THE WILD WORLD OF GUINEA WORMS: TRANSMISSION DYNAMICS AND PHYLOGENETICS OF THE AFRICAN AND NORTH AMERICAN GUINEA WORMS

by

CHRISTOPHER AARON CLEVELAND

(Under the Direction of Michael J. Yabsley)

ABSTRACT

Dracunculus medinensis, or Guinea worm (GW), causes a painful and debilitating infection in people. The Guinea Worm Eradication Program (GWEP) has successfully reduced human GW cases from 3.5 million in 21 countries in 1986 to 11 in four remaining endemic countries in 2018. In 2011, an unprecedented increase of GW infections in domestic dogs was reported and incidence has increased annually to 942 in 2018. Epidemiologically, it was posited that transmission may not occur via the classical route (i.e., ingestion of water containing infected copepods), but instead, via a paratenic host. My goals included 1) review *Dracunculus* species in wildlife and use these data to inform experimental transmission trials, 2) explore novel transmission routes under laboratory conditions, 3) conduct field-based surveillance of potential paratenic hosts in Chad, and 4) investigate the ecology of the North American Guinea worm, *D. insignis,* in wildlife and domestic animals in the United States. I showed that *D. medinensis* and *D. insignis* can experimentally infect several amphibian species, amphibians serve as paratenic hosts and natural *D. medinensis* infections were found in three frog species in

Chad. Although fish did not become infected (as paratenic hosts), fish were able to serve as transport hosts. These data illustrate novel transmission routes with the latter causing great concern as fish are a heavily relied upon in Chad as a food source. Also, villagers feed their cats and dogs fish entrails; our data suggest this could lead to infection. Our U.S.-based studies documented numerous dog and cat infections and that raccoons (*Procyon lotor*) and opossums (*Didelphus virginiana*) were common wildlife hosts. The high prevalence of infection in opossums was previously unreported. Based on partial cytochrome c oxidase gene sequences, little diversity was noted among worms from dogs and wildlife and no geographic or host-associations were noted. We also detected *D. insignis* larvae in musculature of frogs, further confirming that paratenic hosts may be playing a role in transmission of *Dracunculus*. Collectively, these data have filled several knowledge gaps in the *Dracunculus* life-cycle, resulting in the development of new interventions to assist the GWEP in endemic countries.

INDEX WORDS: *Dracunculus insignis*, *Dracunculus medinensis*, Dracunculoidea, Amphibians, Fish, Paratenic hosts, Transport hosts, Guinea worm, Eradication

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DEDICATION

The work herein is representative of efforts far beyond the scope of an individual. The amount of sacrifice, time, energy, hard work, and devotion cannot be attributed to myself, but to countless people who over the years have been supportive, have been emotional and intellectual strongholds, and without whom I wouldn't be facing down the completion of this work.

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~To the Demise of the Worm.

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CHAPTER 1

INTRODUCTION

Understanding the life cycle and transmission pathways of parasites is imperative for effective control, prevention and eradication. Parasitic nematodes infecting livestock and humans cause high medical, educational and economic burden and management efforts for several key species have been initiated (Hotez et al. 2008). Yet, successful elimination of parasites with complex life cycles is challenging and complicated by potential shifts to novel hosts, behavioral patterns of hosts, and the opportunity for multiple pathways of transmission to be involved (Webster et al. 2017).

One of the most well-known nematodes targeted for eradication is *Dracunculus medinensis* (Order Spirurida), a member of parasitic nematodes within the Superfamily Dracunculoidea which infect mammals, fish, reptiles, amphibians and birds (Chabaud, 1960; Petter and Planelles, 1986). *Dracunculus medinensis* has been associated with people for a very long period of time and although mortality is rare, morbidity can be severe and debilitating, causing an overall loss of productivity.

A global eradication campaign was initiated to alleviate the effects of dracunculiasis (Hopkins et al. 2017). In 1986, the World Health Assembly alongside The Carter Center, with support from the World Health Organization (WHO), Centers for Disease Control and Prevention (CDC), and additional partners began the global Dracunculiasis Eradication Program (DEP). At the time, there were an estimated 3.5

million cases in 21 countries in Africa and Asia (Hopkins et al. 2017). At the end of 2017, only 30 human cases of dracunculiasis were reported from two countries (Chad and Ethiopia). However, Chad, Ethiopia and Mali all reported an increase in dracunculiasis among domestic dogs. This poses serious challenges to the eradication campaign, especially considering the increasing number and geographical occurrence of dog infections in Chad (Hopkins et al. 2017).

In 2010, after a ten-year hiatus of zero reported infections in Chad, ten human dracunculiasis cases were documented. Cases continued to be discovered, and Chad was declared endemic again for guinea worm in 2012 (Hopkins et al. 2017). Also, in 2012, the first reports of dracunculiasis in dogs were documented (Eberhard et al. 2014). The geographic and temporal co-occurrence of dog infections and human cases led to the question of whether dogs were acting as a reservoir for *D. medinensis*.

Dog infections also complicated the modality of transmission occurring in Chad, as only a single or few human cases would be documented in a single area, yet numerous dog infections in the same area would be observed (Eberhard et al. 2014). This pattern does not support the typical point-source infection seen historically with classical water-borne transmission. There were many hypotheses developed to understand this including 1) dogs and people were infected with different strains of the parasite and 2) another method of transmission may be involved, such as a paratenic host (Eberhard et al. 2014).

The hypothesis that an alternate transmission route may be occurring with *D. medinensis* was well founded, based on the life-cycle of a closely related nematode, *Dracunculus insignis,* found in North American wildlife. First described as *Filaria insignis* (Leidy, 1858), the parasite was moved to the genus *Dracunculus* and, in fact,

once was considered conspecific with *D. medinensis*, but is now accepted as a unique species (Chitwood, 1933; Chandler, 1942). A comprehensive review of *D. insignis,* as well as other species within the genus that infect wildlife, is presented in Chapter 2 of this dissertation.

To better understand the transmission of *D. medinensis* to dogs, several experimental and field experiments on both *D. medinensis* and *D. insignis* (as a model system) were conducted. The specific goals included 1) conduct a literature review of known species of *Dracunculus* species and identifying key knowledge gaps, 2) investigate the role of fish as a paratenic or transport host, 3) conduct surveillance for *D. medinensis* third-stage larvae in aquatic animals in Chad, Africa, and 4) study the transmission, host diversity and phylogenetics of *D. insignis* at a site in the southeastern United States with a high prevalence of infection.

The results of this work will provide insights on how transmission might be occurring in Chad. The inclusion of either paratenic or transport hosts in the life-cycle of *D. medinensis* had not previously been described, and our work could highlight the role that these animals perform in transmission, especially among dogs. We also planned to identify how sylvatic transmission for *D. insignis* occurs, as consumption of an amphibian paratenic host is a pathway which has been suggested but never proven (Anderson, 2000).

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CHAPTER 2

THE WILD WORLD OF GUINEA WORMS: A REVIEW OF THE GENUS *DRACUNCULUS* IN WILDLIFE.[1]

[1]**Cleveland, C.A.,** Garret, K.B., Cozad, R.A., Williams, B.M., Murray, M.H., and M.J. Yabsley. 2018. Open Access. *International Journal of Parasitology: Parasites and Wildlife* 7 (3): 289-300. Reprinted here with permission of publisher.

Abstract

Nematodes are an extremely diverse and speciose group of parasites. Adult dracunculoid nematodes (Superfamily Dracunculoidea) occur in the tissues and serous cavities of mammals, fish, reptiles, amphibians and birds. Of the dracunculid group, perhaps best known is *Dracunculus medinensis*, the human Guinea Worm. Considerable work has been done on *D. medinensis*; however recent infections in peri-domestic dogs and the finding of naturally-infected paratenic hosts (previously unreported for *D. medinensis*) indicate we still have much to learn about these parasites. Furthermore, among eight species in the Old World and six species in the New World there is a lack of general life history knowledge as well as questions on species occurrence, host diversity, and transmission dynamics. Herein, we provide a comprehensive review of the genus *Dracunculus*, in order of a theoretical evolutionary progression from reptilian to mammalian hosts. Species descriptions, where available, are provided but also show where gaps occur in our knowledge of various species. Additionally, many first reports of *Dracunculus* spp. were done prior to the development and use of molecular tools. This is especially important for this group of parasites as speciation based on morphology is only applicable to males of the genus, and males, given their size, are notoriously difficult to recover from definitive hosts. Therefore, we also discuss current molecular tools used in the investigation of this group of parasites. Given recent host-switching events, the dracunculids are of increasing importance and require further work to expand our understanding of this genus.

1. Introduction

Adult dracunculoid nematodes (Superfamily Dracunculoidea) occur in the tissues and serous cavities of mammals, fish, reptiles, amphibians, and birds (Chabaud, 1960; Petter and Planelles, 1986). Many of the species in this group are similar in that females fill with large numbers of first-stage larvae that are released when females come into contact with water. The Family Dracunculidae contains two genera: *Dracunculus*, parasites of mammals and reptiles, and *Avioserpens*, parasites of birds. There are 14 valid species of *Dracunculus* but most knowledge about these parasites stems from research on the medically important *D. medinensis*, also known as the African Guinea worm. Considerable literature and reviews exists for this species (Muller, 1971, 1976; Cairncross et al., 2002; Ruiz-Tiben and Hopkins, 2006; Eberhard et al., 2014). The life cycle of *D. medinensis* has been well studied and documented, due in large part to the long history of human infections and an eradication campaign initiated by the World Health Assembly in 1981 and spearheaded in 1986 by The Carter Center (Ruiz-Tiben and Hopkins, 2006). However, despite a long history of epidemiologic and public health research, recent infections in peri-domestic dogs in several remaining endemic countries in Africa and the finding of naturally infected paratenic hosts (previously unreported for *D. medinensis*) indicate we still have much to learn about these parasites (Eberhard et al., 2014, 2016a,b; Cleveland unpublished data). This review focuses on *Dracunculus* species other than *D. medinensis*, with an emphasis on *D. insignis*, which is being used as a model parasite for studies to assist the Guinea Worm Eradication Program.

1.1 Genus *Dracunculus*

Nematodes in the genus *Dracunculus* are large subcutaneous parasites of mammals and reptiles (snakes and turtles) with most described species being from snakes. The females of *Dracunculus* spp. are some of the longest nematodes with recorded lengths up to 100 cm (Cairncross et al., 2002). Morphologically, female *Dracunculus* spp. are very similar and molecular characterization is needed for definitive identification. Males are considerably smaller (16–40 mm), but they have several morphological features that can be used to distinguish the different parasite species (Crichton and Beverly-Burton, 1973; Cairncross et al., 2002). Unfortunately, males are rarely detected and have never been described for some species. This is particularly problematic for hosts that may be infected with more than one dracunculid (e.g., river otters (*Lontra canadensis*)) or with parasites detected in novel hosts.

1.2. Species of *Dracunculus*

The highest diversity of described *Dracunculus* species occurs in the Old World. To date, eight species have been described with seven occurring in snakes endemic to Europe, Africa, Asia, and Australia (Table 1). Although most *Dracunculus* species described are from snakes, the most widely known and studied species is *D. medinensis*, the human Guinea worm, which also happens to be the only Old World mammalian species (Eberhard et al., 2014). Although *D. medinensis* was historically widespread in Africa and South Asia, through considerable management and eradication efforts, the total number of countries with endemic transmission in either humans or dogs has been reduced from 21 to 3 resulting in a decrease of human cases from 3.5 million in 1986 to 30 in 2017 (Molyneux and Sankara, 2017; https://www.cartercenter.org/health/guinea_worm/case-totals.html). Numerous studies and reviews on recent

developments related to this parasite have been published (Eberhard et al., 2014; Eberhard et al., 2016a; b; Cleveland et al.,2017).

In the New World, a lower diversity of *Dracunculus* species has been reported, but more species have been described from mammals (Tables 1 and 2). There are at least 2 species of *Dracunculus* that infect snakes (*D. ophidensis* and *D. braziliensis*), 1 from a snapping turtle *(D. globocephalus*), and 3 from mammals (*D. insignis, D. lutrae,* and *D. fuelleborni*).

1.3. General life cycle

Adult female *Dracunculus* mature in the subcutaneous tissues of the definitive vertebrate host where they will form blisters primarily on the distal extremities; however, reports of these lesions are rare in reptile hosts (Crichton and Beverley-Burton, 1977; Brackett, 1938). When the host places the affected area in water the blister will erupt and the female release nearly 500, 000 first stage larvae (L1) into the water column (Muller, 1971). The release of larvae can occur multiple times during different events.

Cyclopoid copepods are a required intermediate host in the life cycle of *Dracunculus* species, although few naturally or experimentally competent species have been identified (Table 1). The intermediate host range appears broad as Old World copepod species can become infected with *D. insignis* and New World copepods species can become infected with *D. medinensis* (Sullivan et al., 1991). However, there was differential mortality among different copepods species following infection with *D. medinensis* suggesting some copepod species are better intermediate hosts than others (Bimi et al., 2005). Once L1s are consumed by copepods they develop to the infective third stage larvae (L3) (Fedchenko, 1871; Crichton and Beverley-Burton, 1975).

Dracunculus medinensis and *D. insignis* L1 molt to L3 within 21–25 days at 24 °C (Fedchenko, 1871; Crichton and Beverley-Burton, 1975).

Definitive hosts can become infected by ingesting L3s within copepods when drinking water or, for some species, consumption of frog paratenic hosts or fish transport hosts (Fig. 1) (Brackett, 1938; Crichton and Beverley-Burton, 1977; Eberhard and Brandt, 1995; Anderson, 2000; Eberhard et al., 2016a,b; Cleveland et al., 2017). Once ingested, L3s migrate to subcutaneous and intramuscular connective tissues of the thoracic and abdominal musculature where they undergo an additional 2 molts. Male and female nematodes fully mature to adults after 60–70 days, with fertilization occurring after maturation (Anderson, 2000). Males and unfertilized females remain in subcutaneous musculature of the abdomen and thorax, while fertilized females begin to migrate through the subcutaneous tissues, primarily to the distal extremities. Once fully gravid and larvigerous, females create a nodule and an ulcerative-type lesion. This lesion occurs when the female releases uterine fluid (which may contain a few larvae), initiating an immune response from the definitive host and leading to a “thinning” of epidermis through which she can emerge (Muller, 1976; Cairncross et al., 2002).

When these lesions are exposed to water, larvae are expelled from the female into the environment and consumed by the intermediate cyclopoid copepod host (Fig. 1). Female nematodes will then senesce and may be pulled out of tissue by the affected animal or will retreat subcutaneously and calcify. Male and unfertilized female nematodes can survive for 330 days; however, it is proposed that this estimate is conservative (Crichton and Beverley-Burton, 1973, 1974; 1977; Brandt and Eberhard, 1990).

1.4. Diagnostic features

Dracunculus spp. adults are large filiform pale yellow-white nematodes that have marked sexual dimorphism with females being considerably larger than males (Table 1). Both females and males have an atrophied intestine and females have atrophied vulva and vagina. Gravid female worms are almost completely occupied by a uterus distended with L1. The anterior end is rounded, the buccal cavity is reduced, the mouth opening is round and surrounded by variable numbers of papillae, and a thick and clearly defined peribuccal ring is present. Anterior constriction just anterior to the nerve ring may be present. The esophagus is very long with a short muscular portion and a long glandular portion. Tails are generally conical with a sharply pointed tip. Males have either equal or subequal spicules and most species have a gubernaculum (Brackett, 1938; Chandler, 1942a; Crichton and Beverly-Burton, 1975; Cairncross et al., 2002; Moravec and Little, 2004).

First stage larvae released from female worms have extremely long, tapered tails and their overall length can vary between species (i.e., 0.3–0.9 mm), but there is considerable overlap in ranges for many species (Fig. 2). When available, length of L1s for valid *Dracunculus* spp. is provided in Table 1. When larvae develop to the infectious L3 stage in copepods, they lose the long tapering tails, become shorter and broader (i.e., 0.24–0.608 mm, although lengths of most species are unknown), and develop a trilobed tail (Fig. 2) (Anderson, 2000; Muller, 1971).

2. *Dracunculus* species of squamates

Currently there are nine formally described *Dracunculus* species in snakes. The highest diversity has been reported in Eurasia and Africa where *D. coluberensis, D. alii,*

D. houdemeri, *D. doi*, *D. dahomensis*, and *D. oesophageus* occur. A single species, *D. mulbus*, has been reported from Oceania. In the Americas, two species (*D. ophidensis* and *D. brasiliensis*) have been described. Also, worldwide, several uncharacterized species have been reported from numerous snake species.

2.1 Squamate *Dracunculus* species by region

2.1.1 *Dracunculus ophidensis* (Brackett, 1938) and other species from North America

Dracunculus ophidensis was first described from garter snakes (*Thamnophis sirtalis*) from southern Michigan and subsequently found in Minnesota (Brackett, 1938). Nematodes were found in the subcutaneous tissue, serous membranes, and body cavity of infected snakes, and during summer months, gravid females were present in visible subcutaneous swellings (Brackett, 1938; Moravec, 2006). Infections were only detected during the summer months, with signs of infection disappearing by fall or early winter (Brackett, 1938). The arrangement of genital papillae and length of spicules in males are the most reliable characteristics used to differentiate *D. ophidensis* from other *Dracunculus* species. Spatial variation has been noted in *D. ophidensis* prevalence with a higher prevalence found in Minnesota compared with Michigan (Brackett, 1938).

Parasites identified as *D. ophidensis* have been reported from the mesentery of the Blackbelly garter snake (*Thamnophis melanogaster*) from several states in Mexico, indicating that this parasite may be widespread in garter snakes (Pérez-Ponce de León et al., 2001; Jiménez-Ruiz et al., 2002; Moravec, 2006). *Dracunculus ophidensis* also has been reported from northern water snakes (*Nerodia sipedon*) from Maryland, Minnesota, and Pennsylvania, and a plain-bellied water snake (*Nerodia erythrogaster*) from

Michigan. However, confirmation in these other species requires molecular characterization or detection of male nematodes (Mirza and Roberts, 1957; USNPC 1362086; Mirza, 1957; Moravec, 2006).

Unidentified *Dracunculus* spp. have been detected in a captive gopher snake (*Pituophis catenifer*) and a captive Florida kingsnake (*Lampropeltis getula floridana*) from The National Zoological Park (Washington D.C.) (Mirza and Roberts, 1957; USNPC 1342730).

2.1.2 *Dracunculus braziliensis* (Moravec, 2009) and other species from Central and South America

A single subgravid female and part of a gravid female recovered from subcutaneous tissue and mesentery of one anaconda (*Eunectes murinus*) from Brazil were used to describe *D. brasiliensis* (Moravec and Santos, 2009). Although most female *Dracunculus* spp. are morphologically similar, the shape of the female caudal end of *D. brasiliensis* differs from other *Dracunculus* spp. in that it is broad with a widely-rounded tip and the excretory pore located just posterior to the nerve ring. Additionally, the L1 of *D. brasiliensis* are considerably larger than most snake-infecting *Dracunculus* spp. (Moravec and Santos, 2009). Recently, *D. brasiliensis* was reported in a Brown-banded water snake (*Helicops angulatus*) from Matto Grosso state, Brazil (Quirino et al., 2018). Description of a male of this species is still needed.

Uncharacterized *Dracunculus* spp. have been reported from a number of free-ranging Central American, South American, and Caribbean snake species including free-ranging anaconda from Brazil and Venezuela, Pearl Island boa (*Boa constrictor sabogae)* from Panama, and boas (*B. constrictor* and reported as *Corallus enydris*) from Trinidad

(Muller, 1971; Calle et al., 1994; Moravec and Santos, 2009; USNM 1345047 and 1345052). In addition, unidentified *Dracunculus* nematodes have been recovered from several captive snakes that had been imported from South America including boas (*B. constrictor and B. mexicana*), an anaconda, and a bushmaster (*Lachesis muta*) (Kotlan and Raitsits, 1923; Moravec, 1966; USNM 1338673 and 1335517).

2.1.3 *Dracunculus* spp. from Eurasia

The highest diversity of squamate *Dracunculus* spp. have been reported from Eurasia, including *D. oesophageus* (Desportes, 1938), from Europe and *D. coluberensis*, *D. alii* (Deshmuck, 1969), and *D. houdemeri* (Hsü, 1933) from Asia (Table 1). Many of these remain poorly described and studied, with the latter three having only been described based on individuals of one sex. Furthermore, from the checkered keelback snake (*Xenochrophis* (=*Natrix*) *piscator*), *D. alii* was described from only male nematodes in India and *D. houdemeri* was described from only subcutaneous female nematodes in Vietnam (Hsü, 1933; Deshmukh, 1969). No other life history traits are known, and additional morphological and molecular work is needed to better define the validity of these two species from keelback snakes.

The best studied of this group is *D. oesophageus* which was described from colubrid snakes (Moravec, 2006). A male specimen of *D. oesophageus* originally described as a *Filaria oesophagea*, was detected in the esophagus of a viperine snake *Natrix maura* (=*N. viperina*), thus the specific epithet. Based on morphologic analysis of parasites detected in the grass snake (*Natrix natrix persa*), the parasite was transferred to the genus *Dracunculus* and renamed *D. oesophageus* (Desportes, 1938, Deshmukh, 1970). Parasites from *N. natrix* described as *Pesteria inglisi* by Tadros (1966) are

presumed to be *D. oesophageus*. The copepod *C. fuscus* is a confirmed experimental intermediate host (Desportes, 1938). The tails of the L3 have the typical tricuspid tail similar to *D. medinensis*. This species is also the only squamate *Dracunculus* spp. for which there is any genetic data available; phylogenetic analysis of ~1,600bp of the 18S rRNA gene from two specimens from the mesenteries of *N. natrix* from Slovakia indicated that this parasite was related to *D. medinensis* and the fish parasite *Philometra obturans* which is in the same order as *Dracunculus* (Wijová et al., 2005).

An unidentified female *Dracunculus* species was reported emerging from the head of a cobra (*Naja tripudians*) that was held in captivity. Recovered larvae were used to infect copepods and underwent growth (from 300µm to 600µm). Subsequent inoculation of snakes with the unidentified *Dracunculus* species failed, possibly due to the use of copepods that had only been infected for six days and larvae that had not yet molted to the infectious L3 stage (Turkhud, 1920).

The only report of *Dracunculus* in a lizard species was by Mirza and Basir (1937), but this report is suspect. In that study, 60% of monitor lizards (*Varanus* sp.) in India were infected in the body cavity and in subcutaneous tissues with up to 15 worms. However, identification was based only on one damaged female worm measuring 68cm long. Although originally identified as *D. medinensis*, no additional morphologic or molecular work was conducted so it is possible that this parasite is one of the commonly reported filarial worms (e.g., *Oswaldofilaria*, *Hastospiculum*) in *Varanus* spp. (Bolette, 1998; Rataj et al., 2011). For example, in 2017 several large subcutaneous nematodes were collected from *V. niloticus* in Chad, Africa and were identified as filarial worms

(Onchocercidae) based on molecular analysis of the cytochrome *c* oxidase I (COI) (Cleveland and Yabsley, unpublished data).

2.1.4. *Dracunculus* spp. from Oceania

The only species from Oceania is *D. mulbus*, which was originally described from nematodes collected from the body cavity of the water python (*Liasis fuscus*) in northern Australia (Table 1) (Jones and Mulder, 2007). This parasite was found in 22% of pythons examined and a maximum of 14 individuals were detected; males were collected from around the heart, lungs, and liver and females from the mesenteries. This parasite has also been reported from the Papuan olive python (*Apodora papuana*) from Papua New Guinea (Moravec and Gibson, 2007).

2.1.5. *Dracunculus* spp. from Africa

There are two described *Dracunclus* species from Africa (Table 1). *Dracunculus doi* (Chabaud, 1960) was originally described using male specimen from the Madagascar ground boa *(Acrantophis madagascariensis*) (Chabaud, 1960). Females of this parasite were subsequently detected in a captive Madagascar tree boa (*Sanzinia madagascariensis*) in a Paris Zoo (Vaucher and Bain, 1973). Larvae from the tree boa were infective to *Cyclops strenuus* and developed to L3 in 13 days at 22-25 ^{o}C. These infective larvae were given to a ball python (*Python regius*), which developed an infection with two male nematodes (Vaucher and Bain, 1973; Moravec, 2006).

Dracunculus dahomensis (Neumann, 1895) Moorthy, 1937 was originally described from the lymphoid tissues of a captive African rock python (*Python sebae*) from Benin (then Dahomey) (Neumann, 1895). Males of this species were redescribed by Moorthy (1937). No other life history traits are known for this parasite (Moravec, 2006).

2.2 Intermediate and paratenic hosts of squamate *Dracunculus* spp.

As in experimental infection trials with other *Dracunculus* species, copepods have been used as experimental intermediate hosts (Brackett, 1938; Muller, 1971) (Table 1). Emergence of adult female nematodes from snake hosts has rarely been reported; however, adult females are present in subcutaneous masses and Brackett reported that gravid *D. ophidensis* responded to water in the same manner as *D. medinensis*. Therefore, it is likely that transmission to copepods is similar to the mammalian *Dracunculus* spp. (Brackett, 1938). Experimentally, an undescribed *Dracunculus* sp. detected in boas from Trinidad developed in *C. vernalis* to the infective third stage larvae in 12-14 days at 25 ^{o}C and *D. doi* developed to L3 in *Cyclops strenuus* in 13 days at 22-25 ^{o}C, thus development appears to be similar to that of mammalian *Dracunculus* spp. (Muller, 1971; Vaucher and Bain, 1973).

Experimental studies show that paratenic amphibian hosts may be involved in the life cycle of some squamate *Dracunculus* spp. Copepods infected with *D. ophidensis* were readily ingested by tadpoles of an unreported frog species and larvae were noted in the tadpole body cavity (Brackett, 1938). Feeding of these tadpoles to two garter snakes and a water snake (*N. sipidon*) resulted in infection of one snake of each species (Brackett, 1938). However, there have been no reports of natural infections in aquatic paratenic hosts.

3. *Dracunculus* species in chelonians

To date, *D. globocephalus* is the only species reported from chelonians. The only two known hosts are the common snapping turtle (*Chelydra serpentina*) in the United States and the South American snapping turtle (*Chelydra acutirostris*) in Costa Rica.

However, the report from Costa Rica was based on morphology of a single worm of unknown sex (Bursey and Brooks, 2011).

Dracunculus globocephalus was first described by Macklin (1927) from the mesenteries and body cavity of common snapping turtles from Oklahoma and Illinois, USA. Since its first description, *D. globocephalus* has been re-described by two groups and detected in numerous states (Table 2.1) (Williams, 1953; Gatschet and Schmidt, 1974; Moravec and Little, 2004). Morphologic features that can be used to distinguish *D. globocephalus* from other *Dracunculus* spp. are the presence of markedly uneven spicules (0.8mm and 0.2mm in length), the absence of a gubernaculum, and the placement of male caudal papillae (Moravec and Little, 2004).

4. *Dracunculus* species in mammals

4.1.1 *Dracunculus insignis*

Dracunculus insignis was initially described as *Filaria insignis* by Leidy in 1858 from a female nematode in the foot of a raccoon (*Procyon lotor*) from Pennsylvania, USA (Chandler, 1942a). Morphologically similar parasites detected in raccoons from Texas were noted to be similar to *Dracunculus fuelleborni* from a big-eared opossum (*Didelphis aurita*) in South America, based on the size and cephalic structures of the female nematodes. At that time, the morphologic characteristics of males, which are needed for definitive identification, were not available (Travassos, 1934; Chandler, 1942b). A male specimen of *D. insignis* was first detected in a raccoon in Dorchester County, Maryland (Chitwood, 1950) and was noted to be morphologically like *D. medinensis* but was differentiated based on the number and arrangement of genital papillae and length of gubernaculum (Chandler, 1942a; Chitwood, 1950). Although this

review focuses on wildlife hosts, *D. insignis* also infects domestic dogs and domestic cats in the United States and Canada. A recent review was published (Williams et al., 2018).

4.1.1 Intermediate hosts for *Dracunculus insignis*

To date, there have been no reports of wild *D. insignis*-infected copepods; however, the intermediate host range appears to be large as both *Acanthocyclops vernalis* and *Cyclops bicuspidatus* from North America as well as *Cryptocyclops linjanticus* and *Mesocyclops aequatorialis similis* from Cameroon, Africa were experimentally susceptible (Sullivan et al., 1991). Susceptibility can vary as *Mesocyclops leuckarti leuckarti* from Pakistan and *Thermocyclops emini* from Cameroon were partially refractory to infection (Sullivan et al., 1991). *Dracunculus insignis* larvae develop to L3 in 21–25 days at 24 °C (Crichton and Beverley-Burton, 1975). As noted earlier, development is regulated by water temperature and infected copepods kept at 8 °C and 15 °C showed no development 60 days post infection (DPI) (Crichton and Beverley-Burton, 1975).

4.1.2 Natural infections of *D. insignis* in wildlife

4.1.2.1 Infections in wild raccoons

Raccoons are the most common host for *D. insignis* (Cheatum and Cook, 1948; Long, 2003). Although the classic transmission route for *D. medinensis* is the ingestion of copepods infected with L3 in drinking water, it has been suggested that this is unlikely to be the primary route for *D. insignis* among raccoons (Muller, 1971; Crichton and Beverley-Burton, 1977). Instead, transmission via consumption of an amphibian paratenic host or fish transport host containing *D. insignis* L3 seems more

likely, although this has not been evaluated beyond showing the capability of transmission (Fig. 2.1) (Crichton and Beverley-Burton, 1977; Anderson, 2000). Most reports of *D. insignis* in raccoons have been based on detection of adult females which cannot be identified to species (Fig. 2.2); thus, these infections cannot be definitively said to have been with *D. insignis*. To date, however, no other *Dracunculus* sp. has been detected in raccoons (either by identification of males or genetic characterization) (Chitwood, 1950; Crichton and Beverley-Burton, 1975; Elsasser et al., 2009). Infections have been noted in raccoons in numerous states in the United States (primarily East of Texas/South Dakota line) and in Ontario province, Canada (Table 2.2). The prevalence of infections in raccoons from Ontario, Canada, where a good proportion of surveillance work has been conducted, was 69% (154/223) (Crichton and Beverly-Burton, 1974). In the southeastern United States at a site with endemic *D. insignis* transmission in raccoons, a prevalence of 36% (35/98) has been detected (Cleveland and Yabsley, unpublished data).

Several studies have noted a marked seasonality in the prevalence of infection and stage of development of *D. insignis*. The highest prevalence has been reported in the spring (April–June) at multiple locations throughout the United States (Texas, New Hampshire, Tennessee, and Kentucky) and Canada (Ontario). However, the lower prevalence noted in other seasons may be related to the lack of detection of males and immature females, as female worms in the fall are mostly in the subcutaneous tissues of the abdomen or thorax and are small and immature (Chandler, 1942a; Siegler, 1946; Crichton and Beverly-Burton, 1974; Crichton and Beverley-Burton, 1977; Diters and Ryan, 1980; Smith et al., 1985). A study of *D. insignis* in raccoons at a site in the

southeastern United States with endemic transmission found that naturally-infected individuals sampled in February–March had gravid females subcutaneously or had post emergence scarification, indicating a late winter to early spring emergence (Cleveland and Yabsley, unpublished data).

4.1.2.2. Infections in other wild species

In addition to raccoons, infections with parasites presumed to be *D. insignis* have been reported in multiple wild carnivore species (e.g., skunks (*Mephitis* spp.), coyotes (*Canis latrans*), foxes (*Vulpes* spp.)), Virginia opossums (*Didelphis virginiana*), and rarely rodents (i.e., muskrat (*Ondatra zibethicus*) and North American beaver (*Castor canadensis*)) (Table 2.2). Except for a study in Ontario Canada, none of the worms detected in these other wild species have been confirmed to species by examination of males or through molecular confirmation. In Ontario, fisher (*Martes pennanti*), mink (*Neovison vison*), and North American river otter (*Lontra canadensis*) have been confirmed as hosts for *D. insignis* through molecular characterization (Elsasser et al., 2009). This lack of species confirmation may be important for species that are known to harbor more than one *Dracunculus* sp. (e.g., river otters), are hosts for other subcutaneous filarid worms (e.g., *Filaria taxidae* in mustelids and raccoons), or unusual hosts (e.g., rodents, none of which were infected with mature larvigerous females so may be dead-end hosts) (Gibson and McKiel, 1972; McKown et al., 1995).

4.2 Experimental infections of hosts with *D. insignis*

Several species, including raccoons, rhesus macaques (*Macaca mulatta*), mink, and domestic ferrets (*Mustela putorius furo*), have been experimentally-infected with *D. insignis* (Beverly-Burton and Crichton, 1976). Regardless of the inoculation route or

dose, not all individuals of host species developed patent infections in these studies. Furthermore, a relatively low percentage of L3 used to expose definitive hosts develop into adult worms. Exposure of two domestic dogs (*Canis lupus familiaris*) and a single marten (*Martes americana*) to *D. insignis*-infected *Cyclops vernalis* (n = 250, 210, and 220 respectively) did not result in infection; however, natural infections have been reported in both hosts (Beverly-Burton and Crichton, 1976).

4.2.1 Raccoons

Because raccoons are considered to be the primary wildlife host for *D. insignis*, experimental trials have been conducted to document the transmission, pathologic lesions associated with infection, and possible changes in behavior of infected raccoons (Miller et al., 1946; Cheatum and Cook, 1948; Wilson, 1958; Crichton and Beverley-Burton, 1977). In one study, captive bred raccoons were inoculated with L3 recovered from digested copepods (Crichton and Beverley-Burton, 1975). Raccoons were euthanized at various time points to examine parasite migration. Overall, 30 of 33 (87%) raccoons became infected and only 4.3% of 9,320 larvae administered to the raccoons were recovered with the average number recovered being 12.3 worms/raccoon. At 7 h post inoculation, 2.3% of inoculated larvae were detected within the duodenum and stomach (a similar percent recovery was noted at 19 h) but of the 16 larvae recovered, 1 was from the duodenum whereas the other 5 worms were located in the abdominal cavity. At 4 days post inoculation, all worms were recovered from the abdominal cavity while some worms were detected in diaphragm and intercostal muscles on day 5. By days 6 and 7, worms were detected in the subcutaneous tissues of the thorax and abdomen. At 19 days the single larvae detected was a L4 and was 1,220 μm long with a blunt tail. By 34 DPI,

females were 4.5–10.3 mm long and males were 7.6–8.4 mm long and had developed spicules and a gubernaculum.

By 60 days, the fourth stage cuticle was nearly shed, tails had 10 small conical projections, and the intestine had become dark brown. Males had already shed their L4 cuticle and the seminal vesicle contained sperm. By 77 days, females were 87–125 mm long and mated (vaginal plug present). The intestine in males were not well developed by day 120. By 90, days females contained ova which had developed to L1 by 120 days. By 270 days, females were 200–310 mm in length. From 300 to 365 days females began to create lesions into which the uterus would sometimes rupture releasing larvae into the lesion (Fig. 2.2). Females died after larvae were released and some were resorbed by the host, while others became calcified. Males were also found, indicating that males could likely persist between transmission seasons. At 480 days, a small (95 mm) immature female was found in the subcutaneous tissue of the trunk providing evidence that not all female worms mature and become gravid and these unfertilized females likely do not migrate to the extremities.

Experimental studies have also been conducted to examine the ability of frogs to transmit infection to raccoons. Larvae of *D. insignis* recovered from experimentally infected paratenic hosts (*Lithobates pipiens* and *L. clamitans*) were fed to a captive-born raccoon. Upon necropsy, the infected raccoon had larvigerous female *D. insignis* present in all legs and lesions from female worms preparing for larval release (Crichton and Beverley-Burton, 1977). Localized edema, inflammation, and thickened cells typically surrounded ulcer formation in the host. Upon death of worms subcutaneously, the affected extremities bore small superficial scars (Crichton and Beverley-Burton, 1977).

Bouts of inactivity (30–60 min) and distress, difficulty moving, and favoring a leg were all observed in the infected individual.

4.2.2 Mink

In the same study in which raccoons were inoculated with *D. insignis*, Crichton and Beverley-Burton (1975) similarly exposed captive-bred mink. Compared with the raccoons, a lower percentage of exposed mink became infected (18/31, 58%), the number of larvae recovered was lower (53/4895 larvae, 1.1%) and the worm burdens were lower (2.9 worms/infected mink). In addition, development of most worms was slower and fewer worms matured and became larvigerous. A male was detected in a mink euthanized at day 365. Collectively, these data show that mink can be definitive hosts for *D. insignis* but fewer ingested larvae develop to larvigerous females.

4.2.3 Rhesus macaques

A Rhesus macaque was experimentally exposed to 400 L3 of *D. insignis* via a stomach tube (Beverly-Burton and Crichton, 1976). The primate was necropsied at 180 DPI and nine gravid female nematodes from the subcutaneous tissues and a single male nematode from the connective tissue were recovered (Beverly-Burton and Crichton, 1976). This finding in a non-human primate raises the possibility that *D. insignis* has the potential to be zoonotic; however, no infections in humans have ever been reported.

4.2.4 Domestic ferret

Because raccoons, mink, and rhesus macaques are not amenable to laboratory studies on *Dracunculus* due to husbandry costs and handling difficulty, domestic ferrets were evaluated as a suitable animal model (Eberhard et al., 1988; Brandt and Eberhard,

1990, 1991; Broderson et al., 1991). Collectively, these studies show that domestic ferrets are appropriate definitive hosts for both *D. insignis* and *D. medinensis* (Eberhard et al., 1988, 2016; Brandt and Eberhard, 1990).

Initially, ferrets were exposed to *D. insignis*-infected copepods to evaluate susceptibility (Eberhard et al., 1988). Ten of the 18 (56%) ferrets developed infections with 44 worms (Eberhard et al., 1988). Gravid females were recovered as early as 128 DPI and by 190 DPI, 93% (14/15) of female worms contained larvae. Most (87%, 13/15) worms were recovered from the legs (Eberhard et al., 1988). Examination of various inoculation routes showed that intraperitoneal (IP) inoculation resulted in the highest recovery rate of adult nematodes compared to gavage and subcutaneous inoculation routes (Brandt and Eberhard, 1990). Therefore, another study was conducted to examine the IP route of transmission using a low number (n = 10) of *D. insignis* L3 (Brandt and Eberhard, 1991). Of 10 ferrets exposed, one died of unrelated causes early in the study, and 67% (6/9) became infected. Recovery rate increased from 6.5% to 21% compared to the Eberhard et al. (1988) study. Although the IP route of exposure does not mimic natural infections, this work further establishes that a small number of *D. insignis* larvae are sufficient to establish infection in definitive hosts.

Using the ferret model system, Eberhard et al. (1990) investigated possible chemoprophylactic treatment on *D. insignis* infections with the goal of informing the eradication efforts of *D. medinensis* in Africa. Five compounds (diethylcarbamazine (DEC), albendazole (ALBZ), ivermectin (IVER), metrifonate (INN), and amocarzine (CGP 6140)) were evaluated on ferrets exposed to 100 infected copepods. Ferrets were treated at 60 and 90 DPI. Necropsies were performed between 7 and 11 months post-

infection and no significant difference of worm recovery rates was found between treatment and control groups. To date, there has been no effective anthelminthic preventative or treatment identified for use on *Dracunculus* species.

4.3 Paratenic hosts of *D. insignis*

Until recently, no natural infections of a paratenic host for *D. insignis* had been identified despite data from several experimental studies suggesting that amphibians, and possibly fish, may serve as paratenic hosts (Fig. 2.1) (Eberhard et al., 2016a, Eberhard et al., 2016b; Cleveland et al., 2017). Interestingly, the first evidence suggesting that amphibians can serve as paratenic hosts came from an experimental study with *D. ophidensis*—one of the more poorly studied *Dracunculus* species (Brackett, 1938). Several potential paratenic hosts have been experimentally evaluated for *D. insignis* including several species of crayfish, fish, and amphibians (Crichton and Beverley-Burton, 1977; Eberhard and Brandt, 1995; Eberhard et al., 2016a, Eberhard et al., 2016b; Cleveland et al., 2017).

4.3.1 Fish

Exposure trials with various species of fish suggest they are generally refractory to infection, but some species may act as paratenic or transport hosts (Cleveland et al., 2017). In one study, of the seven species of fish (white suckers (*Catostomus commersonii*) rainbow trout (*Oncorhynchus mykiss*) common shiner (*Luxilus cornutus*) brown bullhead (*Ameiurus nebulosus*) bluntnose minnow (*Pimephales notatus*), stonecat catfish (*Noturus flavus*) and brindled madtom catfish (*N. miurus*)) exposed to 10–200 *D. insignis* L3s, 40% (2/5) white suckers and 33% (1/3) rainbow trout had 1-2 larvae

recovered 6–11 DPI; however, sample sizes were generally low (Crichton and Beverley-Burton, 1977). Similarly, the single study that exposed eight Northern Clearwater crayfish (*Orconectes propinquus*) with 10–20 *D. insignis* L3s failed to establish any infection (Crichton and Beverley-Burton, 1977). These data contrast sharply with adult amphibians (*Lithobates* spp. and African clawed frogs (*Xenopus* spp.)) which generally have high infection rates and 45–90% recovery rate of *D. insignis* larvae. Regardless, these data suggest a need for further investigation into the role of certain fish species to serve as paratenic hosts.

Recently, work has been conducted to investigate the possibility that fish may serve as a short-term transport host in the transmission of *Dracunculus* spp. (Cleveland et al., 2017). Three species of fish were allowed to feed on *D. insignis*- and *D. medinensis*-infected copepods; Mosquitofish (*Gambusia affinis*) Fathead minnows (*Pimephales promelas*) and Nile tilapia (*Tilapia niloticus*)). The fish were euthanized and fed to domestic ferrets within 3 hours of ingesting copepods. Three ferrets were given *D. insignis*-fed fish and one ferret was given *D. medinensis*-fed fish. Two of the *D. insignis* ferrets became infected, as did the single *D. medinensis*-exposed ferret. The results of this study were important to the three remaining endemic countries where *D. medinensis* is still transmitted as The Carter Center Guinea Worm Eradication Program has suggested fish entrails be buried or burned to prevent their ingestion by dogs (Eberhard et al., 2014). These results indicate yet another potential route of transmission for *Dracunculus* species.

4.3.2 Amphibians

Several studies have shown that tadpoles and adult frogs of numerous species are experimentally susceptible to *D. insignis*, including initial work that was conducted via oral inoculation on Northern leopard frog (*Lithobates pipiens*) and Green frog (*Lithobates clamitans*) tadpoles and adults (Crichton and Beverley-Burton, 1977). The low recovery rate of *D. insignis* larvae from tadpoles (0–7%) was attributed to difficulty in orally inoculating small tadpoles. A subsequent study which allowed African clawed frogs (*X. laevis*) and American bullfrog (*L. catesbeianus*) tadpoles to ingest *D. insignis*-infected copepods from small dishes of water, a more natural route of infection, obtained similar results (Eberhard and Brandt, 1995).

To investigate the ability of larvae to survive and persist through metamorphosis, *Xenopus* spp. tadpoles were infected with *D. insignis* and then held for 2–4 weeks at which time larvae were recovered from tissues of adults (Eberhard and Brandt, 1995). Similarly, examination of experimentally-infected adult *X. laevis* at regular time periods indicated live *D. insignis* could be recovered up to eight months post infection (Cleveland and Yabsley, unpublished). In contrast to tadpoles, oral inoculation of adult frogs resulted in a larval recovery rate of 45–90%, further supporting the role of amphibians as potential hosts of *D. insignis* (Eberhard and Brandt, 1995). One study also noted that L3s recovered from *L. pipiens* were longer (570–698 μm, mean 629 μm) than L3s recovered from copepods (434–605 μm, mean 554 μm); however, this finding has not been further investigated (Crichton and Beverley-Burton, 1977).

Larvae recovered from tadpoles were capable of developing in two species of vertebrate definitive hosts; raccoons and ferrets. Larvae (n = 250 L3s) from orally-

inoculated *Lithobates* spp. were given to a captive-reared raccoon, which harbored infection with 13 male and 27 female mature *D. insignis* 167 DPI (Crichton and Beverley-Burton, 1977). Similarly, one of two ferrets fed tadpoles exposed to *D. insignis*-infected copepods became infected with one male worm after seven months (Eberhard and Brandt, 1995).

Collectively, these data indicate amphibians can act as experimental hosts for *D. insignis* and that infection can be maintained through metamorphosis and for at least eight months post infection. However, critical data on the potential role of amphibians in the natural cycle of *Dracunculus* are generally lacking. Despite the experimental findings to support their role, the occurrence of natural infections is likely rare and thus difficult to detect. However, surveillance has been limited and further study is needed to identify sylvatic transmission of *Dracunculus*. In one study in Ontario, Canada, 45 wild-caught *L. pipiens* and *L. clamitans* were negative (Crichton and Beverley-Burton, 1977). However, a natural infection was recently detected in one of eight southern leopard frogs (*L. sphenocephalus*) from central Georgia/upper coastal plains Georgia where the prevalence of *D. insignis* in raccoons 36% (Cleveland and Yabsley, unpublished data). Additionally, two amphibians of 240 surveyed from Chad, Africa were detected harboring *D. medinensis* L3s (Eberhard et al., 2016a, Eberhard et al., 2016b; Cleveland and Yabsley, unpublished data). These findings support the role of amphibians as paratenic hosts for *Dracunculus* and will hopefully stimulate further research.

4.4 Dracunculus lutrae

Dracunculus lutrae was first described from specimens collected from otters from Ontario, Canada (Table 2) (Crichton and Beverly-Burton, 1973). A molecular study

conducted on *Dracunculus* from various wildlife species in Ontario, Canada revealed that 18 otters were infected with *D. lutrae* but two other otters were infected with *D. insignis* (Elsasser et al., 2009). Recently, 184 river otters (*Lontra canadensis*) from Arkansas were examined for the presence of *Dracunculus* species (Tumlison and Surf, 2018). Twelve otters were found to have cysts on the distal extremities consistent with *Dracunculus* infections. An individual nematode from each otter was genetically characterized and all were *D. insignis*. These data indicate that the distribution of *D. lutrae* and *D. insignis* in otters may be constrained by a latitudinal gradient, with *D. lutrae* occurring above 45° N and *D. insignis* occurring below 45° N. Few studies have assessed the prevalence and distribution of *D. lutrae* in otters, but unidentified *Dracunculus* spp. or molecularly unconfirmed *D. lutrae* have been reported in several locations in North America including Kentucky, Alabama, New York, and Ontario, Canada (Table 2) (Cheatum and Cook, 1948; Crichton and Beverly-Burton, 1974; Fleming et al., 1977; Kimber and Kollias, 2000; Elsasser et al., 2009; Barding and Lacki, 2015). To date, no studies have been conducted on possible paratenic hosts, but based off research conducted on *D. insignis*, it is assumed that fish and/or frogs could be involved in transmission.

4.5 *Dracunculus fuelleborni*

Dracunculus fuelleborni was first reported from the subcutaneous connective tissue of a big-eared opossum from Rio, Brazil (Travassos, 1934). Both male and female nematodes were obtained and, morphologically, the female resembled *D. insignis*, yet was nearly twice the length of recorded *D. insignis* specimens (Table 1). Morphometrics of spicules and gubernaculum of males confirmed *D. fuelleborni* was a distinct species.

However, this parasite has not been genetically characterized so the relationship between *D. fuelleborni* and other *Dracunculus* spp. has yet to be documented. Despite erroneous older reports that assumed worms detected in opossums were *D. fuelleborni*, it is not known to occur in North America. Our recent genetic data supports this assertion as opossums from Georgia (USA) were infected with *D. insignis* (Alexander et al., 1972; Cleveland unpublished data). Thus, to date, there have been no confirmed reports of *D. fuelleborni* since its initial description.

5. Molecular epidemiology of *Dracunculus* spp.

Molecular characterization has allowed for the investigation of the phylogenetic relationships of many parasites, including nematodes. However, among the parasitic representatives there are some groups, including the Dracunculoidea, that are underrepresented (Gardner, 2001; Bimi et al., 2005; Wijová et al., 2005; Elsasser et al., 2009). The superfamily Dracunculoidea has at least 166 recognized species, with most species having only morphologic descriptions (i.e., no gene sequence data) and many only having been reported once (see section 3) (Moravec, 2006; Wijová et al., 2005). This is problematic for the Dracunculoidea because of the limited distinguishing morphologic characteristics on female worms and the rare occurrence of male specimens being extracted from definitive hosts.

The first study to investigate the phylogenetic relationships of *Dracunculus* spp. was by Wijová et al. (2005) who included the 18S rRNA sequences from *D. medinensis*, *D. oesophageus* (from *Natrix natrix*) and *Philometra obturans*, a related dracunculid parasite from fish. These three parasites formed a clade within the Spirurida. A subsequent study, also using 18S rRNA gene sequences, included *D. insignis* as well as

many other members of the Dracunculoidea including *Philometra obturans*, *Philometra ovata*, *Philonema oncorhynchi*, *Skrjabillanus scardinii*, and *Molnaria intestinalis* from fish, and *Micropleura australiensis* from the Australian freshwater crocodile (*Crocodylus johnsoni*) (Wijová et al., 2006). The three *Dracunculus* spp. formed a clade with *Philometra* spp. as a sister clade. The 18S rRNA gene sequence has also been obtained for a specimen of *D. lutrae* from an otter from Ontario, Canada and it was identical to *D. insignis*. Additional 18S rRNA sequences of *D. lutrae* from the southeastern US are also identical to *D. insignis* sequences even though these two species are morphologically distinct and were confirmed to be different species based on analysis of the cytochrome *c* oxidase I (COI) gene (Elsasser et al., 2009; Laetsch et al., 2012; Cleveland and Yabsley, unpublished).

These initial studies focused on the 18S rRNA gene which, although useful in examining relationships among large groups of nematodes, could not distinguish some species of *Dracunculus*. Thus, this gene target is not useful for investigating intraspecific variation. An increasingly common target for 'barcoding' is the COI gene, and Elsasser et al. (2009) used this target to study *D. insignis* and *D. lutrae* samples from several hosts (fishers, mink, raccoon and otter) from Ontario, Canada. Sequences were obtained for 82 of the 92 worms investigated. The two species were easily distinguished and it was shown that *D. insignis* was a host-generalist and was detected in all samples species, including otters while *D. lutrae* was specific to otters (Elsasser et al., 2009). In addition, *D. insignis* had minimal intraspecific variability (0.02% (n = 59 worms), especially compared to *D. lutrae* which had 0.33% (n = 23 worms).

6. Conclusions

The human Guinea worm, *D. medinensis*, has afflicted people throughout Africa and parts of Asia for centuries, causing significant morbidity among those infected. Infection results in significant educational and personal losses and has recently been the subject of a highly successful eradication campaign that has reduced the number of endemic countries and the number of annual cases (Molyneux and Sankara, 2017). Yet, despite vast amounts of history and knowledge, this parasite has now been described as having a 'peculiar epidemiology' with infections in domestic dogs becoming more prevalent than at any time in the past. This suggests that there has been a change in transmission mode (Eberhard et al., 2014) and has led to several field and laboratory-based studies that have confirmed the ability of this parasite to utilize amphibian paratenic hosts and the recognition that fish may serve as either paratenic or transport hosts (Eberhard et al., 2016a; b; Cleveland et al., 2017). As noted in this review, several members of the genus were known to infect amphibians, so this new finding for *D. medinensis* is not surprising and many questions remain regarding the importance of amphibians in natural transmission or maintenance of the parasite in the environment (Fig. 1). Although parasites should be studied for their intrinsic value, the emerging concern surrounding *D. medinensis* combined with the discovery of a possible 'novel' transmission pathway (that was highly suggested from past studies with *D. insignis* and other species) should highlight how research on parasites of non-medical importance can be informative to related parasites and should be encouraged.

Because this group of parasites is often overlooked due to the limited pathological importance to their definitive hosts, there are many basic evolutionary and ecological

questions outstanding. This lack of knowledge is especially highlighted by the fact that the historical records of many *Dracunculus* species are still based on morphological identification, typically of females, which is not accurate to distinguish most species. Molecular phylogenetics may hold promise in addressing questions on species diversity and host specificity but is limited because of lack of available samples preserved appropriately for molecular work and the need for extensive field studies to collect new parasite specimens. The application of molecular analyses is especially important among the reptilian species of *Dracunculus* which, given their diversity compared to mammalian-infecting *Dracunculus* species suggests that they may represent the ancestral host of the genus. The breadth of wildlife definitive hosts parasitized by the numerous dracunculids discussed herein, along with the potential role of paratenic hosts in transmission of these nematodes, exemplify unique complex life-cycles highlighting both host-specialist and host generalist nematodes. Considering these intriguing dynamics of transmission and host-parasite relationships, large gaps in basic parasitological knowledge still exist, and further research is needed.

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Table 2.1 Overview of valid *Dracunculus* species from wild animals, including *D. medinensis* for comparison.[a]

Host group	Parasite	Geographic region	Known DH	Known IH	Known PH	Adult length (♂;♀)	Left and right spicule length	Gubernaculum length	Caudal alae	L1 length
Mammals	***D. medinensis***	Historically Africa and Asia	Humans, domestic dogs, domestic ferret (E)[b]	Numerous copepod species	Various amphibians (N,E)		0.42 (0.4-0.52), 0.44 (0.41-0.52)	0.12	Absent	0.581-0.643
	D. insignis	United States, Canada	Raccoons (*Procyon lotor*), mink (*Mustela vison*), fisher, (*Martes pennanti*), Virginia opossum (*Didelphis virginiana*), North American river otter (*Lutra canadensis*), domestic dog, domestic cat, domestic ferret (E)	*Acanthocyclops vernali* (E) and *C. bicuspidatus* (E)	Various amphibians (N,E)	17-22; 200-280 () and 14.2-30.1; 192-275 (Crichton and Beverley-Burton, 1973)	0.46-0.495 each	0.114-0.13	NK[c]	0.664 (0.596-0.857)
	D. lutrae	United States, Canada	North American river otter (*Lutra canadensis*)	NK	NK	36 (32.2-40); 247 (200-290)	0.61 (0.51-0.68), 0.64 (0.59-0.72)	0.17 (0.16-0.18)		0.665 (0.608-0.722)
	D. fuelleborni	Brazil	Big-eared opossum (*Didelphis aurita*)	NK	NK	27-29; 465-490	0.38-0.42 each	0.088-0.1	NK	0.3-0.429
Reptiles	*D. ophidensis*	United States	Common garter snake (*Thamnophis sirtalis*)	*C. viridis* (E)	Tadpoles (species not given)	Up to 16; up to 250	0.523; 0.554	0.09	NK	0.43-0.45
	D. brasiliensis	Brazil	Green Anaconda (*Eunectes murinus*), Brown banded water snake (*Helicops angulatus*)	NK	NK	NG; 130-220mm	NK	NK	NK	0.396-0.429
	D. coluberensis[d,e]	India	Trinket snake (*Coeloghathus* (=*Coluber*) *helena*)	NK	NK	197.5; NG	0.08; 0.07	"small" one present	Absent	NK
	D. alii[e]	India	Checkered keelback snakes (*Xenochrophis* (=*Natrix*) *piscator*)	NK	NK	13.09-24.4; NK	0.22-0.29; 0.23-0.3	0.05-0.07	Absent	NK
	D. houdemeri	Vietnam	Checkered keelback snakes	NK	NK	213	NK	NK	NK	0.33-0.363
	D. doi	Madagascar	Madagascar boa *(Acrantophis madagascariensis*)			28mm; NK	Subequal, ~0.46	0.130	Present	0.37
	D. dahomensis[d,e]	Benin	African rock python (*Python sebae*)	NK	NK	48; NK	0.425; 0.4	NK	Absent	0.40–0.42
	D. oesophageus (=*D. riccii*)	Italy	colubrid snakes (*Natrix viperina, Natrix natrix persa*)	*C. fuscus* (E)	NK	17 (11.7-20); 360	0.297; 0.282	0.065	Absent	0.475-0.570
	D. mulbus	Australia and Papua New Guinea	Water python (*Liasis fuscus*), Papuan olive python (*Apodora papuana*)	NK	NK	23 (17–33); 240 (180-360)	0.4-0.48, 0.4-0.48	0.08–0.11	Present	0.34–040
Turtles	*D. globocephalus*	United States, possibly Costa Rica	snapping turtle (*Chelydra serpentina*)	*C. bicuspidatus* (E)		16-21.7; 90–136	0.963–1.062; 0.186–0.213	Absent	NK	0.666–0.721

[a] Measurements in mm unless otherwise specified.
[b] N=naturally infected, E=experimentally infected
[c] NK = not known
[d] Measurement represents examination of single specimen.
[e] only based on morphology of males.

Table 2.2. The geographic, anatomic location and prevalence of confirmed species *Dracunculus insignis, D. lutrae,* and non-speciated *Dracunculus* in North America.

Species	Host	Geographic location	Anatomic location	Prevalence (%)	Species confirmation method	Reference
D. insignis	Otter (*Lontra canadensis*)	Arkansas, USA	Subcutaneous and intermuscular fascia of carpal and tarsal areas	12/184 (6.5)	molecular	Tumlison and Surf, 2018
	Raccoon (*Procyon lotor*)	Ontario, Canada	NA	NA	molecular	Elsasser, 2009
	Raccoon	Ontario, Canada	Subcutaneous tissues in the inguinal area, thorax, abdomen and fascial layers of the lower legs	253/553 (45.75)	morphology via male specimens	Crichton and Beverly-Burton, 1974
	Raccoon	Ontario, Canada	Subcutaneous tissues in the inguinal area, left and right axillary areas	1/1 (100)	morphology via male specimens	Gibson and McKiel, 1972
	Raccoon	Maryland, USA	Subcutaneaous fascia of legs	1/1 (100)	morphology via male specimens	Chitwood, 1950
D. lutrae	(Otter) (*Lontra canadensis*)	Ontario, Canada	NA	NA	molecular	Elsasser, 2009
	Otter	Ontario, Canada	Subcutaneous tissues of thoracic, abdominal, inguinal areas and intermuscular fascia of legs	178/203 (87.7)	morphology via male specimens	Crichton and Beverly-Burton, 1974
	Otter	Ontario, Canada	Connective tissue beneath latissimus dorsi, subcutaneous tissues of thoracic, abdominal, inguinal areas and intermuscular fascia of legs	NA	morphology via male specimens	Crichton and Beverly-Burton, 1973
Dracunculus sp.	Badger (*Taxidea taxus*)	Iowa, USA	Subcutaneous and intermuscular fascia of carpal and tarsal areas	2/24 (8.3)	NA	Wittrock and Ulmer, 1974
	Beaver (*Castor canadensis*)	Kansas, USA	Connective tissue beneath latissimus dorsi	2/63 (3)	NA	McKown et al., 1995

Fischer (*Martes pennanti*)	New Hampshire, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas	37/748 (4.9)	NA	Carlson and Vito, 1984
Marten (*Martes americana*)	Ontario, Canada	NA	1/405 (0.2)	NA	Seville and Addison, 1995
Mink (*Neovison vison*)	Ontario, Canada	Connective tissue beneath latissimus dorsi, subcutaneous tissues of thoracic, abdominal, inguinal areas and intermuscular fascia of legs	14/42 (33)	NA	Schulte-Hostedde and Elsasser, 2011
Mink	Arkansas, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas	35/507 (6.9)	NA	Tumlison et al., 1984
Mink	Ohio, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas, tail musculature	3/3 (100)	NA	Crites, 1963
Mink	Minnesota, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas	3/3 (100)	NA	Huggins, 1958
Mink	New York, USA	Intermuscular fascial layers of lower leg and ankles; subcutaneous position over pectoralis major	NA	NA	Cheatum and Cook, 1948
Mink	Minnesota, USA	hind leg	2/72 (2.7)	NA	Erickson, 1946
Mink	Iowa, USA	NA	NA	NA	Benbrook, 1940
Mink	Wisconsin, USA	NA	NA	NA	Chaddock, 1940
Mink	Nebraska, USA	NA	NA	NA	Chitwood, 1933
Muskrat (*Ondatra zibethicus*)	Ontario, Canada	Right inguinal region, left and right axillary regions	1/1 (100)	NA	Gibson and McKiel, 1972
Muskrat	Ontario, Canada	NA	NA	NA	Fyvie, 1969

Muskrat	Minnesota, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas	1/1 (100)	NA	Huggins, 1958
Muskrat	Maryland, North Dakota, USA	NA	NA	NA	Dikmans, 1948
Opossum (*Didelphis virginiana*)	Maryland, USA	Subcutaneous fascia	3/64 (4.7)	NA	Alexander et al., 1972
Otter	New York, USA	Intermuscular fascial layers of lower leg and ankles; subcutaneous position over pectoralis major	NA	NA	Cheatum and Cook, 1948
Raccoon	Florida, USA	Subcutaneous and intermuscular fascia	9/54 (16.7)	NA	Keeling et al., 1993
Raccoon	Arkansas	fascia of carpal and metatarsal areas	4/30 (13)	NA	Richardson et al., 1992
Raccoon	Kentucky, USA	Intermuscular fascial layers of lower leg and ankles	10/70 (14.3)	NA	Cole and Shoop, 1987
Raccoon	Tennessee, Kentucky, USA	NA	20/145 (13.8)	NA	Smith et al., 1985
Raccoon	Illinois, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas	2/245 (0.8)	NA	Snyder and Fitzgerald, 1985
Raccoon	Arkansas	Subcutaneous and intermuscular fascia of carpal and metatarsal areas	35/507 (6.9)	NA	Tumlison et al., 1984
Raccoon	Georgia, USA	NA	1/148 (0.7)	NA	Price and Harman, 1983
Raccoon	Connecticut, USA	NA	NA	NA	Diters and Ryan, 1980
Raccoon	Nebraska, Kansas, Missouri, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas, facial areas	4/4 (100)	NA	Ewing and Hibbs, 1966

Raccoon	North Carolina, South Carolina, Florida, USA	Subcutaneous tissue and muscle fascia	14/209 (6.7), 8/34 (23.5), 6/19 (31.6)	NA	Harkema and Miller, 1964
Raccoon	Ohio, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas, tail musculature	3/3 (100)	NA	Crites, 1963
Raccoon	Florida, USA	fascia of carpal and metatarsal areas	2/2 (100)	NA	Layne et al., 1960
Raccoon	South Dakota, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas	1/1 (100)	NA	Huggins, 1958
Raccoon	Michigan, USA	Subcutaneous fascia of thigh	NA	NA	Wilson, 1958
Raccoon	New York, USA	Intermuscular fascial layers of lower leg and ankles; subcutaneous position over pectoralis major	NA	NA	Cheatum and Cook, 1948
Raccoon	New Hampshire, USA	Intermuscular fascial layers of lower leg and ankles	2/4 (50)	NA	Miller et al., 1946
Raccoon	Texas, USA	Subcutaneous and intermuscular fascia of carpal and metatarsal areas	6/15 (40)	NA	Chandler, 1942
Short-tailed Weasel (*Mustela erminea*)	Minnesota, USA	Subcutaneous and intermuscular fascia of tibia on hind legs	1/1 (100)	NA	Goble, 1942
Silver Fox (*Vulpes fulva*)	Iowa, USA	Subcutaneous and intermuscular fascia of carpal and tarsal areas	NA	NA	Benbrook, 1932
Eastern skunk (*Mephitis nigra*)	Ontario, Canada	Subcutaneous and intermuscular fascia of carpal and metatarsal areas	1/125 (0.8)	NA	Webster and Casey, 1970
Eastern skunk	Grand Island, Nebraska, USA	Rear leg	1/1 (100)	NA	Ewing and Hibbs, 1966

Eastern skunk	New York, USA	Intermuscular fascial layers of lower leg and ankles; subcutaneous position over pectoralis major	NA	NA	Cheatum and Cook, 1948
Striped skunk (*Mephitis mephitis*)	Minnesota, USA	skin	1/15 (6.7)	NA	Erickson, 1946

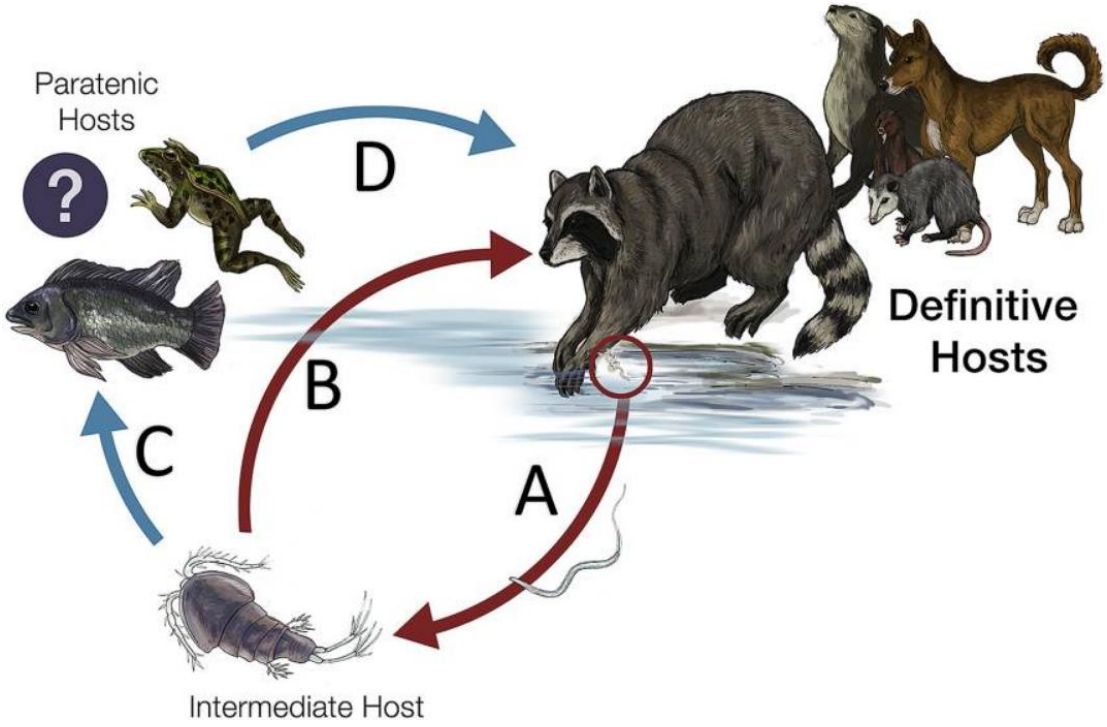

Figure 2.1. Life-cycle of *Dracunculus insignis* in wildlife and domestic dogs. Arrows in red represent transmission from (A) definitive hosts to intermediate hosts (Cyclopoid copepods) and (B) transmission to definitive hosts via consumption of intermediate hosts. Arrows in blue represent (C) transmission from intermediate hosts to paratenic and transport hosts (amphibians and fish) via consumption of infected copepods and (D) transmission to definitive hosts via consumption of paratenic/transport hosts.

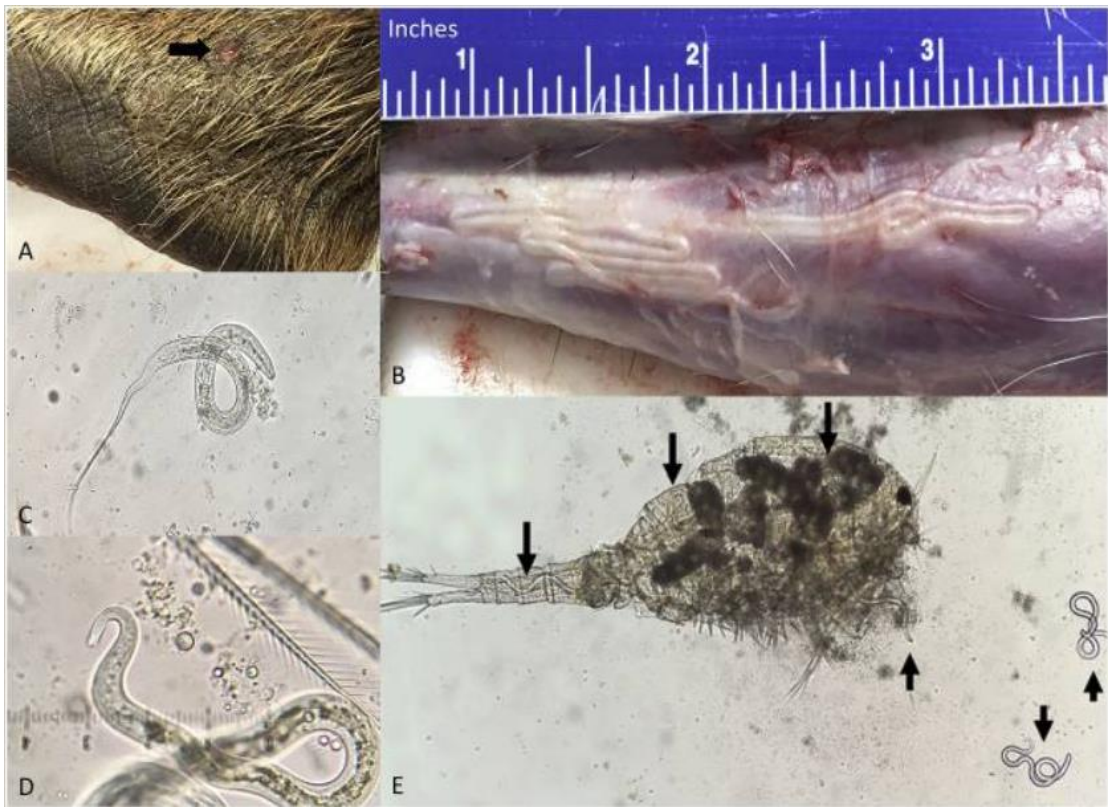

Figure 2.2. *Dracunculus insignis* infection in definitive host raccoon (*Procyon lotor*) and intermediate host (Cyclopoid copepods): (A) lesion from emergence of gravid female *D. insignis* (marked by arrow); (B) *in situ* photograph of patent *D. insignis* infection in forelimb of raccoon; (C) first-stage larvae (L1) of *D. insignis* (0.596-0.857 mm); (D) third-stage larvae (L3) of *D. insignis* (0.434-0.605 mm) with trilobed tail, a distinguishing morphologic characteristic; (E) a copepod infected with *D. insignis* (L3s marked with arrow).

CHAPTER 3

POSSIBLE ROLE OF FISH AS TRANSPORT HOSTS FOR *DRACUNCULUS* SPP. LARVAE.[1]

[1]**Cleveland, C.A.,** Eberhard, M.L., Thompson, A.T., Smith, S.J., Zirimwabagabo, H., Bringolf, R., and M.J. Yabsley. 2017. Open Access. *Emerging Infectious Diseases* 23 (9): 1590-1592. Reprinted here with permission of publisher.

ABSTRACT

To inform *Dracunculus medinensis* (Guinea worm) eradication efforts, we evaluated the role of fish as transport hosts for *Dracunculus* worms. Ferrets fed fish that had ingested infected copepods became infected, highlighting the importance of recommendations to cook fish, bury entrails, and prevent dogs from consuming raw fish and entrails.

INTRODUCTION

The campaign to eradicate *Dracunculus medinensis* infection (Guinea worm disease) has helped 17 of 21 countries interrupt transmission (*1*). Endemic transmission of Guinea worm disease typically occurred via contamination of drinking water sources, resulting in community disease outbreaks. The absence of outbreaks of Guinea worm disease in Chad, coupled with increasing infections among domestic dogs in the transmission cycle (*2*–*6*), led to the hypothesis that transmission was occurring by different means.

Previous work on *Dracunculus* and related spirurids indicates that paratenic hosts might be used to facilitate transmission (*7*,*8*). Recently, an experimental study showed *D. medinensis* worms could use tadpoles as paratenic hosts, and a naturally infected frog was detected in Chad (*9*,*10*). Few data exist on the potential role of fish as paratenic hosts; however, fish are suspected on the basis of epidemiologic data in Chad (*2*). Previous experimental attempts to infect Nile tilapia (*Oreochromis niloticus*) and fathead minnows (*Pimephales promelas*) with *D. medinensis* worms

were not successful (9). However, in trials with *D. insignis* worms, 2 of 7 fish species exposed to high numbers of larvae became infected with low numbers of larvae (*11*). Collectively, these data suggest that fish are generally resistant to infection, need to ingest very high numbers of larvae to establish infection, or have variable species susceptibility.

Alternatively, it is possible that fish might ingest infected copepods and, if consumed by a host within short enough intervals, act as transport hosts. Our objective was to evaluate this potential role by allowing fish to ingest *Dracunculus* worm–infected copepods and then feed them to domestic ferrets to evaluate whether transmission could occur.

THE STUDY

In April 2016, we used first-stage larvae (L1) from gravid *D. insignis* worms from raccoons (*Procyon lotor*) in Georgia (USA) to infect colony-reared cyclopoid copepods (*7*). At 21 days post infection, *D. insignis* larvae were examined to determine if they had developed to the infective third stage (i.e., trifid tail). If ≥25% of copepods were infected, they were used for transmission trials. Gravid *D. medinensis* worms were recovered from naturally infected dogs in Chad, and L1s were used to infect cyclopoid copepods collected from N'Djamena.

We then exposed groups of 5 Nile tilapia, fathead minnows, or mosquitofish (*Gambusia affinis*) to groups of 50 copepods (Table 3.1). We exposed fish to copepods for 3 hours in the first day of the *D. insignis* trial; on subsequent days, we exposed fish for 2 hours. All copepods provided to fish were consumed during

exposures. Individual fish were removed, euthanized by exposure to neutral buffered tricaine methane sulfonate (MS-222) followed by pithing, and dissected. We observed digested copepods and free larvae in the intestine.

We fed the euthanized fish to laboratory-raised ferrets. If fish were not immediately ingested, we mixed the fish carcasses with cat food. Ferrets were fed fish in small batches. For *D. insignis* worms, we conducted exposures using copepods infected with larvae originating from 2 female worms at 2 different times (i.e., 5 fish per day for 3 days in April 2016 and another 5 fish per day for 3 days in July 2016, resulting in exposure to ≈300 copepods) (Table 3.1). Because there was only 1 *D. medinensis* worm, fewer *D. medinensis* worm–infected copepods were available. We exposed only 1 species of fish (mosquitofish). A ferret was given 5 fish per day for 6 days for a total of ≈300 copepods. (Table3.1).

We maintained exposed ferrets for 77–134 days, then anesthetized and humanely euthanized them using 30 mg/kg ketamine followed by sodium pentobarbital. We necropsied the ferrets and examined the recovered *Dracunculus* worms to determine sex and whether females were mated or gravid. All animal procedures were reviewed and approved by the University of Georgia's Institutional Animal Care and Use Committee (no. A2014 11–010).

Of the 3 ferrets we fed fish that had ingested *D. insignis* worm–infected copepods, 2 were infected (Table 3.1). One ferret was infected with 6 *D. insignis* females (5 gravid), the other with 1 male worm (Table 3.1). The 1 ferret fed fish exposed to *D. medinensis* worm–infected copepods became infected with 12

worms; female worms were mated but not gravid because of their young age (Table 3.1).

CONCLUSIONS

The infection of ferrets with *Dracunculus* spp. worms after consuming fish that had eaten infected copepods demonstrates a novel transmission route. The unprecedented increase in the number of *D. medinensis* worm infections in dogs in Chad suggests the potential role of aquatic paratenic hosts (*2*,*12*). Classically, paratenic hosts become infected and facilitate transmission by bridging a trophic level, maintaining long-term infections, or concentrating larger worm burdens in their tissues (*3*). We suggest that fish can serve the role of transport hosts because fish did not have disseminated *Dracunculus* worm infection develop in our initial trials (C.A. Cleveland, unpub. data). Because most cases of Guinea worm disease occur in areas known for intense artisanal fishing and residents' dependence on fish protein, it is likely that fish and other aquatic animals play a role in transmission.

In 2014, preventive measures such as cooking fish thoroughly, burying fish entrails, and preventing dogs from consuming fish entrails were implemented in Chad. By May 2015, interventions were implemented in >50% of at-risk communities (*1*). Although limited, surveys for natural infections in fish from Chad's Chari River have not detected *D. medinensis* larvae (*2*; C.A. Cleveland, unpub. data). However, our findings suggest that the proposed intervention strategies involving fish are relevant and should continue. It is unclear what happens in Chad to small fish caught by fishermen; the fish might be consumed whole without cooking or, more likely, are discarded where dogs could consume them. During previous surveys of fish for *D.*

medinensis worms, large numbers of copepods were observed in their gastrointestinal tracts, supporting their potential role as transport hosts (C.A. Cleveland, unpub. data). Despite the interventions implemented in Chad, sporadic dog and human infections are still reported, suggesting a need for continued educational campaigns.

The recent report of a natural amphibian paratenic host, combined with the results of our study, indicates that the transmission of *D. medinensis* worms is not as simple as once believed (*10*). Despite the highly successful eradication campaign, 4 countries (South Sudan, Mali, Ethiopia, and Chad) still report endemic *D. medinensis* worm transmission. All 4 countries now report infections in dogs, so novel intervention and eradication strategies are needed.

Although our study showed that fish can transmit *Dracunculus* larvae to ferrets, many questions remain. For example, it is likely that different fish species feed on copepods at different rates and have different gastrointestinal tract transit times. This might also explain why individual exposure of mosquitofish to *D. medinensis* or *D. insignis* worms led to infection in only the *D. medinensis*–exposed ferrets; the *D. insignis*–exposed fish were fed to ferrets an hour later than *D. medinensis*–exposed fish. It is possible that mosquitofish transit material through the gastrointestinal tract faster than the other species. Alternatively, the ferrets may not have become infected simply because, as previous work has shown, not all ferrets exposed to *Dracunculus* worms become infected (*12*). Additional data are especially needed for those fish species that might be caught and ingested by humans or dogs in Guinea worm–endemic countries. Furthermore, 2 fish species retained *D. insignis* larvae in their tissues for 7–11 days, demonstrating the need for further

experimental and field work on the role of fish as paratenic hosts for *Dracunculus* spp. worms (6).

BIOGRAPHICAL SKETCH

Mr. Cleveland is a PhD student in the Warnell School of Forestry and Natural Resources and the Southeastern Cooperative Wildlife Disease Study at the University of Georgia. He has broad interests in parasite life cycles and transmission dynamics, wildlife management, and the ecology of infectious diseases.

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Table 3.1. Results of ferret exposure trials with 3 different fish species exposed to copepods infected with *Dracunculus medinensis* or *D. insignis* worms.

Dracunculus sp.	Fish species	Total fish consumed/ total fish offered*	Total No. copepods†	Days until euthanasia of ferret‡	*Dracunculus* infection status of ferret	Total worms and gender recovered§
D. insignis	Mosquitofish *(Gambusia affiinis)*	28/30	300	91 & 134	-	0
D. insignis	Tilapia (*Oreochromis niloticus*)	27/30	300	91 & 134	+	6F¶
D. insignis	Fathead minnow (*Pimephales promelas*)	30/30	300	91 & 118	+	1M
D. medinensis	Mosquitofish *(Gambusia affiinis)*	30/30	300	77	+	1M/11F

* in groups of five fish/day for six days.
† ≥25% of copepods infected.
‡ The *D. insignis* worm-exposed ferrets have 2 entries for days until euthanasia because these animals were exposed to fish at 2 different time points with copepods infected with larvae from 2 different worms.
§ All worms were recovered from the subcutaneous tissues of the limbs.
¶ Of these 6 female worms, 5 were gravid, indicating a male worm was either missed or had died prior to necropsy.

CHAPTER 4

HUNTING TINY DRAGONS: SURVEILLANCE FOR *DRACUNCULUS MEDINENSIS* THIRD-STAGE LARVAE IN AQUATIC ANIMALS, CHAD, AFRICA .[1]

[1]**Cleveland, C.A.**, Eberhard, M.L., Thompson, A.T., Swanepoel, L., Zirimwabagabo, H., Moundai, T., Ouakou, P.T., Ruiz-Tiben, E., and M.J. Yabsley. Submitted to *Scientific Reports,* October 2018.

Dracunculus medinensis, or human Guinea worm (GW), causes a painful and debilitating infection. The global Guinea Worm Eradication Program (GWEP) has successfully reduced human GW cases from 3.5 million in 21 countries in 1986 to only 30 cases in three remaining countries in 2017. Since 2012, an increase in GW infections in domestic dogs and cats has been reported. Because these infections have not followed classical GW epidemiological patterns resulting from water-borne transmission, it has been hypothesized that transmission occurs via a paratenic host. Thus, we investigated the potential of aquatic animals to serve as paratenic hosts for *D. medinensis* in Chad, Africa. During three rainy and two dry season trips we detected no GW larvae in 234 fish, two reptiles and two turtles; however, seven GW larvae were recovered from 4 (1.4%) adult frogs. These data suggest GW infections may occur from ingestion of frogs, but the importance of this route is unknown. Additional studies are needed, especially for other possible routes (e.g., ingestion of fish intestines that were recently shown to be a risk). Significantly, 150 years after the life cycle of *D. medinensis* was described, our data highlights important gaps in the knowledge of GW ecology.

Keywords: *Dracunculus medinensis*, Dracunculiasis, frogs, fish, Guinea Worm, paratenic host, reptiles, transmission, transport host

Introduction

Guinea Worm Disease (GWD), caused by the parasitic nematode *Dracunculus medinensis*, is a painful and debilitating disease of people[1, 2]. The global campaign to eradicate GWD has been an international success and national Guinea Worm

Eradication programs (GWEPs) have reduced the number of human cases from 3.5 million annually in 21 countries to only 30 cases in three remaining countries in 2017[2]. In Chad, infections in dogs, and more recently, cats, have been recorded[2]. Notably, these infections do not typically follow the classic epidemiology of GWD in humans, and it was hypothesized that fish or frogs may be playing a role in transmission of *D. medinensis*[3]. This hypothesis is further supported by the dearth of human cases in many areas where a high incidence of dog infections occur[3]. Furthermore, previous experimental work confirmed that frogs can serve as paratenic hosts for *D. medinensis*, that fish may act as short-term transport hosts, and that both routes (frog and fish) can result in infection of a definitive host[4, 5]. In 2016, for the first time, a natural infection of an amphibian host from the Sarh region of Chad was reported providing field evidence that amphibians may be involved in sylvatic transmission[6].

The purpose of this study was to better understand the possible role of aquatic animals (amphibians, fish, and reptiles) as paratenic hosts for *D. medinensis* in Chad, Africa. These data are critical for the development of new interventions aimed at interrupting Guinea worm transmission, especially now since the numbers of infections in dogs and cats have increased annually since 2012[2]. Our primary objective was to determine the prevalence of *D. medinensis* third-stage (L3) infectious larvae in aquatic animals, with particular attention paid to those species that are likely consumed by humans, those fed to dogs or cats by people, or those that reside in ponds in close proximity to villages where a dog infection or human case has been reported. We also attempted to collect information on which frog species

were consumed by humans, the season in which frogs were harvested, and whether or not frogs or fish were fed to dogs and cats.

Materials and Methods

Study location and animal acquisition

Surveys for *D. medinensis* L3s in aquatic hosts from Chad occurred during five trips during 2016-2018. Regions of Chad reporting human cases and dog infections were prioritized for sampling and included Sarh, Bousso, Guelengdeng, and N'Djamena (Fig. 1). Amphibians were captured by local villagers either by hand or through the use of submersible nets baited with fish tissue. Fish were typically purchased from local fishermen either at the pond or river locale being sampled, or from nearby outdoor markets. Reptiles included in the sampling were provided by local villagers.

Necropsy and sample collection

Necropsies were performed and gastrointestinal (GI) tracts were removed because L3s should only be present in non-GI tissues and muscles[4, 5]. Small tissue samples (<1cm) were taken from every animal and preserved in 70% ETOH for molecular species identification (as described below). For animals <10cm in length, blunt dissections in petri dishes were performed. Skin was removed and muscle tissue was teased apart using forceps and needle tools. The tissue was allowed to soak in water for at least four hours to allow migration of larvae out of tissue. After four hours, water in petri dishes was observed under a dissecting microscope (30x magnification; StereoZoom 4; Optek, USA) for characteristic sinusoidal movement of larvae. For animals >10cm in length, after GI and skin removal, samples were minced

using a metallic mincer that would render muscle into <0.5cm diameter pieces (LEM Products, West Chester, Ohio). This tissue was then placed into a mesh screen inside a Baermann funnel with enough water so that all tissue was submerged, and sedimentation of potential nematode larvae was allowed to occur for at least four hours (Fig. 2). At four hours, 5ml of fluid from the funnel was collected into a petri dish and examined as described above. If the first 5ml were negative for suspect nematode larvae, two subsequent draws of 5ml were examined. Any larvae that were morphologically similar to *Dracunculus* were preserved in 70% ETOH for subsequent molecular analyses.

Molecular identification of host and suspect *Dracunculus* larvae

All suspect samples were observed under a compound microscope for defining characteristics of *D. medinensis* L3s, such as a trilobed tail, striated cuticle, and approximate length of 0.581-0.643mm[7]. Morphologically-compatible larvae and tissue samples were placed in a 0.5 ml microcentrifuge tube and any residual ethanol allowed to evaporate for 12 hours. DNA was extracted using a commercial DNA extraction kit (DNeasy, QIAGEN, Valencia, California) following manufacturer's instructions for tissue. However, larvae were allowed to incubate at 56°C with proteinase K and Buffer ATL for six-eight hours to ensure full digestion. For species identification of animal samples, the 16s rRNA gene target was amplified for fish[8] and a portion of the cytochrome-*c* oxidase I (COI) gene was amplified for amphibians[4]. To identify larvae, a partial (COI) gene target was amplified using a cocktail of six M13-tagged primers[9]. Amplicons were purified from a 0.8% agarose gel stained with gel red (Biotium Inc., Hayward, California, USA) using a

commercial gel-purification kit (QIAGEN). Bi-directional Sanger sequencing was conducted on amplicons at the University of Georgia Genomics Facility (Athens, Georgia) or Genwiz (South Plainfield, New Jersey). Chromatograms were analyzed in Geneious R7 (Auckland, New Zealand) and consensus sequences were compared to sequences in the GenBank database.

Identification of frog use by villagers

Where possible, local villagers were queried regarding use and consumption of amphibians as food sources. These questions were as follows: (1) Do you catch and eat frogs?; (2) If you catch frogs, during what season and how?; (3) Can you describe the frogs you catch?; (4) Are frogs cooked or eaten raw?; (5) Do you own a cat or dog, and if so, do you feed them frogs? If individuals stated that they captured frogs, they were shown photos of *Hoplobatrachus occipitalis* (African Crowned bullfrog locally referred to as black-backed, white-bellied river frog) and *Pyxicephalus edulis* (Giant Bullfrog locally referred to as black-backed, yellow-bellied frog) and asked to identify which frogs they capture and during which time of the year (Fig. 3).

Ethical approval and informed consent:

All animal procedures were reviewed and approved by the University of Georgia's Institutional Animal Care and Use Committee (A2016 07-024). In addition, all methods were performed in accordance with the relevant guidelines and regulations within the aforementioned University of Georgia's Institutional Animal Care and Use Committee (A2016 07-024).

Results

During the five trips, we sampled 170 individual fish representing eight different species, 280 individual amphibians representing six species, two African helmeted turtles (*Pelomedusa* sp.), and two Nile Monitor lizards (*Varanus niloticus*) (Table 1). No *D. medinensis* L3s were detected in the tissues of any fish, turtles, or Nile monitor lizards. Of particular note, when skinning the monitor lizards, we found large subcutaneous nematodes on the ventral surface extending along the intercostal muscles and into the thoracic cavity (Fig. 4). Previous work had suggested that *Varanus* spp. could be hosts for adult *D. medinensis*[10]; however, the morphology of these nematodes clearly indicated they were not *Dracunculus* and molecular characterization using the same primers as used for larvae showed these nematodes were most similar to *Ochoterenella* spp. (Onchocercidae).

We recovered a total of 363 larvae from amphibians, 90 of which met morphological criteria warranting molecular identification. Of those, seven (8%) were confirmed as *D. medinensis* with sequences 100% similar to the previously accessioned cytochrome *c* oxidase subunit I, *D. medinensis* HQ216219.1 (Genbank). The first trip was conducted June-July 2016 in Marabe (Moyen Chari region, Kyabe district), and a single individual larvae was found in an African puddle frog (*Phrynobatrachus francisci*) as previously reported[6]. On two separate trips (Jan. 2017 and April 2018), we recovered six *D. medinensis* L3's; two of the six were found in a single *H. occipitalis* bullfrog during Jan. 2017 from the village Sidi-1in the Bousso area (Fig. 1) while the remaining four were detected in April 2018 from Tarangara village in the Sarh area (three larvae from a *H. occipitalis* and one larva from another

H. occipitalis) (Fig. 1). Additional nematodes were obtained from the amphibian samples, although we did not enumerate or identify all specimens. Of those identified, we found *Raillietnema* spp., *Cosmocercoides* spp., and Spirurida species.

When collecting samples in villages (Fig. 1), numerous villagers answered the questionnaire about frog hunting and consumption. Villagers stated that frogs were captured and consumed; however, some villagers did not eat frogs for various reasons. Two species of frogs (*H. occipitalis* and *P. edulis*) were the targets of frog catchers. When interviewed, frog catchers were able to describe both aforementioned species prior to seeing photos, then accurately picked photos of each species when shown. No one who admitted to eating frogs said that they consumed frogs raw, all stated that they cook frogs very well (Fig. 5) , similar to fish, and they do not feed frogs to dogs for fear that the dogs would become sick.

Discussion

Annual increases of GW infections since 2012 among domestic dogs and more recently cats, and the absence of classical water-borne outbreaks of GWD among humans led to the hypothesis that transmission was not occurring via the classical route of ingesting water containing copepods infected with L3s. Instead, it was posited that a paratenic host may be involved and that domestic animals were being infected via consumption of undercooked flesh or viscera of aquatic animals[3]. Our previous experimental studies showed that various amphibian species are susceptible to infections; however, in order to be important in the natural transmission cycle of *D. medinensis*, natural infections must occur. Thus, the goal of this study was to investigate what aquatic animals may be infected with *D. medinensis* L3s (as a

result of ingestion/consumption of copepods containing L3s). Our intensive surveys in areas of concern in Chad identified several infected frog species including two species that are known to be eaten by people. No *D. medinensis* larvae were found in any of the sampled fish, *Varanus*, or turtles, although only a limited number of the latter two groups were sampled.

Many Chadian villages rely heavily on fish protein during off-seasons of agricultural production (July-November). Villages in close proximity to the Chari river that identify as fishing villages are at highest risk for *D. medinensis* transmission based on biological, epidemiological, and environmental investigations by the Centers for Disease Control and Prevention and The Carter Center Guinea Worm Eradication Program[2]. The absence of detection of *D. medinensis* L3s from any of the fish samples obtained from villages actively fishing the same species from local ponds and river tributaries suggests a limited role of fish as paratenic hosts for *D. medinensis*. Our detection method (using Baerman funnels and sedimentation) has been widely used in detection of larvae specimens from muscular tissues of hosts[11, 12]. However, this method likely lacks sensitivity as it requires larvae to migrate out of tissues. When considering the role of fish, data from experimental studies indicate fish have been challenging to infect with *D. insignis* (commonly found in North American raccoons (*Procyon lotor*)), and *D. medinensis* infection in fish has not occurred in Chad [4, 13].

We did not recover any larvae from fish; however the importance of fish to the transmission of *D. medinensis* in Chad remains uncertain. Although it appears unlikely that fish act as a paratenic host in the wild, a recent study using fish species

found in Chad showed that fish may serve as short term transport hosts[5]. In this situation, a transport host would be a smaller fish that feeds on copepods infected with *D. medinensis* L3s and is subsequently consumed by a definitive host prior to complete digestion or passage of parasites thereby "transporting" potential infection to definitive hosts[5]. Larger fish may also ingest copepods so allowing dogs or cats to ingest any fish intestines could pose a risk[5]. The methodology for larval recovery is different than that of paratenic hosts, and L3s would need to be recovered from the gastrointestinal tract. The role of fish as transport hosts in natural settings has yet to be determined and warrants investigation, especially considering increasing incidence of dog and cat infections in Chad along the Chari river corridor where there is significant fishing pressure[2].

We recovered *D. medinensis* L3s from several amphibians, but the prevalence and burden of infection per individual frog was low (1-2 larvae/infected individual); however, our sampling may have missed infections due to low sensitivity of our recovery method or because infections in amphibians may be temporally and/or spatially clustered. For example, had we sampled tadpoles or frogs soon after they emerged from a water body known to have been contaminated with *D. medinensis*, the prevalence or worm burdens may have been higher. In North America, *D. insignis* has been used as a model parasite to evaluate potential transmission routes for *D. medinensis*. Additionally, field-based studies on *D. insignis* transmission have been conducted and we recently detected *D. insignis* L3s in several southern leopard frogs (*Lithobates sphenocephala*) at a site where prevalence of *D. insignis* is very high in raccoons (*Procyon lotor*) and one adult frog contained 45 larvae in its musculature

(Cleveland, unpublished data). This finding could support the association among a single, heavily infected paratenic host and if ingested by a definitive host, lead to infection with numerous adult nematodes.

In Chad, there have been dog infections in which >50 worms emerge, but the average infection per dog is 2 worms with a range of 1-79 (The Carter Center)[14]. For those dogs with high worm burdens, a large number of L3s must be ingested, either from a fish acting as a transport host, a number of frogs acting as paratenic hosts, ingestion of water containing infected copepods, or a combination of these possible routes. Certain individual dogs may also exhibit behaviors that put them at higher risk for infection, such as preferentially predating and consuming amphibians or living in households that feed large numbers of raw fish or fish entrails to their animals. Although there are now several hypothesized alternative transmission routes, classical transmission by ingestion of water with infected copepods remains a possibility. A recent experimental study shows that dogs are capable of ingesting copepods during a normal drinking event; however, low numbers were ingested per drinking event and in cases where larger number of copepods were ingested, the density of copepods was typically higher than what is normally seen in Chadian water-bodies[15]. Instances of cat infections in Chad further complicate identifying the type of transmission occurring, and studying the diets and movement of cats that have been infected or live in villages with dog infections could be informative.

Integration of the potential transmission routes may be advanced using mathematical modeling. For example, a combination of transmission routes in avian influenza[16] and Ebola[17] help to explain phenomena including pathogen invasion and

persistence. Uncertainty in model structure, including the existence of certain transmission routes, as well as parametric uncertainty, especially the rates and probabilities associated with transmission routes, can be explored using mathematical models in conjunction with sensitivity analyses[18]. These approaches allow an assessment of which features of transmission are most likely to interrupt the overall transmission cycle[19]. In the case of *D. medinensis* in Chad, transmission via transport and paratenic hosts may be combined with waterborne transmission in a model to both explore the likely impact of reducing or blocking one of these routes on overall transmission, and to estimate key parameters by fitting such models to data.

The importance of additional data on the role of paratenic and transport hosts as food sources can be used to strengthen model development as well as in the design and implementation of interventions in Chad. For example, *H. occipitalis* (Fig. 3), which was infected with *D. medinensis* L3s, was confirmed as a human food source through questionnaires conducted in numerous villages among various local ethnicities. Additionally, *P edulis* (Fig. 3), was also identified as a human food source, and although we did not detect *D. medinensis* in this species, only three individuals of this species have been sampled to date so additional testing is needed. It is important to note that people consume frogs commonly and consistently report cooking them (Fig. 5), which would kill any parasites present. They also alleged they did not purposely feed frogs to dogs, but bringing the frogs into the village could provide a scavenging opportunity for dogs and Carter Center researchers have observed children feeding tadpoles to dogs (unpublished data). However, ingestion of

both paratenic and transport hosts could occur independently of human activities during foraging and scavenging events by animals.

While we have been successful in supporting an alternative method of transmission for Guinea worm, our results should be considered a conservative estimate of the number of amphibians infected with Guinea worm L3s. Two of the surveys occurred outside of the peak transmission season (January-June), and may not be representative of the prevalence in amphibians or possible host diversity. An additional important consideration is that detection of *D. medinensis* L3s via our methods is likely insensitive, and it is possible that larvae were missed during the process of necropsy and microscopy. Also, the application of Abate® (Temephos) to control copepod populations post potential contamination of water-bodies with Guinea worm could affect the number of L3s recovered from an amphibian paratenic host.

The role of amphibians is not yet fully defined, but there are additional challenges based on life-stages of amphibians. For example, tadpoles are very likely the key life-stage wherein ingestion of infected copepods occur and if tadpoles consume large numbers of infected copepods, they could be acting as a sink for infection if tadpole/amphibian ingestion by dogs or people is ultimately shown to be low. Also, if the species of tadpoles consuming the copepods is a fully aquatic amphibian, such as *Xenopus*, then it is less likely to be scavenged or hunted by a cat or dog, despite having potential infective L3s in its tissue. Conversely, the possibility for amphibians to act as long-term paratenic hosts must be considered. Data from an experimental study showed that motile larvae of *D. insignis* and *D. medinensis* were

recovered from frog tissues (*Xenopus* spp.) for up to 8 months and 2 months, respectively (Cleveland and Yabsley, unpublished data).

Conclusions and Management Implications

Stopping transmission of Guinea worm and achieving eradication of *D. medinensis* is the goal of the global Guinea Worm Eradication Program. Alternative transmission routes for *D. medinensis* to humans and animals are a concern for eradication. While we have identified *D. medinensis* infection in frogs, we have not received reports nor collected data on dogs or cats consuming frogs. Consumption of frogs by peri-domestic animals may occur, and further studies are needed to identify the location and frequency of these behaviors. Furthermore, while our data do not provide evidence that fish are paratenic hosts, there has been no research yet conducted on the presence of *D. medinensis* larvae in the intestines of fish in Chad; however, large numbers of copepods have been observed in the digestive tracts of small fish (Cleveland, unpublished data). A recent case-control study conducted by The Centers for Disease Control and Prevention personnel in Chad investigating risk-factors contributing to infection with *D. medinensis* reported a statistically significant association between provisioning of fish entrails to dogs and infections among dogs (S. Roy, The Centers for Disease Control and Prevention, personal communication).

Understanding how transmission is occurring among dogs and cats would allow for implementation of timely and more effective preventative measures to be devised. In order to ensure compliance among village residents, the intervention(s) must be simple and straightforward. The owners of peri-domestic animals in Chad must understand the importance of limiting the ability of dogs and cats to scavenge or

consume frog flesh and fish entrails. It should also be noted that occurrence of *D. medinensis* transmission in Chad primarily occurs along the Chari river and tributaries, but, there are few human cases and animal infections from communities closer to the Logone river (Fig. 1), a major tributary of the Chari river (H. Zirimwabagabo, The Carter Center-Chad, personal communication). Furthermore, dog, cat and baboon (*Papio anubis*) infections have been documented in Ethiopia and dog infections in Mali, therefore potential animal behaviors and transmission route(s) may differ[20]. Therefore, careful field investigations in each region will be essential during, what is hoped to be, the tail end of the Guinea worm eradication program.

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Author contributions:

CC, MY, ME, HZ, PO, TM and ERT planned, designed, and conducted surveys in Chad. CC, AT, LS, and KG conducted molecular analyses. CC and MY wrote the main manuscript text and prepared tables and figures. All authors reviewed the manuscript.

Competing interests statement: The authors of this work declare no competing interests.

Data availability statement: Any data discussed herein will be made available upon request.

Table 4.1: Number and species of fish, amphibians, and reptiles surveyed for *D. medinensis* L3s in Chad, Africa.

		No. Sampled (No. positive, %)					
Group	Species	June 2016	January 2017	July 2017	April 2018	August 2018	Total
Fish	*Alestes* spp.	21	0	0	0	0	21
	Barbus spp.	4	0	0	0	0	4
	Barbus baudoni	1	0	0	0	0	1
	Brycinus spp.	3	0	0	0	0	3
	Chrysichthys spp.	5	0	0	0	0	5
	Coptodon spp.	51	0	0	0	0	51
	Coptodon zilli	10	0	0	0	0	10
	Hemichromis spp.	30	0	0	0	0	30
	Hydrocynus spp.	5	0	0	0	0	5
	Labeo spp.	9	0	0	0	0	9
	Lates niloticus	7	0	0	0	0	7
	Malapterurus spp.	1	0	0	0	0	1
	Micralestes spp.	5	0	0	0	0	5
	Oreochromis spp.	11	0	0	0	0	11
	Oreochromis aureus	1	0	0	0	0	1
	Pangasius spp.	6	0	0	0	0	6
	Parachanna spp.	1	0	0	0	0	1
	Pareutropius spp.	5	0	0	0	0	5
	Petrocephalus spp.	7	0	0	0	0	7
	Pollimyrus spp.	1	0	0	0	0	1
	Synodontis sp.	8	42	0	0	0	50
	Fish Total	192	42	0	0	0	234

Amphibian	*Hoplobatrachus occipitalis*	17	36 (1, 2.8%)*	32	28 (2, 7.1%)*	12	125 (3, 2.4%)
	Phrynobatrachus francisci	8 (1,12.5%)*	28	0	0	0	36 (1, 2.8%)
	Ptychadena spp.	44	47	5	0	0	96
	Pyxicephalus edulis	0	0	3	0	0	3
	Rana galamensis	2	1	2	0	0	5
	Xenopus fischbergi	2	1	8	0	0	11
Reptile	Lizard (*Varanus niloticus*)	0	2	0	0	0	2
	Turtle (*Pelomedusa* sp.)	0	0	2	0	0	2
	Amphibian/reptile Total	73	115	52	28	12	280

* Species positive for *D. medinensis L3s*

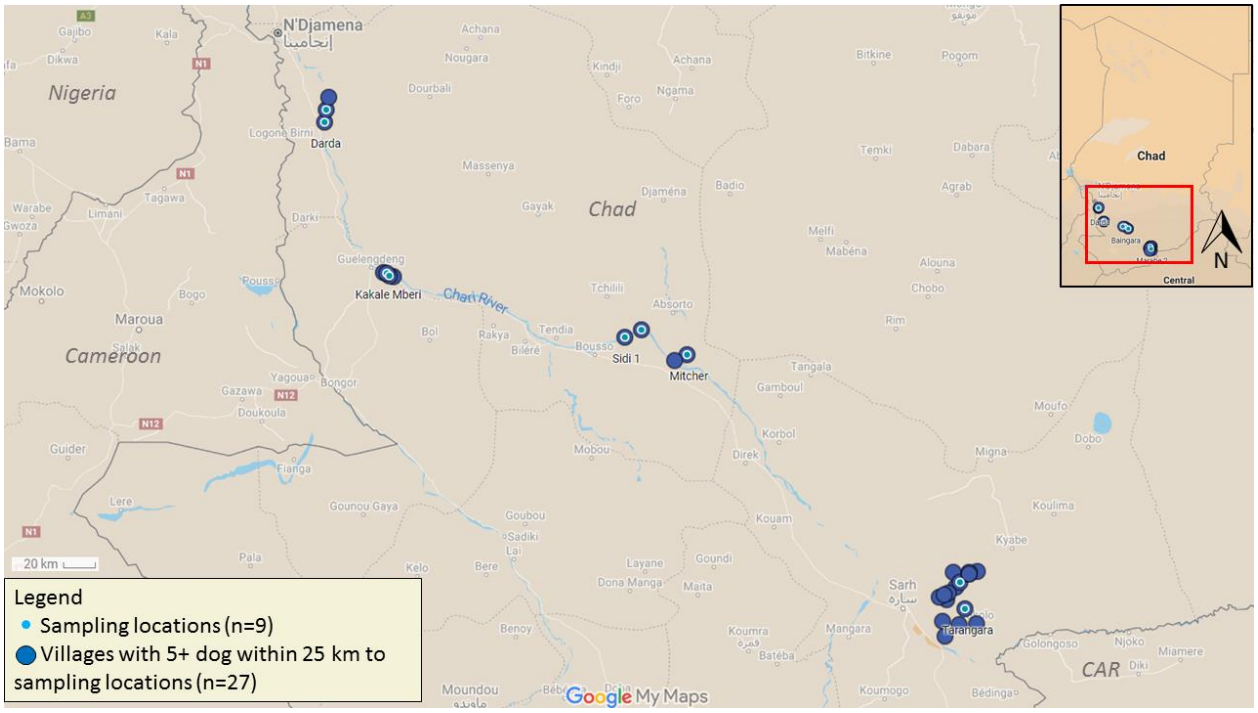

Figure 4.1: Map of nine sampling site locations and villages within 25km reporting five or more dog infections in Chad, Africa during 2016-2018 for surveys of *D. medinensis* third-stage larvae (L3) in amphibians and fish.

Figure 4.2: Modified Baermann funnels for sedimentation and recovery of suspect *D. medinensis* larvae from muscle tissues of fish and amphibians in Chad, Africa.

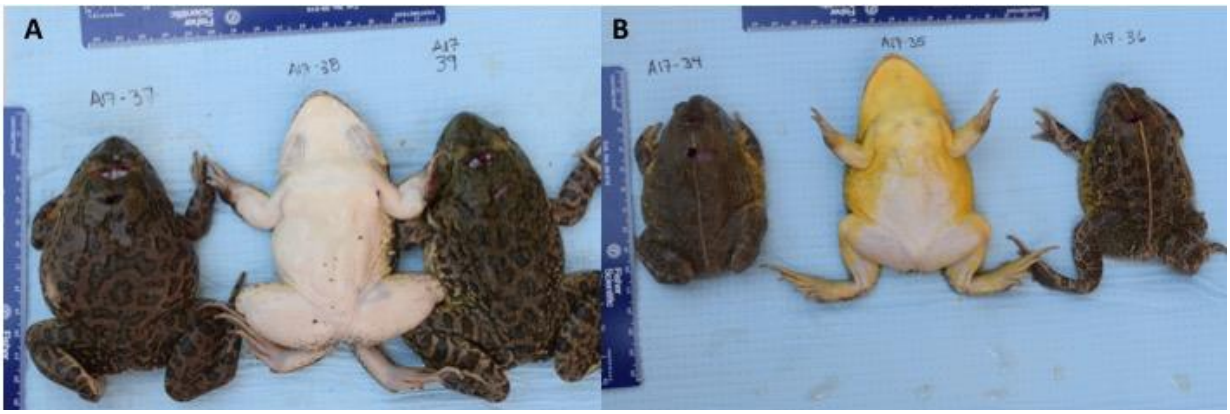

Figure 4.3: Specimens of *Hoplobatrachus occipitalis* (A) and *Pyxicephalus edulis* (B) sampled for the presence of *D. medinensis* third-stage larvae (L3) from Chad, Africa and shown to villagers during surveys of frog catching and consumption.

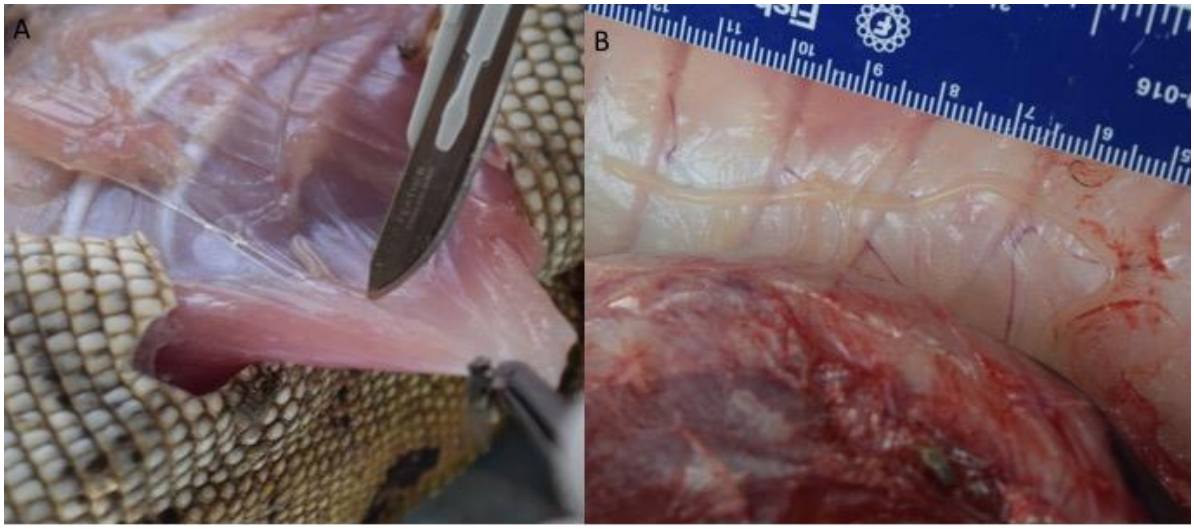

Figure 4.4: Necropsy of Nile monitor lizard (*Varanus niloticus*) from Bousso, Chad with subcutaneous infection of *Ochoterenella* spp. visible on external surface (A) and internal surface (B) of thoracic cavity resembling *D. medinensis*.

Figure 4.5: Image of frogs being cooked on grill from Doba, Moissala district, Chad, Africa taken April 2017 (photo courtesy of H. Zirimwabagabo).

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CHAPTER 5

PREVALENCE, GENETIC DIVERSITY AND PARATENIC HOSTS OF *DRACUNCULUS INSIGNIS* IN MESO-MAMMALS FROM DI-LANE PLANTATION, WAYNESBORO, GEORGIA, UNITED STATES AND IMPLICATIONS FOR THE GUINEA WORM ERADICATION PROGRAM IN CHAD, AFRICA.[1]

[1]**Cleveland, C.A.,** Eberhard, M.L., Garrett, K.B., Thompson, A.T., Swanepoel, Miller, E.A., Stephens, O.L. and M.J. Yabsley. Submitted to *Journal of Parasitology*, October 2018.

Abstract:

The prevalence and diversity of parasitic nematodes in wildlife has been well studied for certain species, yet for others there exist considerable gaps in knowledge. The parasitic nematode, *Dracunculus insignis*, infects North American wildlife and past research has led to increased understanding of the potential host diversity and transmission of the closely related human Guinea worm, *D. medinensis* (which is currently the focus of a global eradication program). Many definitive hosts have been documented for *D. insignis*, however, the life-cycle has only been studied in laboratories and not proven in the field. Additionally, only one phylogenetic study has been conducted on *D. insignis* from Canada. The goal of this study was to investigate the prevalence of *Dracunculus* infections among wildlife at a single site (Di-Lane Plantation) in the southeastern United States, evaluate the genetic diversity of parasites at this site, and investigate the role of paratenic hosts in transmission. Over the course of 3 years, we sampled 228 meso-mammals, reporting an overall prevalence of infection with *Dracunculus insignis* of 20% (46/195). Sampling of 63 individual fish of a single species and 68 frogs representing 5 species from water bodies in the same geographic area as infected meso-mammals resulted in recovery of *D. insignis* third stage larvae (L3s) from 2 species of amphibians, but all fish sampled were negative. The phylogenetic analysis of the partial cytochrome-*c* oxidase I (COI) gene shows very little diversity of *Dracunculus* at Di-Lane; however, we did recover a single nematode from a Virginia opossum (*Didelphis virginiana*) that falls outside of the *D. insignis* clade, more closely aligns with *D. lutrae*, and may represent an undescribed species. This work increases life-cycle knowledge on a previously

undocumented transmission pathway in nature at a single site with endemic transmission of *D. insignis* among raccoons and opossums. Furthermore, phylogenetic analysis illustrates sustained localized transmission, yet also indicates that there may be sporadic introductions of novel *Dracucunculus* sp. into this system, the distribution and prevalence of which are unknown. When applied to the global Guinea Worm Eradication Program, and Chad, Africa in particular, this work increases our knowledge of the potential role of aquatic animals in transmission of *Dracunculus* species and informs on potential intervention strategies that may be applied to the eradication of Guinea worm in Africa.

Key Words: amphibians, *Dracunculus*, fish, Georgia, Guinea worm, nematode, Virginia opossums, raccoons, transmission

Dracunculus spp. are subcutaneous nematode parasites (Family Dracunculidae, Order Spirurida) of numerous reptile and mammalian species. Although natural history data are limited for reptile-infecting species, considerable data are available for *Dracunculus medinensis* that infects people and causes Guinea Worm disease (GWD) in Africa. There is also considerable data available for *Dracunculus insignis* (that infects wildlife species such as raccoons (*Procyon lotor*), mink (*Neovison vison*), skunks *(Mephitis mephitis*), weasels (*Mustela* spp.), and North American river otters (*Lontra canadensis*) in North America (Crites 1963; Crichton and Beverly-Burton, 1974; Anderson, 2000; Cleveland et al., 2018; Williams et al., 2018). *Dracunculus* infections have been documented in a variety of hosts from multiple regions of North America; however, identification in most studies was limited to morphology of female worms, which cannot be reliably identified to species without

genetic characterization. Although male worms are morphologically distinguishable, they are rarely detected (Cleveland et al., 2018). The only previous study that examined the host-range of confirmed *D. insignis* (by sequence analysis) was conducted in Canada and found that *D. insignis* recovered from raccoons, mink, fishers (*Martes pennanti*), and river otters had minimal sequence divergence among hosts, supporting the theory that *D. insignis* is a host generalist (Elsasser et al., 2009). By contrast, *D. lutrae* was found to be a host specialist for river otters but exhibited a high degree of genetic diversity and several individual otters were infected with multiple parasite lineages (Elsasser et al., 2009).

Dracunculus insignis has increasingly been used as a surrogate parasite to inform eradication efforts of the related parasite, *D. medinensis*, for several reasons including close genetic relationship, the presumed similarities in life-cycles, the ability to acquire local specimens, and a history of successful experimental infections (Beverly-Burton and Crichton, 1976; Eberhard et al., 1988; Eberhard and Brandt, 1995; Elsasser et al., 2009). This has become increasingly important because there has been a recognized change in the epidemiology of *D. medinensis*. Since 2012, there has been an increase in the number of peri-domestic dog and cat infections in Chad, Ethiopia, and Mali, Africa (Eberhard et al., 2014; Molyneaux and Sankara, 2017). The geographic distribution and increasing number of animal infections led to the theory that aquatic paratenic hosts (e.g., fish and amphibians) may play a role in transmission of *D. medinensis*. This was recently supported by experimental work and the detection of a *D. medinensis* third-stage larvae (L3) in a wild caught frog in Chad (Eberhard et al., 2014, 2016a, b; Cleveland et al., 2017). However, little work

has been done on the natural cycle of transmission of *D. medinensis* in Chad among dogs and no research has been conducted on potential susceptible wildlife. Understanding the natural history and genetic diversity of *D. insignis* in wildlife in North America may assist the eradication efforts for *D. medinensis* because, if amphibians are a reliable source of infection to definitive hosts, then this information can provide valuable insight into devising and implementing interventions to prevent infections of *D. medinensis* in Chadian dogs and cats.

This study was conducted to better understand the prevalence and genetic diversity of *Dracunculus* spp. in various meso-mammals in Georgia. To date, no multi-host surveys have been conducted in the southeastern United States and the single study that investigated the genetic diversity of *D. insignis* was conducted on worms collected from throughout Ontario, Canada (Elsasser et al., 2009). In addition, little work has been done examining the natural transmission cycle. Although it has been hypothesized that *Dracunculus* can use aquatic paratenic host(s) (likely an amphibian), to date there are no natural infections of paratenic hosts reported for *D. insignis* and only a single frog infected with *D. medinensis* (Crichton and Beverly-Burton, 1977; Anderson, 2000; Eberhard et al., 2016). Thus, we conducted surveillance of local fish and amphibians for *D. insignis* to identify any paratenic hosts potentially involved in transmission at a site with a high prevalence of the parasite in raccoons.

MATERIALS AND METHODS

Sampling Site

Di-Lane plantation is a 3278-ha wildlife management area located in Burke County, Georgia (N 32° 58’ 12.2, W 82° 03’ 34.0) managed by the Georgia Department of Natural Resources for early successional habitat with an emphasis on bobwhite quail (*Colinus virginianus*). In addition to habitat management, supplemental feeding and predator control programs are currently being conducted in an effort to increase bobwhite quail populations. The plantation contains upland hardwoods, loblolly pine (*Pinus taeda*) uplands, and dove field plantings. There are ephemeral water sources, permanent impoundments, and streams throughout the property.

Animal collection

Meso-mammals:

Predator removal at Di-Lane plantation was conducted by USDA APHIS Wildlife Services and we examined adult animals (based on size) captured from 2015-2017. Each year of trapping, 120 live capture traps (Tomahawk Live-trap Company, Tomahawk, Wisconsin, USA) and 120 double coil spring offset jaw foot-hold traps (MB-550, Minnesota Trapline Products Inc., Pennock Minnesota, USA) were used during 2 trapping sessions occurring from late February to mid-March and from mid-May to June (between 14-21 trapping nights per session). Trapping sessions were either right before or during peak time of emergence of female *D. insignis* (Cleveland et al., 2018). Each trap was checked beginning at sunrise. Any caught animal was humanely euthanized following the American Veterinary Medical Association’s

guidelines for humane euthanasia of animals (AVMA, 2013). The examination of animals for pathogens was included in a protocol reviewed and approved by UGA's IACUC committee (A2018 02-010).

Paratenic hosts:

Amphibians and fish were caught at several permanent ponds at Di-Lane plantation using dip-nets. Each animal was euthanized via cervical dislocation, identified to species, eviscerated, and skinned. The remaining muscle tissue was bluntly dissected, facilitating release and recovery of any *D. insignis* larvae present as previously described (Eberhard et al. 2016). Tissue was placed in petri dishes with Dulbecco's Phosphate Buffered Saline (DPBS) for a minimum of 4 hours until microscopy was conducted for larval detection (Eberhard and Brandt, 1995). Capture and sampling of aquatic hosts was reviewed and approved by UGA's IACUC committee (A2018 02-010).

Parasite collection and identification:

Meso-mammals were necropsied immediately or were frozen at -20°C until they were necropsied. Peritoneal cavities and subcutaneous tissues were examined for parasites. Subcutaneous parasites were stored in either 70% ethanol (EtOH) immediately or in DPBS overnight to allow third-stage larvae (L3s) to exit for use in experimental infection trials (Cleveland et al., 2017). After larvae recovery, nematodes were stored in 70% EtOH for subsequent molecular analyses. Adult females were classified as *Dracunculus* sp. based on general characteristics such as location in definitive host, size, morphology and the presence of larvae (Anderson, 2000; Cleveland et al., 2018). Males were not recovered during this study.

After tissues from amphibians and fish were soaked, the DPBS was examined for larvae. Any larvae exhibiting gross morphologic similarity to *Dracunculus* spp. were removed, assessed under a compound microscope for the presence of a blunt, trifid tail, and were then placed individually in 1.5 ml microcentrifuge tubes with 70% EtOH for molecular characterization. Unpaired t-tests with a 95% confidence interval were used to compare differences of parasite burden between hosts and among sexes of hosts.

Molecular characterization:

Adult nematodes were removed from EtOH and several small (1-2mm) pieces were placed into a microcentrifuge tube which was left open for 12 hours to allow ethanol to evaporate. Suspect larvae were processed the same as adult nematodes except whole larvae were extracted and the 18S gene was amplified as described (Bimi et al., 2005). DNA was extracted from adult nematodes and larvae using a commercial DNA extraction kit (DNeasy, QIAGEN, Valencia, California) following manufacturer's instructions for tissue. Partial cytochrome-*c* oxidase I (COI) gene was amplified using a cocktail of six M13-tagged primers as described (Prosser et al., 2013). Amplicons were purified from a 0.8% agarose gel stained with gel red (Biotium Inc., Hayward, California, USA) using a commercial gel-purification kit (QIAGEN). Purified amplicons were bi-directionally sequenced at the University of Georgia Genomics Facility (Athens, Georgia). Chromatograms were analyzed in Geneious R7 (Auckland, New Zealand) and consensus sequences were generated and compared to sequences in the GenBank database. Sequences were aligned using ClustalW (Thompson et al., 2002) in MEGA. Phylogenetic trees were constructed in

MEGA X using maximum-likelihood algorithms with partial deletion and 1,000 bootstrap iterations (Kumar et al., 2018). Sequences from this study were deposited into Genbank (Accession #'s MK085893-MK085902). For comparison, sequences from *D. medinensis* (AP017682, HQ216219), *D. lutrae* from Canada (EU6456494, EU646593, EU646600, EU646602), and *D. insignis* from Canada (EU646534, EU646535, EU646559, EU646569) were obtained from Genbank and included in the phylogenetic analysis with *Philometroides sanguinensis* (NC024931) and *Procamallanus slomei* (MG948463) as outgroups to root the tree.

RESULTS

Meso-mammals

A total of 228 meso-mammals were sampled for *Dracunculus* (Table 1), of which 46 were infected with subcutaneous nematodes grossly identified as *Dracunculus* sp. The highest prevalence was noted in raccoons (32%, 38/122) followed by opossums (*Didelphis virginiana*) (9.9%, 8/81). All coyotes (*Canis latrans)*, bobcats (*Lynx rufus*), and armadillos (*Dasypus novemcinctus*) were negative, but sample sizes were low (n=11,7 and 7 respectively). A total of 90 female *Dracunculus* were recovered, 77 from raccoons and 13 from opossums. Of the 90 recovered specimens, 55/77 were found in the hind limbs of raccoons, and 7/13 were on hind limbs of the opossums. We recovered 5 *D. insignis* from opossums that were located on the abdominal (3) and pectoral (2) musculature instead of distal extremities. Prevalence was significantly higher in male raccoons and male opossums (M=0.32, SD=0.21) compared to females (M=0.06, SD=0.06), $t(8)=2.7$, p 0.02. Also, prevalence was significantly higher in male raccoons (M=0.41, SD=0.19) compared

to female raccoons (M=0.07, SD=0.07) $t(4)=2.9$, p=0.04. The highest worm burden was recorded in a female raccoon (n=9), and female raccoons had a higher average worm burden (4.3) and range (n=1-9) compared to male raccoons (average 2.7 worms, range n=1-7). Male opossums had an average of 2.2 worms with a range of 1-5, whereas we only recovered single *D. insignis* female worms from female opossums.

Paratenic hosts

During peak transmission season (March-June) of *D. insignis*, we sampled 68 frogs representing 5 species (Table 2). *Dracunculus* larvae were detected in two species, *Rana catesbeiana* (6/43) and *Rana sphenocephala* (5/11). Intensity was generally low (mean of 1.6 and 15, respectively) but one *R. sphenocephala* harbored 45 larvae. A single larva from each positive frog was confirmed to be *D. insignis*; partial 18S gene sequences were 100% identical to one another and 99% similar to *D. insignis*. We also sampled 68 *Centrarchus macropterus* (Flier sunfish) from ponds on Di-Lane plantation and all were negative for *Dracunculus* larvae.

Molecular and phylogenetic analyses

A total of 50/90 adult female nematodes recovered from definitive hosts (38 raccoons, 12 opossums) yielded useable sequence via Sanger sequencing and were genetically characterized. All were confirmed to be *Dracunculus* species. The maximum-likelihood phylogenetic tree revealed no geographic or host clustering of *D. insignis* sequences. Sequences from all but one worm from Di-Lane were 99.9% similar to one another and to *D. insignis* sequences derived in various hosts from Canada (Elsasser et al., 2009). A single worm from an opossum (MK085893) was

only 92% similar to *D. insignis* and 91% similar to *D. lutrae* (our study and from Elsasser et al. (2009). The unique *Dracunculus* sequence from the opossum clustered with other sequences of *D. lutrae* outside of the *D. insignis* clade (Fig. 1).

DISCUSSION

Dracunculus insignis has been well documented in raccoons across a wide geographic area of North America (Crichton and Beverly-Burton, 1974, 1977; Cleveland et al. 2018); however, detailed investigation into the range of susceptible wildlife, associated phylogenetic relationships, and the role of paratenic and transport hosts in the transmission cycle at endemic locations had yet to be investigated. Our study supports the role of raccoons as the most common definitive host for *D. insignis* (Crichton and Beverly-Burton, 1974) but also indicates the potential involvement of opossums in supporting sylvatic transmission. The diet of opossums is similar to raccoons, therefore transmission of *D. insignis* via consumption of a paratenic host could occur. Our finding of *D. insignis* in *R. catesbeiana* and *R. sphenocephala* provides further support that paratenic hosts may be important in the life-cycle of *Dracunuclus* spp. as suggested by experimental studies and a recent finding in Chad, Africa (Eberhard et al., 2016a, 2016b; Cleveland et al., 2017).

The significantly higher infection prevalence in male raccoons and opossums compared with females could be a result of behavioral differences during the seasonality of sampling (spring); raccoon and opossum females are with young during these sampling periods and may have decreased movement while increasing localized foraging bouts. Male raccoons and opossums often have larger territories than females (Holmes and Sanderson, 1965; Lotze and Anderson, 1979; Gerht and

Fritzell, 1998) and could be foraging more broadly across the landscape which may increase risk of infection. This is also supported by well documented behavior of raccoons utilizing water sources to "wash" their prey, resulting in raccoons at water sources and in close proximity to amphibian populations (Lyall-Watson, 1963; Lotze and Anderson, 1979). Female raccoons also exhibit greater site fidelity than males (Gerht and Fritzell, 1998), and it is possible that the presence of infected paratenic hosts (either infection in intermediate hosts was not established or there are other macroinvertebrates predating on *D. insignis* larvae) may vary between waterbodies, potentially accounting for the difference in males versus females.

We found no infections in coyotes and bobcats, but sample sizes were low. Both species may consume appropriate paratenic or transport hosts and are possibly unrecognized hosts of *D. insignis* (Cleveland et al., 2018). A recent review of *Dracunculus* infections in domestic dogs and cats highlights that canids and felids are susceptible to infection (Williams et al., 2018). Furthermore, given the diversity of known hosts for *D. insignis,* it would seem that infection with *D. insignis* in coyotes and bobcats is possible and warrants further investigation. Phylogenetic analysis of *Dracunculus* species recovered from raccoons and opossums showed little sequence divergence, further supporting that *D. insignis* is a host generalist as suggested by Elsasser et al. (2009).

The life-cycle of *D. medinensis* was first determined in 1871 (Fedchenko, 1871) and since that time it was considered a human parasite with only rare spill-over events to animals. However, the changing epidemiology in Chad, Africa led to the suggestion that aquatic hosts could be involved based on previous experimental data,

indicating tadpoles (*R. pipiens*, *R. clamitans*, *R. catesbeiana*) are susceptible to infection with *D. insignis* (Crichton and Beverly-Burton 1977; Eberhard and Brandt, 1995, Eberhard et al., 2014). Despite these experimental data, one published report has documented a single frog in Chad was found infected with *D. medinensis* (Eberhard et al., 2016b), and similar findings in two additional frog species in Chad have been documented (C.A. Cleveland, unpublished). Our finding of *D. insignis* L3s in 11 frogs provides strong support that paratenic hosts may be important in the life-cycle of *Dracunculus* spp. (Eberhard et al., 1995, Anderson, 2000). The high number of L3s (n=45) recovered from the tissue of a frog from Di-Lane may also partially explain high worm burdens that may occur in some raccoons and opossums (Table 1).

In contrast to frogs, we did not find any larvae in tissues of the sampled flier sunfish. This species is the most abundant fish species at our site and the fish were caught near the shore in the same water bodies as the sampled amphibians. The lack of natural infection supports previous experimental work highlighting the difficulty of experimentally infecting fish (Crichton and Beverly-Burton, 1977). However, our experimental work does show that fish may have a role as transport hosts instead of paratenic hosts (Cleveland et al., 2017). The ability of a fish to act as a transport host could be a result of the short transit time of larvae and copepods (the intermediate host) through fish gastrointestinal (GI) tracts or the narrow probability of detection within short intervals of fish predating upon infected copepods. Previous experimental work has shown that beyond three hours from initial ingestion, various species of fish will either digest copepods and larvae or they will pass through the GI system (Cleveland et al., 2017). To date, no fish investigated in the wild have had true

infection in musculature with *D. insignis* L3s. The role of fish in transmission of *Dracunculus* to definitive hosts continues to be an important research interest due to the high numbers of fish that are consumed by humans, dogs, and cats in Chad, Africa.

Field studies on *Dracunculus* spp. are complicated by the fact that the parasite can only be diagnosed by euthanizing an animal and completely skinning the carcass. On very rare occasions and during a short time period of the year, worms may emerge or create a swelling that can be detected without euthanizing an animal, but this method of surveillance has limited sensitivity. Additionally, animals are unlikely to leave a worm hanging from a leg and may bite or tear at an emergent worm leading to missed infections. This highlights an issue facing the Guinea Worm Eradication program: infection in definitive hosts, such as dogs and cats, may be detected by owners in Chad; however, there is a lack of surveillance in wildlife because of many logistical issues which may be resulting in a missed group of reservoir hosts.

The goals of this study were to investigate the diversity of hosts that may be infected with *D. insignis* at a single site, and to evaluate the genetic differences of *D. insignis* recovered from those infected animals. Overall, we found that infections occurred in 2/5 species (raccoons and opossums) and that there was very little genetic difference among the *D. insignis* recovered, yet we were also able to identify a potentially undescribed species of *Dracunculus*. Furthermore, we were able to recover *D. insignis* L3s from two species of amphibians (*R. catesbeiana* and *R. sphenocephala*) that may be acting as a common food source among raccoons and

opossums. This points to the maintenance of transmission and life-cycle of *D. insignis* at Di-Lane plantation among raccoons and opossums.

The questions we investigated were chosen to help assist the Guinea Worm Eradication Program in Chad, Africa by providing information on transmission of parasites that is closely related and appears to have a similar natural history. Of concern is that sylvatic transmission of *D. medinensis* may be occurring in wildlife, and this could be responsible for the increasing prevalence of infections in dogs and cats despite comprehensive intervention strategies focused on people and domestic animals. The finding of naturally occurring paratenic hosts for *D. insignis*, coupled with the previous report of a naturally infected frog in Chad (Eberhard et al., 2016b) provide evidence that transmission to dogs, cats, and wildlife in Chad may be possible outside of the classical route of ingestion of infected copepods from drinking water. If eradication of *D. medinensis* is to be achieved, consideration of a wildlife reservoir with transmission routes similar to that of *D. insignis* should be considered and investigated.

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Table 5.1: Prevalence of adult female *Dracunculus* in meso-mammals sampled at Di-Lane Plantation, Waynesboro, GA, USA during 2015-2017

	Raccoon (*Procyon lotor*)		Virginia Opossum (*Didelphis virginiana*)	
	Male	Female	Male	Female
2015	58.8% (10/17)	0% (0/1)	n/a	n/a
2016	45% (18/40)	8.3% (1/12)	33.3% (5/15)	0% (0/25)
2017	20.7% (6/29)	13% (3/23)	5.6% (1/18)	8.7% (2/23)
Overall Prevalence	39.5% (34/86)	11.1% (4/36)	18.2% (6/33)	4.2% (2/48)
Total Prevalence	32.1% (38/122)		9.9% (8/81)	

Table 5.2: Prevalence of *D. insignis* third-stage larvae (L3) recovered from fish and amphibians at Di-Lane Plantation, Waynesboro, GA, USA during 2015-2017.

	Species	No. sampled	Prevalence (pos/total)	No. larvae (range, mean)
Fish	*Centrarchus macropterus* (Flier sunfish)	68	0% (0/68)	NA
Amphibians	*Rana catesbeiana* (Bullfrog)	43	14% (6/43)	10 (1-4, 1.6)
	R. sphenocephala (Southern Leopard frog)	11	45% (5/11)	61 (2-45, 15)
	Acris crepitans (Northern Cricket frog)	3	0% (0/3)	NA
	Hyla cinerea (Green Treefrog)	7	0% (0/7)	NA
	Psuedacris crucifer (Spring peeper)	4	0% (0/4)	NA

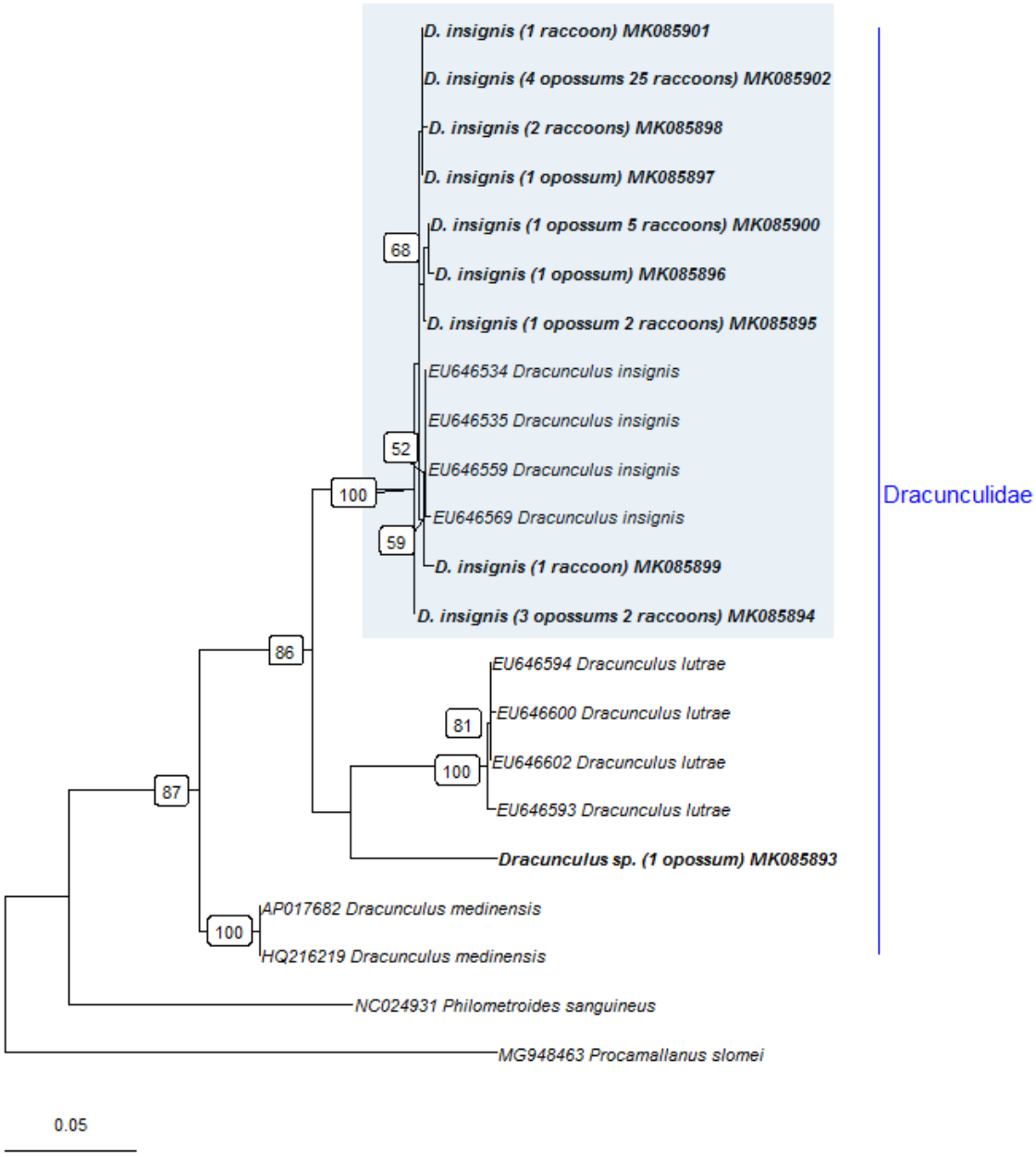

Figure 5.1: Genetic relationships of 49 *Dracunculus* adult females from racoons (n=38) and Virginia opossums (n=12) from Di-Lane plantation (Georgia, USA) compared with other *Dracunculus* spp. based on partial cytochrome oxidase subunit 1 gene sequences.

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CHAPTER 6

CONCLUSIONS

From both a public health and wildlife parasitology perspective, there are significant knowledge gaps concerning the genus *Dracunculus*. The work herein was conducted to fill some of these gaps and data were used to develop new questions for future investigations. While working towards the overall goal of informing Guinea worm eradication efforts, we uncovered several other species of *Dracunculus* that lack substantial descriptive life-cycles or molecular and morphological description. Additionally, this work showed that multiple pathways for transmission of *Dracunculus* are possible and warrant consideration in the scope of eradication efforts in the remaining Guinea worm endemic countries. As the eradication campaign moves forward, we should attempt to characterize and learn as much as possible about *D. medinensis* prior to its intentional extinction.

Study 1 (Chapter 2)

Prior to our undertaking, there had not been a comprehensive review of the genus *Dracunculus* in wildlife. Our literature review highlights the definitive host diversity of *Dracunculus* among mammals and reptiles. In Africa, we were able to shed light on a variety of *Dracunculus* species infecting reptiles. These data suggest that ancestral forms of *Dracunculus* originated from reptilian hosts and radiated out to infect mammalian definitive hosts. In North America, this review compiled historical and contemporary references to illustrate host diversity and geographic distribution,

allowing for further discussion on our lack of knowledge surrounding the life-cycles of *D. globocephalus* in snapping turtles (*Chelydra serpentine*), *D. ophidensis* in garter snakes (*Thamnophis sertalis*), and *D. lutrae* in river otters. As future investigations of *D. medinensis* in Africa occur and increased surveillance is undertaken on the role of wildlife in transmission of *D. medinensis*, having a strong basis for comparison from a life-history and molecular background will be useful.

Study 2 (Chapter 3)

Experimental infection trials have provided a strong background for informing field-based surveillance for *D. medinensis* (Eberhard et al., 2016a, 2016b). The work exploring the role of fish in transmission of *D. medinensis* in Chad was a key step for identifying intervention strategies that could be deployed to interrupt transmission. Infecting fish and exploring their role as paratenic hosts had been previously attempted (Crichton and Beverly-Burton, 1977), yet no work had been done assessing the role of fish as a short-term paratenic host. With the information that fish can harbor infective third-stage *D. medinensis* larvae in their guts, and that this is a method of infection to definitive hosts, surveillance and interventions in Chad can be more focused. Although the initial intervention of burying fish entrails and cooking fish thoroughly had been previously pursued in Chad, compliance may not be as high as needed. Furthermore, when working in villages with dog and cat infections, villagers often stated that they would feed their animals fish entrails, or witnessed their animals scavenging from overflowing fish entrail burial holes (Cleveland, unpublished). Field surveillance investigating fish entrails and the detection and recovery of *D. medinensis* larvae from villages with associated animal infections has

yet to be done but is a key step in identifying how transmission is occurring in Chad among dogs and cats.

Study 3 (Chapter 4)

Alternative pathways of transmission for *D. medinensis* have been suggested and proven in laboratory conditions (Eberhard et al. 2016, Cleveland et al. 2017), but surveillance for infective third-stage larvae (L3s) in fish entrails is needed in Chad, Africa, where transmission is occurring among domestic dogs and cats. The goal of this study was to conduct surveys for *D. medinensis* L3s in aquatic animals in Chad. Sampling locations were located within 25km of villages reporting 5 or more animal (domestic dogs and cats) infections. We investigated 514 total animals representing 26 species (234 fish, 276 amphibians, 2 turtles, 2 Nile monitors). We found no larvae in the fish, turtles, or Nile monitors, but did recover L3s from 4 individual frogs of two different species (*P. francisi* and *H. occipitalis*). The finding of a paratenic host infected with *D. medinensis* larvae in Chad rewrites 150 years of accepted knowledge surrounding Guinea worm transmission and highlights the importance of comprehensive surveillance moving forward with the eradication campaign. Although the prevalence of infection in the amphibians was only 1.45% (4/276), identifying alternative transmission routes is exceedingly important in the face of eradication. Finally, fish do not appear to be paratenic hosts for Guinea worm, yet the role of fish as transport hosts in Chad is a priority for future research efforts.

Study 4 (Chapter 5)

Despite a long history documenting infection in a variety of fur-bearer species in North America, data on the life-cycle and transmission of *D. insignis* is still

lacking. This parasite is closely related to the human Guinea worm, *D. medinensis*, and has also been used as a surrogate study-parasite to replicate experimental infection trials to inform Guinea worm eradication efforts in Chad, Africa. Our study investigated infection among meso-mammals (raccoons, opossums, coyotes and bobcats) at a single site with endemic transmission of *D. insignis*. Our goal was to identify the host diversity exploited by *D. insignis* in the southeastern United States, but also to explore the role of fish and amphibians in the natural cycle of *D. insignis*. Additionally, we conducted a phylogenetic analysis to evaluate the genetic differences of worms from a variety of hosts. We found that only raccoons and opossums were infected with *D. insignis,* and raccoons had the highest prevalence of infection, 32.1% (38/122). We report the unusual finding of a high prevalence of *D. insignis* infections in opossums (9.9%, 8/81). We also recovered *D. insignis* L3s from two species of amphibians (*R. catesbieana* and *R. sphenocephala*), the first report of such a finding. Genetically, we showed very little difference among adult female worms from raccoons and opossums (based on evaluation of the mitochondrial cytochrome *c* oxidase gene). However, a single worm from an opossum, was phylogenetically distinct from the *D. insignis* clade, and was more closely related to *D. lutrae* from North American river otters. This parasite could potentially be a previously undescribed species, and warrants further study. Ultimately, this work informs on the life-cycle and transmission of *D. insignis* in the southeastern United States, but also highlights the importance of considering wildlife in the maintenance of *D. medinensis* transmission domestic animals and potentially wildlife in Chad, Africa.

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