular characteristics amongst the taxa of these three groups, demonstrates a need for revision of the subfamilial boundaries (Lefoulon et al., 2015).

There is a lack of resolution from phylogeny reconstructions in the current study as the sequence data show relatedness to two different species i.e., COI supports relatedness to *C. quiscali* and ITS2 supports relatedness to *M. perstans*. However, the well-supported bootstrap value within the ITS2 phylogeny provides the most accepted outcome. Interestingly, a similar finding was reported by Bravo-Barriga et al. (2016) who detected *Mansonella* sp. with 71% identity, albeit with low quality sequences, in the head and abdomen of *Cx. pipiens* with the same ITS2 primer pair used in the current study. The previous study similarly concluded that the mosquitoes contained *Mansonella*-like nematodes and that no further conclusions could be drawn even after molecular confirmation using COI primers (Bravo-Barriga et al. 2016).

The use of COI molecular markers has been accepted as the gold standard however, limitations of reconstruction of phylogenetic trees is present, as evident in the lack of definitively homologous identification in the current study, thus, employment of other

molecular markers, such as ITS2 molecular markers has become commonplace (Bourke et al., 2013). According to Rishniw (2006), the amplification of ITS2 gene, is capable of detecting several filarial nematode species, six of which were confirmed namely, *Acanthocheilonema reconditum, A. drucunculoides, Brugia pahangi, Dirofilaria repens* and *D. immitis*. Using the primers pairs, the expectation was that one or both known endemic species, *Acanthocheilonema reconditum* and *Dirofilaria repens* in KZN would have detected in mosquitoes if present (Schwan 2009). However, the lack of detection of these species could be due to the low prevalence in the study area.

It is concluded that, when dealing with filariae that are not well studied, phylogenetic reconstructions using sequence data do not necessarily conform to the current taxonomic classification of Onchocercidae, as demonstrated in this study. This disparity may be due to the lack of broad molecular taxonomy and phylogenetic data currently available. In order to produce accurate and specific identifications, a combination of morphological, molecular and biological data is required to add extensive phylogenetic knowledge to the family, Onchocercidae (Lefoulon et al., 2015).

To the authors' knowledge, this is the first time that Onchocercidae sp. was detected in mosquitoes in South Africa. Also, this is the first time that *Cx. pipiens* was implicated as a possible vector of microfilariae in the country.

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CHAPTER 5: General Discussion, Conclusion and Recommendations

5.1 General Discussion

The adult and larval stages (microflariae) of mosquito-borne endoparasitic filarial nematodes reside in mammals, birds, reptiles and amphibians, reportedly resulting in moderate to high pathogenicity, depending on the damage inflicted by the nematodes in the host. The prevalence of filaroids has been established in South Africa, as a previous study demonstrated the incidence of the endemic species i.e., *Dirofilaria repens and Acanthocheilonema reconditum* in domestic animal hosts, with KwaZulu-Natal (KZN) Province possessing the highest prevalence. The current state of knowledge regarding microfilariae in mosquito vectors is predominantly unknown in South Africa. To address the gaps in information, a study was conducted to investigate the presence, prevalence and phylogenetics of microfilariae found in mosquitoes in a major municipal metropolitan of Durban (eThekwini), KZN

It was established that there is a very low prevalence of microfilariae in mosquitoes found in Durban. No microfilariae were found in mosquitoes through dissection and microscopy. It was concluded that microfilariae prevalence in mosquitoes was below 4%, much lower than originally thought, given the previous overall prevalence of 14.21% of microfilariae in domestic animals in South Africa and Maputo province of Mozambique (Schwan, 2009).

Fifteen mosquito species were found during the study period with particularly high abundances of *Culex pipiens s.l.* and *Mansonia africana*. The moderate activity of *Cx pipiens* was noticed all year round, most likely due to the suitability of artificial breeding sites constantly available in the municipality. *Mansonia africana* abundances peaked in November to January (wet season) when temperature and rainfall is high, coinciding

with peak microfilariae infectivity. These species have also been characterised as exhibiting high transmission potential in other parts of subtropical Africa.

The attempt to detect and classify microfilariae, resulted in the implication of *Cx. pipiens* as a possible vector in Durban. The amplification of the COI and ITS genes of microfilariae DNA did detect presence in the mosquitoes however, failed to molecularly characterise microfilariae to genus or species-level. It is suspected that species that were not yet characterised in molecular libraries were detected. The phylogenetic hypotheses proposed matched current taxonomy, however, with a few diversions. The phylogenetic analysis based on the ITS2 gene produced the most accepted identification, concluding that the positive pool was a filarial species in the family Onchocercidae closely related to *Mansonella*.

This study is possibly the first to investigate and document microfilariae in mosquitoes in South Africa. The study provides fundamental knowledge concerning the role of mosquito populations in the known filarial nematode species in the country.

5.2 Conclusion

The research conclusions are as follows:

1. For Objective 1, it was concluded that no microfilariae were found in dissected mosquitoes of various genera in Durban, KZN. The negative result is possibly due to the very low prevalence of microfilariae in both the animal (definitive) hosts and in turn, the mosquito (intermediate) vectors which bite and feed upon the animals present at CROW and the Umgeni Bird Park in Durban.

2. For Objective 2, it was concluded that two mosquito species, *Culex pipiens* s.l. and *Mansonia africana* are potential mosquito vectors for microfilariae in Durban, South Africa.
3. In addition, with regards to the large diversity of filarial nematodes that are not well-studied, the use of COI and ITS2 genes of filarial nematode DNA are capable of broadly detecting presence of the parasites. However due to the lack of complete molecular libraries, determining identification of such species at genus and species level is difficult at this stage.

5.3 Recommendations

The following recommendations are given based on the research findings:

1. Given the conclusion that no microfilariae in mosquitoes were detected in this study, to confirm prevalence, there is a need for further investigation of larger sample sizes of mosquitoes. In addition, it is recommended that the most common species found in this present study, including *Culex pipiens*, *Mansonia africana*, *Aedes aegypti*, *Culex neavei*, *Coquilettidea microannulata* and *Eretmapodites quinquevittatus* are the primary focus in further regional studies of presence, prevalence, biology and genetics of microfilariae in Durban, South Africa.
2. Further studies are recommended to investigate the ability of *Culex pipiens* to support the development of microfilariae by assessing mosquito mouthparts, the location in which infective larvae accumulate in preparation for transmission. A combination of manual dissection and molecular techniques should be used to obtain morphological, morphometric, biological and genetic data to formulate a

comprehensive understanding of the life cycle, vectors and host ranges of filarial nematodes.

5.4 References

Schwan, E. V. 2009. Filariosis of domestic carnivores in Gauteng, KwaZulu-Natal and Mpumalanga provinces, South Africa, and Maputo province, Mozambique. Doctoral dissertation. University of Pretoria, pp. 1-155.

APPENDIX I

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

13 May 2022

CONDITIONALLY APPROVAL

Ethics Reference No	**REC071-21**
Protocol Title	**Detection of microfilariae (Nematoda: Filarioidea) in mosquito vectors trapped at animal shelters in Durban, South Africa**
Principal Investigator	**Ms N Govender**
Supervisors	**Dr MC Marufu**

Dear Ms N Govender,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC071-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Conditionally approved (pending obtaining ALL other relevant approvals).

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

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APPENDIX II

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

2 June 2022

Approval Certificate
New Application

AEC Reference No.: REC071-21
Title: Detection of microfilariae (Nematoda: Filarioidea) in mosquito vectors trapped at animal shelters in Durban, South Africa
Researcher: Ms N Govender
Student's Supervisor: Dr MC Marufu

Dear Ms N Govender,

The **New Application** as supported by documents received between 2022-04-06 and 2022-05-30 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-05-30.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Lower invertebrates - mosquitoes	1200
Samples	Number
various Culicidae species - pools of mosquitoes trapped at animals shelters	150

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-06-02.
3. Please remember to use your protocol number (REC071-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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APPENDIX III

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development Private Bag X138, Pretoria 0001
Enquiries: Ms Marna Laing • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/12 (2285KL)

Ms Natasha Govender
Durban Natural Science Museum Research Centre
Tel: 012 539 8827
E-mail: chris.marufu@up.ac.za; Natasha.Govender2@durban.gov.za

Dear Ms Govender

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application dated 18 January 2022 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The research project is approved as per the application dated 18 January 2022 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
3. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;

4. Only the following may be used in this study:

   4.1. Mosquitoes trapped at Umgeni Bird Park and the Centre for the Rehabilitation of Wildlife in Durban for which a state veterinary letter has been supplied to DALRRD. It is the responsibility of the researcher to contact the local state veterinarian and ensure that the abovementioned properties are not under veterinary restriction before sampling is conducted on these properties;

   4.2. An amendment to this permit may be obtained to allow sampling in other areas. Please apply in writing to MarnaL@dalrrd.gov.za;

5. All incidentally trapped vectors and other insects must be killed at the Durban Natural Science Museum Research Centre and disposed of by a waste removal company registered to remove biohazardous waste, in this case Compass Medical Waste;

6. No live animals may be used in this research project;

7. All insects and mosquitoes must be packaged and transported in compliance with the National Road Traffic Act 1996 (Act no 93 of 96) and/or IATA requirements;

8. Mosquitoes must be immobilized and killed at the Durban Natural Science Museum Research Centre. No live mosquitoes may be transported to the Department of Veterinary Tropical Diseases, Onderstepoort;

9. Only microfilariae organisms may be screened for in this research project under this section 20 permit using PCR and sequencing;

10. Any incidence or suspected incidence of a controlled or notifiable animal disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84), must be reported to the local state veterinarian immediately;

11. All documents or publications that may be made available to the public and that contain the outcomes of the testing of controlled and notifiable animal diseases in South Africa in terms of the Animal Diseases Act 1984 (Act no 35 of 84), must be screened and approved by the Director: Animal Health prior to their release and/or publication. These must be sent to MarnaL@dalrrd.gov.za and the Section 20 permit reference number and research project title must be included in the email;

12. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the research project;

13. Only a waste disposal company registered for the disposal of biohazardous waste may be used for the removal of all potentially infectious waste from the research project;

14. It is the responsibility of the researcher and relevant laboratory or facility managers to ensure that the human safety aspects of this research project are adequately addressed;

15. Records must be kept for five years for auditing purposes;

Title of research/study: Detection of microfilariae in mosquito vectors trapped at animal shelters in Durban, South Africa
Researcher: Ms Natasha Govender
Institutions: Durban Natural Science Museum Research Centre, eThekwini; Molecular Diagnostic Laboratory, Department of Veterinary Tropical Diseases, Onderstepoort.
Permit Expiry Date: 31 December 2025
Our ref Number: 12/11/1/12 (2285KL)
Your ref: REC071-21

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2022 -02- 23

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984) 12/11/1/12 (2285KL)*