INVESTIGATING THE ROLE OF TRANSPORT AND PARATENIC HOSTS IN THE TRANSMISSION OF *DRACUNCULUS* SPECIES

by

ERIN KENNEDY BOX

(Under the Direction of Michael J. Yabsley)

ABSTRACT

Dracunculus medinensis (GW) is a parasitic nematode that causes the disease dracunculiasis in humans. Other *Dracunculus* species infect wildlife hosts. GW is transmitted via ingestion of infected copepods (intermediate host). GW is targeted by an eradication program that has decreased cases >99.99% since the 1980s. In Chad, Africa, GW infections in dogs are increasing and pose a challenge to eradication efforts. It is unlikely that animal hosts become infected by directly ingesting copepods; thus, I investigated other infection routes: paratenic and transport hosts. Copepods were readily ingested by fish (34/50 [68%]) and frogs (18/50 [36%]) over 24 hours, supporting their roles as transport or paratenic hosts, respectively. I found that four amphibian, two lizard, and one fish species are susceptible to infection with GW or *Dracunculus insignis* larvae. Infections persisted up to eight months. These findings inform future efforts to eradicate GW and understand *Dracunculus* species transmission to wildlife.

INDEX WORDS: *Dracunculus*, Transmission, Intermediate Host, Paratenic Host, Transport Host, Reservoir Host, Copepod, Amphibian, Fish, Reptile

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DEDICATION

I dedicate this work to my parents, Elizabeth and Craig Box, who have supported me through this and every endeavor in life.

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I would like to thank Michael Yabsley for allowing me to volunteer at SCWDS and for letting me stick around. When I first walked into SCWDS, I had no idea how impactful my time here would be on my academic and professional life. I also want to thank Christopher Cleveland for helping me since my first day at SCWDS and allowing me to work with him on the Guinea worm project. The mentorship and guidance that you both have provided through my undergraduate, young professional, and graduate career has been invaluable. I truly could not have done any of this without you both.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Understanding the life cycles and transmission pathways of parasitic nematodes is important, as many, including those in the genus *Dracunculus*, have a significant impact on the health of humans and animals (Anderson, 2000). Although disease caused by *Dracunculus medinensis* (human Guinea worm) infection, also called dracunculiasis, is not typically lethal, it is painful, can be debilitating, and can lead to the development of secondary bacterial and fungal infections (Muller, 1971; Cairncross et al., 2002).

When the British colonized West Africa in the early 20th century, control efforts were implemented to decrease *D. medinensis* transmission, as dracunculiasis negatively impacted the health of British troops and the supporting labor force (Tayeh et al., 2016). These efforts focused on ensuring access to clean drinking water, thereby ceasing transmission which occurs via ingestion of aquatic copepod intermediate hosts containing *D. medinensis* larvae (Tayeh et al., 2016). Over time, *D. medinensis* infections decreased in many countries as water infrastructures improved (Tayeh et al., 2017). In the 1980s, a global dracunculiasis eradication effort was launched by the United Nations (UN), the World Health Organization (WHO), and the Carter Center (Tayeh et al., 2017). Since 1986, there has been a >99.99% decrease in global case numbers and many countries have been certified free of dracunculiasis (Molyneux and Sankara, 2017). Currently, there is endemic transmission of *D. medinensis* in only five countries (Angola, Chad, Ethiopia, Mali, and South Sudan) (WHO, 2020a).

In 2010, after a decade with no reported cases of *D. medinensis*, and during the process of becoming certified dracunculiasis free, a human case was reported in Chad (Eberhard et al., 2014). In 2012, a *D. medinensis* infection was reported in a peridomestic dog (*Canis lupus familiaris*) from Chad (Eberhard et al., 2014). Reports of dog infections from Chad continued to increase and epidemiological patterns were unlike those seen in previous, human-only outbreaks (Eberhard et al., 2014). Since then, infections have been reported in other animals, although the majority of animal cases are dogs (Eberhard et al., 2014; Cleveland et al., 2019). Animal infections have been reported from Chad (dog, and cat [*Felis catus*]), Ethiopia (olive baboon [*Papio anubis*], leopard [*Panthera pardus*], dog, and cat), Mali (dog), and Angola (dog) (WHO, 2017; WHO, 2020a). The parasites recovered from dogs and other animals were determined to be the same species (*D. medinensis*) that infects humans (Thiele et al., 2018; Durrant et al., 2021).

Current control efforts (e.g. providing water filters, access to bore wells, and chemical treatment of water bodies) have been successful in eradication of Guinea worm from the majority of countries, but may be insufficient to completely eradicate the parasite if dogs or other animals are serving as reservoir hosts (Molyneux and Sankara, 2017). In order to understand how to most effectively interrupt transmission of *D. medinensis* to animals, it is vital to understand how they are becoming infected. Previous research with *D. medinensis* and a related species of *Dracunculus* (*D. insignis*) determined it is possible that alternative transmission pathway(s) may play a role in animal infections (Brandt, 1938; Eberhard et al., 2014; Eberhard et al., 2016a; Cleveland et al., 2017).

It is believed that aquatic animals can serve either as paratenic or transport hosts in the transmission of *Dracunculus* spp. to dogs and other animals. Most of the studies suggesting a

possible role of amphibians in transmission is based on laboratory studies; however, three studies have confirmed natural infections. Eberhard et al. (2016a) recovered a *D. medinensis* L3 from a wild frog (*Phrynobatrachus francisci*) in Chad, confirming that frogs can become infected with *D. medinensis* larvae in the wild. Cleveland et al. (2019) detected additional *D. medinensis* L3s from wild Chadian frogs (*Hoplobatrachus occipitalis* and *P. francisci*). Then, Cleveland et al. (2020) reported the recovery of *D. insignis* L3s from wild frogs (*Lithobates catesbeiana* and *Lithobates sphenocephala*) from Di-Lane Plantation, GA, USA further supporting the possible role of frogs in the life cycle of these parasites (Cleveland et al., 2020). Although there is some experimental and field evidence that amphibians have the capacity to act as paratenic hosts of *Dracunculus* spp., we still do not know how important they are for transmission in the wild. In order to better understand *Dracunculus* transmission to animal definitive hosts, data are needed on these alternative transmission pathways.

To address this knowledge gap, I investigated two specific objectives:

1. To evaluate the rates of copepod ingestion by several species of amphibians (frogs, tadpoles, and newts) and small fish.
    a. Hypothesis: Fish will consume more copepods than amphibians because of general diet preferences and more active feeding behavior.
2. To assess the susceptibility of different species of amphibians, lizards, and fish to infection with *D. insignis* and *D. medinensis* and to assess the long-term persistence of *Dracunculus* spp. larvae in amphibians.
    a. Hypothesis: Amphibians will become most readily infected, and lizards and fish will develop few if any infections.

b. Hypothesis: Larvae will persist for several weeks or longer within infected paratenic hosts.

LITERATURE REVIEW

The genus *Dracunculus*

Dracunculus parasites (Nematoda: Dracunculoidea) are a group of large, subcutaneous nematodes that can infect a diversity of reptile and mammal hosts (Cleveland et al., 2018). Adult females are large, pale, and filiform worms and can be almost a meter long when mature, while males are reported to reach up to four centimeters in length (Figure 1.1) (Cairncross et al., 2002; Cleveland et al., 2018). The greatest number of described species are from reptile hosts which have 10 formally described species. Only four species infect mammals, including the most well-known member of this genus, *D. medinensis* (the human Guinea worm) (Cleveland et al., 2018). *Dracunculus medinensis* primarily infects humans, but is also recovered from other host species (Cairncross et al., 2002; Thiele et al., 2018). Historically, this is the only species confirmed to infect humans, although there is a recent report of a *Dracunculus* sp. from a human in Vietnam (Cleveland et al., 2018; WHO, 2020a).

Humans and *D. medinensis* have a long, intertwined history, with infections recorded as early as 1550 BC in the Ebers Papyrus (Miller, 1989). The Ebers papyrus describes winding an emergent Guinea worm on a stick, a technique which is still used today (Miller, 1989). There are also historical, sporadic reports of what was believed to be *D. medinensis* infections in some animals, including domestic dogs (*Canis lupus familiaris*), cats (*Felis catus*), and baboons (*Papio* spp.) (Muller, 1971). These reports of infections were infrequent and would cease when

the parasite was eradicated from the region, suggesting that animals may not maintain transmission of the parasite (Muller, 1971).

All of the other *Dracunculus* species are parasites of wildlife, although a few of them have also been reported from domestic animals (Cleveland et al., 2018; Williams et al., 2018). In the New World, the most commonly reported species is *Dracunculus insignis,* which has been reported from numerous mammalian hosts in the United States and Canada (Cleveland et al, 2018). Common wildlife hosts include raccoons *(Procyon lotor*), river otters (*Lontra canadensis*), Virginia opossum (*Didelphis virginiana*), minks (*Mustela vison*), and fishers (*Martes pennanti*). Infections have also been reported in domestic dogs and cats in North America (Lucio-Forster et al., 2014; Williams et al., 2018). Another widespread species, *D. lutrae,* is also found in the United States and Canada, but appears to be a host-specialist, infecting only river otters (Crichton and Beverly-Burton, 1973; Elsasser et al., 2009). Recently, using molecular techniques, a novel genotype (or possible species) of *Dracunculu*s was detected in a Virginia opossum from Georgia (USA) (Cleveland et al., 2020). Subsequently, a worm that was genetically similar to this opossum worm was reported in a dog from Toledo, Spain (Diekmann et al., 2020). The only other named *Dracunculus* species in the New World is *D. fuelleborni,* which infects big-eared opossum (*Didelphis aurita*) in Brazil; however, an uncharacterized *Dracunculus* sp. has been reported in several dogs in Argentina (Hoyos et al., 1995; Bono Battistoni et al., 2011).

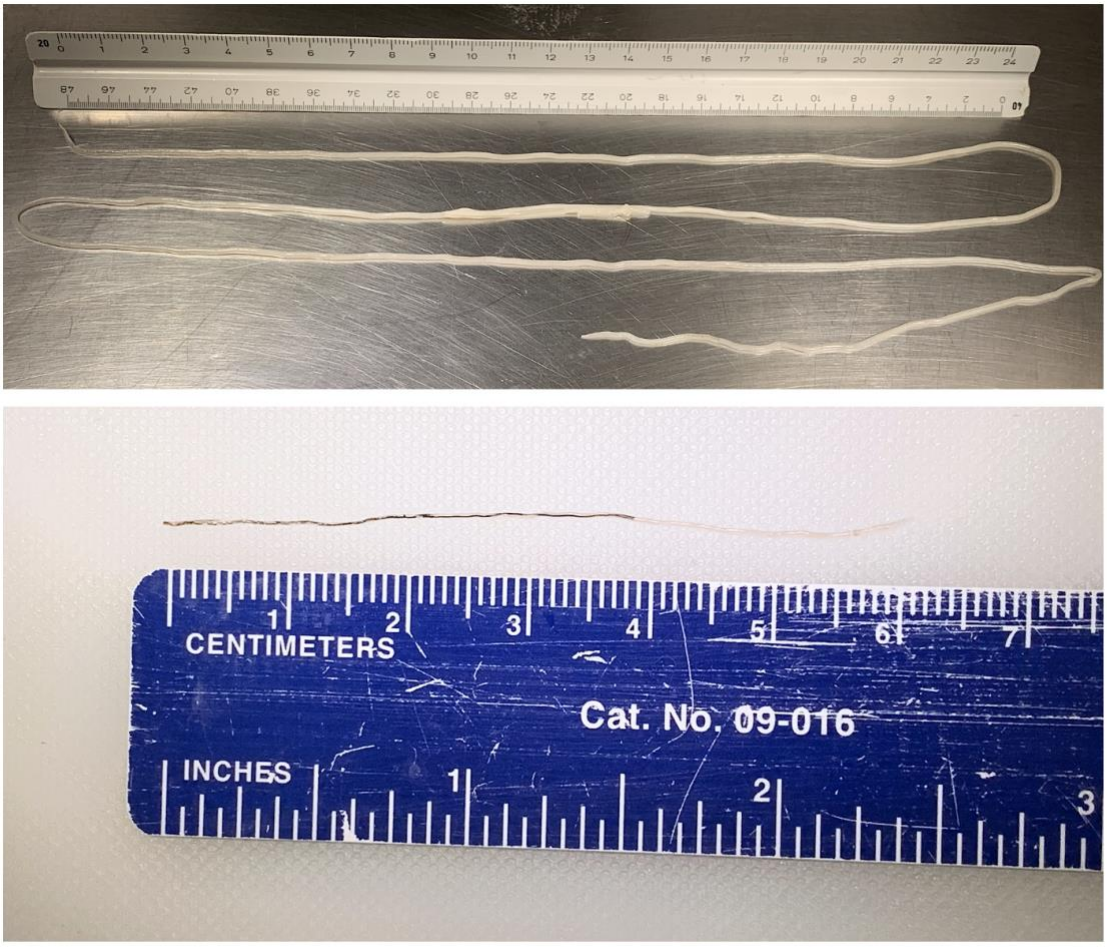

Figure 1.1. An adult female *Dracunculus medinensis* (top) and an adult male *D. medinensis* (bottom) recovered from experimentally infected ferrets. (Images courtesy of K.B. Garrett)

The remainder of the described *Dracunculus* species infect reptiles (Cleveland et al., 2018). Nine species of *Dracunculus* are described from snakes, although the lack of molecular confirmation of recovered males for morphological identification makes an exact determination of species challenging (Cleveland et al., 2018). Most species descriptions of *Dracunculus* from snakes are based on a single recovered male or only female worms (Cleveland et al., 2018). Although numerous *Dracunculus* species are described from snakes in Eurasia, very little

research has been done on these species (Cleveland et al., 2018). It is also likely that there are many additional species yet to be recognized, as sporadic reports are made of *Dracunculus*-like worms emerging from unusual hosts or in unusual locations, and genetic analysis is often required to definitively differentiate *Dracunculus* species (Cleveland et al., 2018; Diekmann et al., 2020; WHO, 2020a). The most thoroughly studied species of *Dracunculus* which infect snakes are *D. oesophageus* (in Europe) and *D. ophidensi*s (in North America) (Brackett, 1938; Wijová et al., 2005; Cleveland et al., 2018). Only one species of *Dracunculus*, *D. globocephalus*, is reported from turtles (Macklin, 1927; Moravec and Little, 2004). *Dracunculus globocephalus* has been recovered from the common snapping turtle (*Chelydra serpentina*) in North America and the South American snapping turtle (*Chelydra acutirostris*) in Costa Rica; although, the Costa Rican case was described from only a single worm (Bursey and Brooks, 2011; Cleveland et al., 2018).

The *Dracunculus* life cycle

The life cycles of the few dracunculids that have been studied are similar (Figure 1.2) (Brackett, 1938; Crichton and Beverley-Burton, 1975; Anderson, 2000). Large, gravid, female *Dracunculus* typically emerge from the lower extremities of an infected definitive mammalian host, although they can emerge from any region (Cairncross et al., 2002). Unlike mammals, reports of emergence and associated lesions are rare in reptile hosts, thus emergence in reptiles is poorly understood (Brackett, 1938). As the worm prepares to emerge, some larvae are released beneath the skin of the infected host (Muller, 1976). These larvae trigger a host immune response, causing a painful blister to form, from which the adult worm will eventually emerge (Muller, 1976). In human dracunculiasis cases, the blister is described as quite painful, causing a burning sensation (Cairncross et al., 2002). If the blister is submerged in water, it will rupture

and the exposed anterior end of the female worm will burst, releasing many thousands of free-swimming first stage (L1) larvae into the water (Muller, 1976; Cairncross et al., 2002). A female worm can contain one to three million larvae which are released over the course of worm emergence (Cairncross et al., 2002; Cleveland et al., 2018). It often takes a few weeks for the worm to fully emerge from the infected host (Cairncross et al., 2002).

Once in the water, free-swimming *Dracunculus* L1s are consumed by cyclopoid copepods, the obligate intermediate host (Cleveland et al., 2018). Numerous copepod species can serve as intermediate hosts for *Dracunculus*; however, which copepod species are involved in the life cycle of specific *Dracunculus* species is not fully known, especially for non-*D. medinensis* species (Cleveland et al., 2018). The *Dracunculus* L1s molt twice and mature to the infectious third stage (L3) inside the copepod (Fedchenko, 1871; Crichton and Beverley-Burton, 1975). Classical transmission to a definitive host occurs when copepods containing infectious L3 larvae are consumed directly by a definitive host via contaminated drinking water (Muller, 1971). Once ingested, the copepods die, releasing the L3s to migrate into the musculature of the thorax or abdomen (Anderson, 2000). Once there, the larvae molt two additional times and begin to grow and mature into adults (Anderson, 2000). Mating also occurs in the thorax or abdomen (Anderson, 2000). After females are fertilized, they migrate into subcutaneous tissues, eventually reaching the location from which they will emerge (Crichton and Beverley-Burton, 1977; Cairncross et al., 2002). Time from infection to parasite emergence is approximately 10-14 months after infection (Cairncross et al., 2002).

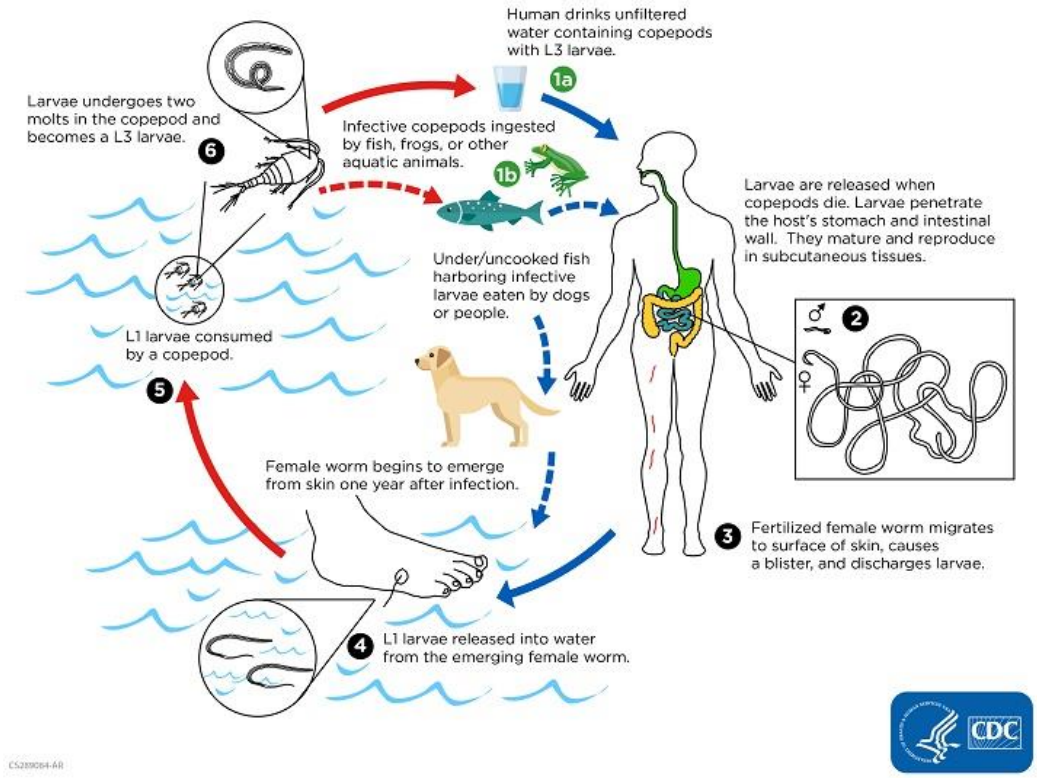

Figure 1.2. The life cycle of *Dracunculus medinensis*. (Image source: CDC)

Significance to human and animal health

Humans

While dracunculiasis is not typically a direct cause of mortality in infected individuals, it does cause significant discomfort and disability (Muller, 1971; Cairncross et al., 2002). The blister from which the worm emerges can remain open for many weeks, as the meter-long emergent worm is wrapped around a stick or other item and very slowly removed (Figure 1.3) (Cairncross et al., 2002). During this time, the patient can be in great pain, may be debilitated, and is at risk for secondary bacterial or fungal infection at the site of the wound (Muller, 1971). If the worm is ruptured during the extraction process, a subcutaneous abscess could form

(Muller, 1979). Additional complications can arise if a gravid female worm migrates to or releases larvae in a location other than the subcutaneous tissues (e.g., in a joint or wrapped around a nerve or tendon) (Muller, 1976). People often become infected with low numbers of worms (typically 1-3), although there are reports that some patients have had dozens of worms emerge (Muller, 1971; Muller, 1979; Ruiz-Tiben et al., 1995). As the site of worm emergence is most often on the foot or lower leg, disability can impede the affected person's ability to work or attend school (Cairncross et al., 2002). Once the worm emerges, most people will recover completely (in about four weeks), although some may take an extended time to recover (10 weeks or more) or experience permanent disability (Muller, 1976).

There are no drugs to prevent or cure dracunculiasis, and prior or current infection does not yield protective immunity against reinfection (Eberhard et al., 1990; Ruiz-Tiben et al., 1995; Ruiz-Tiben and Hopkins, 2006). The historical method of wrapping the emergent worm on a stick to hasten emergence is still common in the management of dracunculiasis cases (Cairncross et al., 2002). Surgical removal is no longer the preferred method of removal due to increased risk of secondary infections and damage caused when trying to remove worms that are deep in the tissue or near sensitive tissues (e.g., nerves, joints, or blood vessels) (Cairncross et al., 2002). Patient care for those suffering from dracunculiasis focuses on pain management and treatment with antibiotics to prevent secondary bacterial infections (Ruiz-Tiben et al., 1995; Ruiz-Tiben and Hopkins, 2006). Anti-inflammatory medications may allow for quicker removal of the worm over a few days versus the typical few weeks it takes to extract a worm (Ruiz-Tiben and Hopkins, 2006).

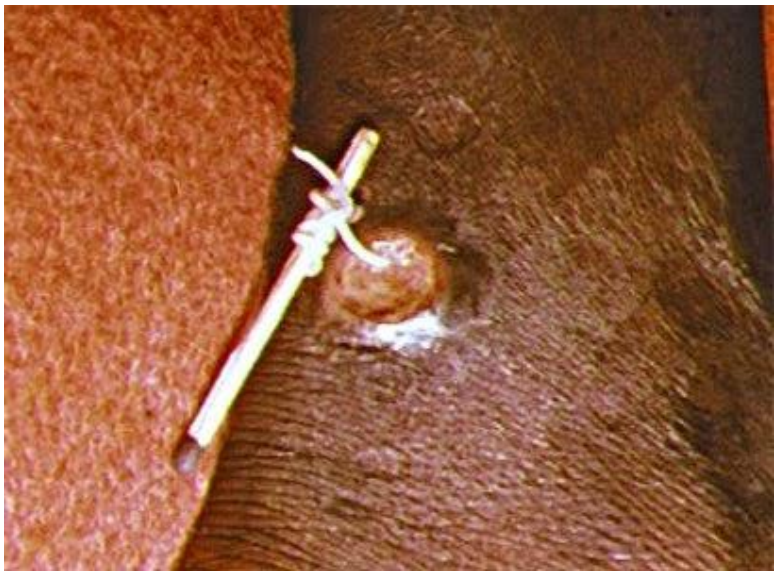

Figure 1.3. Use of a matchstick to carefully remove an adult female *Dracunculus medinensis*. (Image source: CDC)

Domestic animals

Dracunculus insignis nematodes are rarely reported from domestic dogs in North America (Williams et al., 2018). Reported infections of *D. insignis* in cats in North America are even more rare, but do occur (Lucio-Forster et al., 2014; Williams et al., 2018). *Dracunculus medinensis* infections have been reported in domestic and peridomestic dogs and cats throughout the range of the parasite, and reports from dogs are increasingly common in some Sub-Saharan African countries (Figure 1.4) (Eberhard et al., 2014; WHO, 2020a). Suspected *Dracunculus sp.* nematodes have been sporadically recovered from domestic dogs in Argentina, although identification was made from female specimens alone with no molecular confirmation (Hoyos et al., 1995; Bono Battistoni et al., 2011). A *Dracunculus* worm of unknown species (most genetically similar to a worm recovered from a North American Virginia opossum) was recently

recovered from a domestic dog in Spain (Diekmann et al., 2020). *Dracunculus* infections in domestic animals can be misdiagnosed as aberrant subcutaneous emergence of other parasitic nematodes, such as *Dirofilaria immitis* (Williams et al., 2018).

As with human dracunculiasis, there is no drug to treat or prevent *Dracunculus* sp. infection or resulting disease in domestic animals, although preventative anthelmintics continue to be of interest, especially for Sub-Saharan African dogs which become infected with *D. medinensis* (Williams et al., 2018). As long as the entire worm is removed, most *Dracunculus* sp. infections will not have severe or long lasting impacts on animal health (Williams et al., 2018).

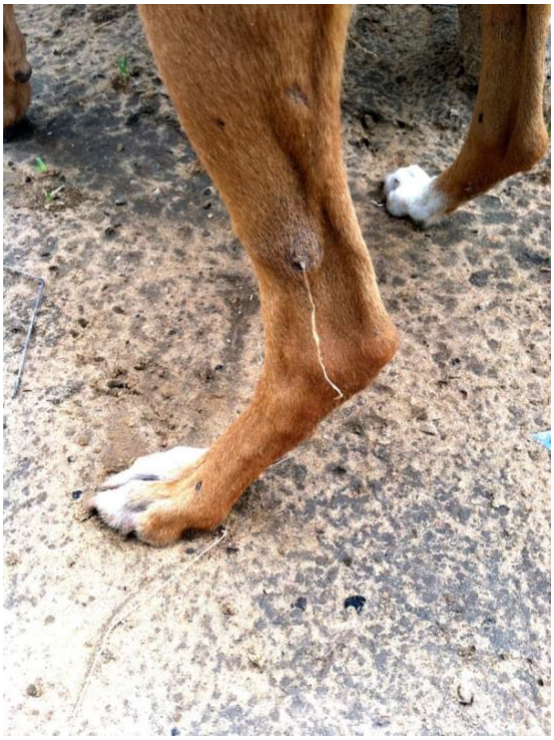

Figure 1.4. *Dracunculus medinensis* worm emerging from a dog in Chad, Africa. (Image source: Eberhard et al., 2014)

Wildlife hosts

Dracunculus infections are rarely noticed in wildlife, and most *Dracunculus* infections found in wild animals are discovered upon necropsy (Alexander et al., 1972; Muller, 1976; Crichton and Beverley-Burton, 1977; Cairncross et al., 2002). Since experimental infection trials with mammal-infecting species of *Dracunculus* often yield a high incidence of infection, it is likely that infection rates in wildlife are far higher than reported (Cairncross et al., 2002). Infected mammals also tend to chew at emerging worms, making detection of the parasite without a necropsy challenging (Muller, 1976). Mammalian carnivores that are infected with *Dracunculus* nematodes appear to experience a similar course of disease to that seen in humans (Crichton and Beverley-Burton, 1975; Muller, 1976). Raccoons infected with *D. insignis* (naturally and experimentally) have been reported to exhibit signs of discomfort (e.g. avoiding bearing weight on the affected limb, swelling of affected limbs, and hair loss and skin damage from scratching at ulcerated lesions), particularly during worm emergence (Crichton and Beverley-Burton, 1977).

Little is known about the impact of *Dracunculus* infection on the health of reptiles (Cleveland et al., 2018). Infection of snakes with gravid female *D. ophidensis* nematodes can be detected in live animals if the worm is coiled below the skin (Brackett, 1938). Wild snakes have been found with cutaneous blisters, under which *D. ophidensis* females were found upon necropsy (Brackett, 1938). Infection with *D. ophidensis* appears to have a minimal impact on the long-term health of the host, as blisters from which the worms emerge heal completely, leaving no trace of previous infection (Brackett, 1938). No disease has been reported in infected turtles, as *D. globocephalus* worms were recovered upon necropsy and females, if present, were not emergent (Mackin, 1927; Moravec and Little, 2004; Bursey and Brooks, 2011).

Control and eradication efforts for human Guinea worm

Despite the challenges posed by the lack of effective drugs or vaccines, as well as the lack of protective immune response, control efforts have been very successful in decreasing cases of human dracunculiasis (Muller, 1979; Tayeh et al., 2017). In the early 20th century, British colonizers in West Africa attempted to decrease *D. medinensis* transmission, because dracunculiasis negatively impacted the health of British troops and their supporting labor force (Tayeh et al., 2016). These early efforts focused on provisioning clean drinking water, thereby ceasing transmission, which was already known to occur via the ingestion of infected copepods (Tayeh et al., 2016). Cases of *D. medinensis* infections decreased in many countries throughout the 20th century, even without specific dracunculiasis control programs, as water infrastructures improved (Tayeh et al., 2017). Still, the parasite persisted in some countries in South Asia and remained especially common in selected regions of Sub-Saharan Africa (Tayeh et al., 2017).

The United Nations included dracunculiasis eradication as an objective in the 1981-1990 clean water decade initiative (Tayeh et al., 2017). Since 1986, the World Health Organization (WHO) and the Carter Center have collaborated to develop a global Guinea Worm Eradication Program (GWEP) to coordinate and provide aid to country GWEPs (Tayeh et al., 2017). Similar to past *D. medinensis* control efforts, this initiative focused on education and providing means to clean drinking water to reduce the transmission of the parasite (Tayeh et al., 2017). The campaign focused mainly on removing infectious copepods by providing filters for drinking water, supporting the building of safer water sources (such as borewells), and killing copepods using chemical treatments such as Abate® (Figure 1.5) (Cairncross et al., 2012; Tayeh et al., 2017).

Figure 1.5. Methods for ensuring the safety of drinking water in the effort to cease the transmission of *D. medinensis*. Top left image demonstrates the use of a LifeStraw® water filter. Top right shows person applying Abate® to a pond. Bottom left image illustrates the use of a classic cloth filter. Bottom right shows a person inspecting a conical, mesh filter. (Images source: The Carter Center)

Overall, *D. medinensis* eradication efforts have been incredibly successful. Since 1986, eradication efforts have resulted in a decrease in human cases by over 99.99% (from 3.5 million cases annually in 1986 to only 53 cases in 2019) (Ruiz-Tiben and Hopkins, 2006; WHO, 2020b). Currently, *D. medinensis* is considered endemic in only five countries (Angola, Chad, Ethiopia,

Mali, and South Sudan), compared to 21 endemic countries in 1986 (WHO, 2020b). Despite this accomplishment, a great challenge still faces the GWEP. As the number of human cases decreased dramatically in recent decades, the number of reported animal *D. medinensis* infections, primarily domestic and peri-domestic dogs, has increased (Eberhard et al., 2014; Eberhard et al., 2016b).

The increase in animal infections is especially notable in Chad, Africa. In 2010, during the dracunculiasis-free precertification process, *D. medinensis* infections were detected in dogs (Eberhard et al., 2014). The epidemiology of these infections was unlike those that had been previously noted in this area (Eberhard et al., 2014). Before this time, dog infections were rarely reported, even in locations where human outbreaks were historically common (Eberhard et al., 2014). In this outbreak, dog cases were more numerous than human cases and had a different geographic and temporal distribution than was historically reported of human cases in the area (Eberhard et al., 2014). Typically, human cases would cluster around a water body that was contaminated the previous year (Eberhard et al., 2014). In contrast, dog infections were not obviously linked to a single water source, suggesting a transmission route other than waterborne (Eberhard et al., 2014).

Animal infections with *D. medinensis* have now been reported from Chad (dog and cat), Ethiopia (baboon, leopard, dog, and cat), Mali (dog), and Angola (dog) (WHO, 2017; WHO, 2020a). Genetic analysis has confirmed that the parasite infecting people and animals in Sub-Saharan Africa is the same species, *D. medinensis* (Thiele et al., 2018; Durrant et al., 2021). These findings support the hypothesis that dogs, and possibly other animals, are serving as reservoir hosts of *D. medinensis* in Chad (Thiele et al., 2018). As humans and animals are becoming infected with the same parasite, understanding how transmission to animal hosts is

occurring is vital to ceasing transmission and achieving the goal of *D. medinensis* eradication. It is important to note that the role of wildlife hosts in the maintenance of this parasite at this time is unknown. Historically, dog cases would stop occurring once the parasite was eradicated from humans in a particular region, so it is unknown if cases in baboons or wild felids would be sustained without transmission among humans or dogs (Eberhard et al., 2014).

Role of paratenic and transport hosts in parasite transmission

Transmission of *Dracunculus* spp. to animal definitive hosts via the direct ingestion of infected copepods has been successful in many laboratory trials (Beverley-Burton and Crichton, 1976; Muller, 1976). However, it is hypothesized that aquatic animals might serve as paratenic or transport hosts in the transmission of *Dracunculus* species to dogs and other wildlife (Eberhard et al., 2014; Cleveland et al., 2018). Generally, a paratenic host is an animal in which parasitic larvae may persist, but do not develop (Anderson, 2000; Eberhard et al., 2016). Paratenic hosts can serve as a bridge host which allows parasites to move more readily between aquatic and terrestrial systems or facilitate transmission up the food chain when a definitive host is unlikely to ingest the intermediate host or infectious stages directly (Mace and Anderson, 1975; Thomas and Ollevier, 1992). The classic definition of a transport host is one on which a parasite merely ‘hitches’ a ride (e.g., contamination of the outside of a fly), although many parasitologists will use the terms paratenic and transport host interchangeably (Graczyk et al., 2000). Throughout this thesis, specifically in regard to *Dracunculus* transmission, I will consider a transport host as a short-term host which, after ingesting *Dracunculus* L3 infected copepods, could transmit the parasite to another host if it is eaten while the larvae are still in the gastrointestinal tract (Cleveland et al., 2017).

Many parasite species include paratenic hosts in their life cycles (Anderson 2000). For example, *Anguillicoloides crassus* (Nematoda: Dracunculoidea), which infects eels (*Anguilla* spp.) in Asia and Europe, uses small fish as paratenic hosts (Thomas and Ollevier, 1992; Anderson 2000). The life cycle of *A. crassus* requires cyclopoid copepods as intermediate hosts, but because adult eels feed primarily on fish and do not intentionally ingest copepods, many species of small fish act as paratenic hosts for the transmission of *A. crassus* to the definitive host (Thomas and Ollevier, 1992). Paratenic hosts are also involved in the transmission of the giant kidney worm (*Dioctophyme renale* [Nematoda: Dioctophymatoidea]) from aquatic intermediate hosts (aquatic oligochaetes) to terrestrial definitive hosts (typically mustelids, although infections commonly occur in other carnivorous mammals) (Mace and Anderson, 1975). Paratenic hosts (fish and frogs) that become infected in the aquatic environment and are then eaten by terrestrial or semi-aquatic hosts and aid in the transmission of this parasite from aquatic intermediate hosts to terrestrial definitive hosts (Mace and Anderson, 1975).

Although the classical mode of *D. medinensis* transmission to humans has been known for over a century, the mode of transmission to animals has mostly been investigated in recent decades and is still not fully understood. As mentioned, many trials have proven that a variety of animals can be successfully infected via the ingestion of *Dracunculus* L3 infected copepods under experimental settings. Despite this, it is deemed unlikely that direct transmission (via copepod ingestion) is the primary mode of natural *Dracunculus* sp. infection in animal hosts, as many of these animal hosts are unlikely to directly ingest enough copepods to become infected with the parasite (Brackett, 1938; Garett et al., 2020). People typically scoop water from a natural source or storage container when they drink, leading to the ingestion of copepods (Cairncross et al., 2002). Animals such as dogs and opossum lap water, and many snake species

suck in water during a drinking event (McManus, 1970; Cundall, 2000; Crompton and Musinsky, 2011). Animals drink water from the top of the water column, while copepods (especially those infected with *Dracunculus* spp.) tend to be lower in the water column (Onabamiro, 1954; Crichton and Beverley-Burton, 1977; Eberhard and Brandt, 1995). The disturbance in the water caused by an animal drinking may also cause copepods to flee from the animal (Eberhard and Brandt, 1995). Garrett et al. (2020) assessed the ability of dogs to ingest copepods while drinking water and found that dogs did consume copepods, although in low numbers, even at high densities in provisioned water containers. Thus, it is unlikely that sustained transmission would occur through the accidental ingestion of infected copepods by suitable definitive hosts. As wild copepods reportedly have a low prevalence of infection, classical transmission would require the ingestion of a large number of copepods (Steib and Myaer, 1988; Garrett et al., 2020). These findings support the hypothesis that aquatic animals which feed on copepods may be important in the transmission of *Dracunculus* spp. to definitive hosts.

Paratenic and transport hosts: Experimental studies with Dracunculus *spp.*

The first experimental infection trials assessing the potential involvement of paratenic hosts in *Dracunculus* transmission was performed by Brackett in 1938. Brackett (1938) conducted experimental trials, investigating the potential for tadpoles to act as paratenic hosts of *D. ophidensis*. Copepods were allowed to feed on *D. ophidensis* larvae and after at least 15 days, the infected copepods were fed to tadpoles (Bracket, 1938). Larvae were recovered from tadpoles two weeks after ingestion of the infected copepods; no larval maturation was noted after ingestion by the tadpoles (Brackett, 1938). Infected tadpoles were then fed to two garter snakes and a water snake (*Nerodia sipedon*) (Brackett, 1938). Upon necropsy four months later, one garter snake and the water snake were found to be infected with adult *D. ophidensis*, the first

confirmation that tadpoles could act as paratenic hosts in the transmission of any *Dracunculus* species (Brackett, 1938).

In a 1977 study by Crichton and Beverly-Burton, tadpoles, frogs, fish, and crayfish were experimentally infected with *D. insignis* L3s. *Dracunculus insignis* L3s were recovered from tadpoles (*Lithobates pipiens*), frogs (*L. pipiens* and *Lithobates clamitans*), and fish (*Catostomus commersonii* and *Oncorhynchus mykiss*) seven to 37 days post-inoculation (Crichton and Beverley-Burton, 1977). The larva recovery rate from infected frogs reached as high as 80-90%, whereas the larva recovery rate from fish was much lower, at only 0.6- 2.0% (Crichton and Beverley-Burton, 1977). No larvae were recovered from *D. insignis* inoculated crayfish (Crichton and Beverley-Burton, 1977). Larvae recovered from paratenic hosts were significantly larger than those recovered directly from infected copepods, although they were still L3s (Crichton and Beverley-Burton, 1977). A raccoon was successfully infected with *D. insignis* when fed L3s recovered from experimentally infected frogs (Crichton and Beverley-Burton, 1977).

Experimental infections of tadpoles (*Lithobates* sp. and *Xenopus laevis*) were also successfully performed by Eberhard and Brandt in 1995. In these trials, the tadpoles were offered copepods infected with *D. insignis* L3s (Eberhard and Brandt, 1995). During this study, researchers found that early Gosner stage tadpoles (those without limb buds) had difficulty ingesting copepods, while later Gosner stages would ingest copepods readily (Eberhard and Brandt, 1995). *Dracunculus insignis* L3s were recovered from the muscle tissues of both species of tadpoles and were found to persist in the tissues of *X. laevis* through metamorphosis, up to 30 days (Figure 1.6) (Eberhard and Brandt, 1995). The viability of the larvae recovered from these tadpoles and frogs was assessed by feeding the infected amphibians to ferrets, from which a

mature *D. insignis* was later recovered, confirming again that tadpoles can successfully serve as experimental paratenic hosts in *D. insignis* transmission (Eberhard and Brant, 1995).

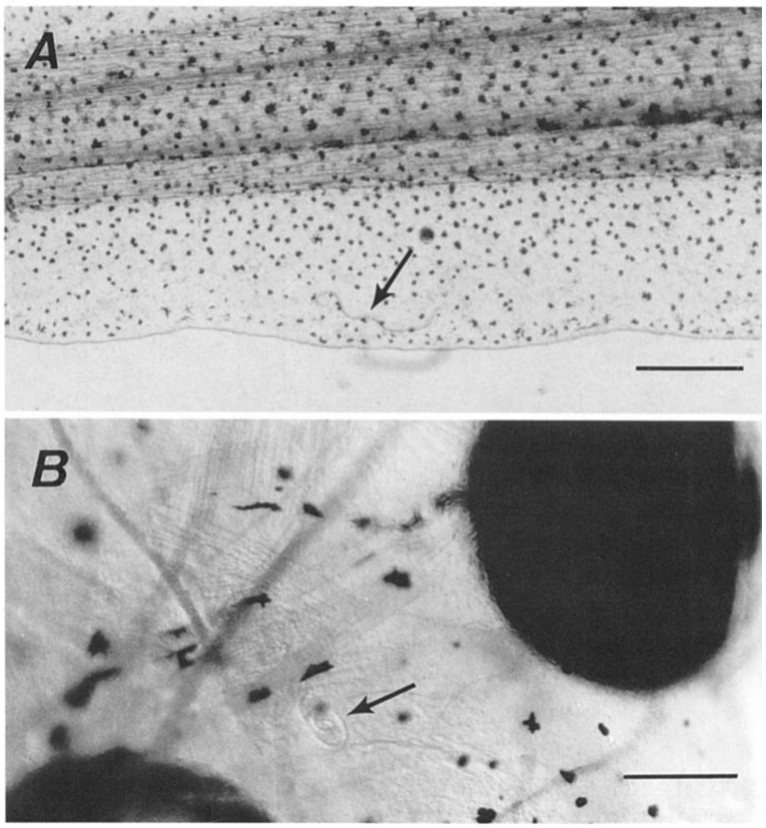

Figure 1.6. *Dracunculus insignis* L3s in the tail (A) and head (B) of experimentally infected *Xenopus laevis* tadpoles. (Image source: Eberhard and Brandt, 1995)

In Eberhard et al. (2016c), *D. medinensis* L3 infected copepods were fed to tadpoles (*Anaxyrus fowleri* and *L. clamitans*) and fish (*Oreochromis niloticus* and *Pimephalis promelas*). One week after this exposure, animals were necropsied and examined for L3s (Eberhard et al., 2016c). Larvae were recovered from four of seven *L. clamitans* tadpoles; it was noted that these

L3s were slightly larger and more active than the L3s recovered directly from infected copepods (Eberhard et al., 2016c). No larvae were recovered from experimentally exposed *A. fowleri* tadpoles or fish during this study (Eberhard et al., 2016c). After examination for larvae, tadpoles and fish were fed to ferrets. Upon necropsy at 70–83 days post-exposure, *D. medinensis* worms were recovered from the ferret which was fed tadpoles, while none were recovered from the ferret which was fed fish (Eberhard et al., 2016c).

Collectively, these studies confirm that amphibians can experimentally function as paratenic hosts of at least three *Dracunculus* species and suggest that fish are poor paratenic hosts of these parasites. Despite being poor experimental paratenic hosts, fish were still suspected of involvement in *Dracunculus* transmission due to the association of fishing villages and *D. medinensis* infections in Chadian dogs (Eberhard et al., 2014; Cleveland et al., 2017; Richards et al., 2020). Thus, the potential for fish to serve as transport hosts in the transmission of *Dracunculus* species was explored (Cleveland et al., 2017). Fish were fed copepods containing *D. medinensis* or *D. insignis* L3s; after ingestion of copepods, fish were euthanized and immediately fed to ferrets (Cleveland et al., 2017). All fish species (*Gambusia affinis, O. niloticus*, and *P. promelas*) were able to successfully infect ferrets, and both *D. medinensis* and *D. insignis* worms were recovered from ferrets (Cleveland et al., 2017). This finding supports the hypothesis that fish may be involved in *Dracunculus* transmission, although, experimentally they are more effective as transport hosts than as paratenic hosts. The role of fish as transport hosts of *D. medinensis* is crucial to investigate further, as potentially infective raw fish and fish entrails are a major food source for Chadian dogs where the majority of *D. medinensis* outbreaks still occur (Eberhard et al., 2014; Guagliardo et al., 2020; Richards et al., 2020). Burying of fish entrails is encouraged by the GWEP, aiming to eliminate them as a potential source of infection

of dogs (Hopkins et al., 2015). This intervention strategy is still being implemented, although efficacy is not known, as the role of wild fish in *D. medinensis* transmission to dogs has not been confirmed (Cleveland et al., 2019; WHO, 2020a).

Paratenic and transport hosts: Field studies with Dracunculus *spp.*

Given the success of experimental trials utilizing amphibian paratenic hosts, an increased effort was made to survey wild aquatic animals (amphibians and fish) for the presence of *Dracunculus* larvae. Eberhard et al. (2016a) surveyed 88 wild frogs in Chad for *D. medinensis* larvae in the viscera and muscle tissues. A single L3 was recovered from a single frog (*Phrynobatrachus francisci*) (Eberhard et al., 2016a). Cleveland et al. (2019) then surveyed wild fish, frogs, and reptiles in Chad for the presence of *D. medinensis* larvae. Animal tissue was screened for larvae after the removal of gastrointestinal (GI) tissue and GI contents. No larvae were found in the 234 fish (21 species) or four reptiles (two species) which were sampled; although, of the 276 amphibians (six species) sampled, four (1.4%; two *Hoplobatrachus occipitalis* and two *P. francisci*) were found to contain *D. medinensis* L3s (Cleveland et al., 2019). Infection intensity ranged from one to three larvae per infected individual (Cleveland et al., 2019).

Cleveland et al. (2020) later surveyed amphibians and fish from Di-Lane Plantation, GA, USA, for the presence of *D. insignis* larvae; methods used were similar to those in Cleveland et al., 2019. No *D. insignis* larvae were recovered from the 68 fish that were assessed (*Centrarchus macropterus*); however, of the 68 frogs (five species) sampled, *D. insignis* larvae were recovered from 11 (16.2%; six *Lithobates catesbeiana* and five *Lithobates sphenocephala*) (Cleveland et al., 2020). The intensity of *D. insignis* infection in these animals ranged from one to 45 larvae per individual, with a mean of 1.6 (Cleveland et al., 2020). Combined analysis of these findings

supports the hypothesis that amphibians may play some role as paratenic hosts in *Dracunculus* transmission to wildlife hosts. However, the significance of that role may vary by amphibian species and parasite species.

Conclusion and knowledge gaps

Current research supports the hypothesis that paratenic and transport hosts likely play some role in the transmission of *Dracunculus* nematodes to definitive animal hosts. However, many unknowns remain, especially an appreciation of the relative importance of these transmission routes (use of paratenic and transport hosts) to *Dracunculus* transmission. Addressing these knowledge gaps will help to develop a better understanding of *Dracunculus* transmission to human and animal hosts and will help GWEP managers to develop methods for controlling *D. medinensis* transmission more effectively.

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CHAPTER 2

COPEPOD CONSUMPTION BY AMPHIBIANS AND FISH AND IMPLICATIONS FOR TRANSMISSION OF *DRACUNCULUS* SPECIES.[1]

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[1]Box, E.K., Cleveland, C.A., Garrett, K.B., Grunert, R.K., Hutchins, K., Majewska, A.A., Thompson, A.T., Wyckoff, S.T., Ehlers, C., Yabsley, M.J. To be submitted to the *International Journal of Parasitology: Parasites and Wildlife.*

ABSTRACT

Nematodes in the genus *Dracunculus* are parasitic and have a complex life cycle (requiring more than one host species). The most well-known *Dracunculus* species, *D. medinensis*, is the causative agent of Guinea worm disease (dracunculiasis). All other confirmed *Dracunculus* species infect non-human animals, primarily wildlife (reptiles and mammals). The classic route of *D. medinensis* transmission to humans is through the ingestion of water containing the intermediate host, a cyclopoid copepod, infected with third-stage larvae (L3s). However, many animal host species (e.g., terrestrial snakes, dogs) are unlikely to ingest a large number of copepods while drinking; therefore, alternative routes of infection (e.g., paratenic or transport hosts) may be responsible for *Dracunculus* transmission to these species. To better understand the role of paratenic and transport hosts in *Dracunculus* transmission to animal definitive hosts, we compared copepod ingestion between aquatic animal species which may serve as paratenic or transport hosts (fish, frogs [tadpoles and adults], and newts). We hypothesized that fish would consume more copepods than amphibians. Our findings confirm that African clawed frogs and fish consume copepods, but that fish ingest significantly higher numbers (34/50 [68%]) than adult African clawed frogs (18/50 [36%]) during the same 24-hour time period. Our results suggest that amphibians and fish have the potential to play a significant role in the transmission of *Dracunculus* to definitive hosts. Further research is required to determine whether, in the wild, fish or frogs are performing as paratenic or transport hosts, thus increasing *Dracunculus* transmission, or whether they may perform simply as dead-end hosts or as means of copepod population control, thus decreasing *Dracunculus* transmission.

1. INTRODUCTION

Dracunculus parasites are a group of subcutaneous nematodes that can infect a diversity of reptiles and mammals (Cleveland et al., 2018). The transmission of *Dracunculus* nematodes to the definitive host occurs through the ingestion of intermediate hosts (cyclopoid copepods) harboring infective third stage larvae (L3s) (Fedchenko, 1870). Copepods become infected when they ingest first stage *Dracunculus* larvae (L1s) that are released into water bodies by gravid female worms that have emerged from vertebrate hosts (Muller, 1971; Cleveland et al., 2018).

The most well-studied species in this genus, *D. medinensis* (the human Guinea worm), causes significant morbidity in patients in the remaining endemic regions of Sub-Saharan Africa (Cairncross et al., 2002). Although eradication efforts have been extremely successful in decreasing the number of human cases (>99.99% in the 1980s to only 54 cases in 2019), some countries are experiencing increasing numbers of *D. medinensis* infections in animals (primarily, dogs [*Canis lupus familiaris*] and cats [*Felis catus*] in Chad, and baboons [*Papio anubis*] in Ethiopia), with the highest increase seen from in dogs from Chad (Molyneux and Sankara, 2017; Cleveland et al., 2019). In 2019, Chad reported only 48 human cases but there were 1,927 infected dogs and 46 infected cats (WHO, 2020a). Despite years of increasing cases in dogs and cats, as of July 2020, the total number of Guinea worm infections in dogs are dramatically lower than at the same point in 2019 (for January-July, only 1,143 infections in 2020 compared to 1,563 in 2019), and human case numbers have also dramatically decreased (for January-July, only nine compared to 38 in 2019) (WHO, 2020b). This may be due to increased numbers of interventions aimed at decreasing transmission among dogs (Cleveland et al., 2019). The current epidemiological patterns of Guinea worm infections among dogs in Chad do not support transmission occurring via the classical route, i.e. drinking water containing infected copepods

(Eberhard et al., 2014). Furthermore, a recent study investigating copepod ingestion by domestic dogs during a drinking event, determined that dogs consume low numbers of copepods when lapping water and that the numbers of copepods consumed were unlikely high enough to maintain currently reported levels of Guinea worm transmission among dogs in Chad (Garrett et al., 2020).

In order to attain Guinea worm eradication, it is necessary to investigate alternative *Dracunculus* transmission routes, in particular, the possible role of aquatic animals as paratenic hosts (an animal in which larvae may persist, but do not develop) or short-term transport hosts (an animal which, after ingesting *Dracunculus* L3 infected copepods, could transmit the parasite if eaten while the larvae are still in the gastrointestinal tract) (Eberhard et al., 2016b; Cleveland et al., 2017). Studies have shown that tadpoles are experimentally susceptible to infection with *D. insignis* (a parasite of many mammal hosts in North America) and *D. ophidensis* (a parasite of garter snakes [*Thamnophis sirtalis*]) in North America (Brackett, 1938; Crichton and Beverley-Burton, 1977; Eberhard et al., 1995). Experimental trials have confirmed the susceptibility of tadpoles to infection with *D. insignis* and *D. medinensis* larvae (Eberhard et al., 2016b; Cleveland et al., 2017). It has also been shown that anurans can retain infections with *Dracunculus* larvae through metamorphosis and that transmission can occur when infected amphibians are ingested by an appropriate definitive host (Eberhard et al., 1995; Eberhard et al., 2016b). Natural infections of amphibians with *D. insignis* and *D. medinensis* L3s have only recently been confirmed (Eberhard et al., 2016a; Cleveland et al., 2019; Cleveland et al., 2020).

Dracunculus insignis larvae have been recovered from experimentally infected fish, although the larval recovery rate was low (0.6- 2.0%) (Crichton and Beverley-Burton 1977). However, the experimental transmission of *D. insignis* and *D. medinensis* using fish as short-

term transport hosts was successful (Cleveland et al., 2017). These findings supported the continued investigation into the role of fish in *Dracunculus* transmission, despite their apparent inability to serve as paratenic hosts.

Although there is experimental evidence that frogs may serve as paratenic hosts and fish may serve as transport hosts, the importance of different host species in the *Dracunculus* life cycle are unknown; a better understanding may be gained from directly comparing copepod consumption by these animals. We evaluated the rates of copepod ingestion by several species of amphibians (frogs, tadpoles, and newts) and small fish. We hypothesized that fish would consume more copepods than amphibians because of general diet preferences and more active feeding behavior (Piasecki et al., 2004; Ibrahim et al., 2015; Ocock et al., 2019). Developing a better understanding of copepod consumption by these potential host species is important, as it may offer insight into *Dracunculus* transmission dynamics, as well as inform future research pertaining to Guinea worm eradication.

2. MATERIALS AND METHODS

2.1. Copepods

Copepods used in this study were from lab-reared colonies of *Macrocyclops* species. Species identification was determined through morphology and analysis of the partial cytochrome *c* oxidase 1 (COI) gene (Pennak, 1963; Folmer et al., 1994). Wild-caught copepods were obtained from ponds in Athens, Georgia, USA, and reared and maintained at the University of Georgia's Aquaculture Biotech Environmental Lab (ABEL) in Athens, Georgia.

2.2. Study Animals (amphibians and fish)

Five species of fish, ranging in size from three to 12 cm in length, were included in the study: channel catfish [*Ictalurus punctatus*], Congo tetra [*Phenacogrammus interruptus*], mosquitofish [*Gambusia affinis*], featherfin catfish [*Synodontis eupterus*], and Nile and blue hybrid tilapia [*Oreochromis aureus* x *Oreochromis niloticus*]). Seven species of tadpoles were included in this study: (American bullfrog [*Lithobates* (*Rana*) *catesbianus*], Fowler's toad [*Anaxyrus* (*Bufo*) *fowleri*], green frog [*Lithobates* (*Rana*) *clamitans*], pickerel frog [*Lithobates* (*Rana*) *palustris*], wood frog [*Lithobates* (Rana) *sylvaticus*], Cope's gray treefrog [*Hyla chrysoscelis*], and African clawed frog [*Xenopus laevis*]). Adult African clawed frogs and one species of adult newt (Eastern red-spotted newt [*Notophthalmus viridescens*]) were also included in this study. Some fish and amphibian species were selected because they (or closely related species) are found in Chad, Africa and may play a role in *D. medinensis* transmission, while other species were selected because they are native to North America and potentially relevant to the transmission of native North American *Dracunculus* species such as *D. insignis*, *D. ophidensis*, or *D. lutrae*.

2.3. Trial Setup

Individual feeding trials were conducted in 2-liter transparent plastic tanks filled with one liter of dechlorinated water at 23°C and outfitted with an oxygenating bubbler in a temperature-controlled room (water temperature: mean= 23°C, SD= 2.27, SE= 0.15). Fifty copepods were added to each tank, representing the average copepod density in bodies of water in Chad (Garrett et al., 2020). Copepods were allowed to acclimate and disperse for five minutes before adding the aquatic animal to be tested. Trials were conducted using a 12-hour day/night light cycle. A total of 305 trial replicates were conducted.

Total length was measured for fish and Gosner stage was determined for tadpoles (Gosner stage is a more accurate measurement than length for tadpoles across different species) before the individual was added to the trial container. One animal was tested per trial container, and time and water temperature were recorded at the beginning and end of each trial.

Each trial was conducted for 24 hours, after which the animal being tested was removed using a large-holed net (to avoid removing copepods). The net and animal were thoroughly rinsed with dechlorinated water to ensure no copepods were inadvertently removed. The rinse water was returned to the trial tank so that any copepods rinsed from animals or nets would be included in the count. Water containing copepods from the feeding trial was poured through a 100-micrometer filter. The tank was rinsed using dechlorinated water to ensure no copepods remained. Finally, copepods were rinsed from the filter into a petri dish and enumerated. Control trials were run with the same methods, but no animal was added to the container.

2.4. Statistical Analyses

Statistical analyses were conducted in R (R Core Team, 2019). We fit an analysis of variance model using the function *aov* (R package "stats" [Chambers at al., 1992]) to determine whether the number of copepods consumed differed between animal types (fish, tadpole, adult African clawed frog, or newt). For all analyses of copepods lost, we included initial water temperature given the potential effects of temperature on fish, amphibian, or copepod activity level. Further, we chose to include initial temperature as opposed to end or average temperatures, because preliminary analyses indicated that all three were highly correlated (Pearson's correlation >0.7) and showed quantitatively similar results.

Next, we examined fish and tadpoles separately. Specifically, we fit separate analysis of variance models to assess whether species of fish and tadpoles differed in the number of

copepods lost. In analyses of fish species, in addition to initial water temperature, we included the length of the fish to account for any potential effects of animal size. In tadpole species analyses, we included initial temperature as well as Gosner stage to account for potential differences between tadpole stages. We employed Tukey post-hoc contrasts (R *multcomp* package) to determine which animal types or species differed from the others.

Ethical approval and informed consent

All animal collections, housing, and experiments were reviewed and approved by the University of Georgia's Institutional Animal Care and Use Committee (A2018 01-010).

3. RESULTS

3.1 Copepods consumed by species

Fish consumed the most copepods during trials. An average of 34 of 50 (68%) copepods were consumed in fish trials compared to African clawed frogs (36% [18/50]), tadpoles (16% [8/50]), and newt trials (17% [8/50]). A small number of copepods were lost in control trials (11% (5/50)).

3.2 Statistical analysis of results

Animal type (fish, tadpole, African clawed frog, or newt) significantly impacted mean copepods consumed, as did water temperature; although, the correlation between water temperature and copepods consumed was very weak (Pearson's correlation <0.08) (Figure 2.1; Table 2.1). Tukey post-hoc contrasts indicated that fish and African clawed frogs consumed statistically different numbers of copepods from each other, as well as from tadpoles and newts ($p<.05$) (Figure 2.1). There was no significant difference in copepod consumption between tadpoles and newts or copepod loss during control trials ($p>.05$) (Figure 2.1).

When comparing copepods consumed by fish, fish species was significant, while water temperature and fish size were not significant (Table 2.2; Figure 2.2). Tukey post-hoc contrasts indicated that all fish species consumed similar numbers of copepods, except Congo tetra which consumed fewer copepods than all other fish species ($p<0.05$).

When comparing copepods consumed by animal types, there was no significant difference in copepods consumed by tadpole species, Gosner stage, or water temperature (Table 2.3; Figure 2.3). Tukey post-hoc contrasts indicated no significant differences in the number of copepods consumed by tadpole species ($p>0.05$) (Figure 2.3).

3. DISCUSSION

The objective of this study was to determine copepod ingestion by several species of amphibians and fish in order to better understand their potential roles in the transmission of *Dracunculus* species. Our data indicate that both fish and adult African clawed frogs consume high numbers of copepods under experimental conditions. Temperature had a negligible impact on copepods eaten during trials.

The most important consumers of copepods in this study were fish, as all species tested ingested a significant number of copepods (on average 34 of 50 [68%]). It has been demonstrated that some fish species (rainbow trout [*Oncorhynchus mykiss*], common shiner [*Notropis cornutus*]) may become infected with *Dracunculus* L3s after exposure to infected copepods (Crichton and Beverley-Burton 1977). However, experimentally, fish are more successful in performing as transport hosts than as paratenic hosts (Eberhard et al., 2016b; Cleveland et al., 2017). As of yet, no *Dracunculus* larvae have been recovered from wild fish (Cleveland et al., 2019; Cleveland et al., 2020). In previous searches for *Dracunculus* larvae in fish, only the

muscle was examined for the presence of larvae; therefore, future studies may benefit from examining the gastrointestinal contents of fish to further evaluate their potential to perform as transport hosts in the wild (Cleveland et al., 2019).

Several species of small fish consumed large numbers of copepods in this study, which provides further support that small fish may play a significant role in the transmission of *D. medinensis* to dogs in Chad. After the re-emergence of *D. medinensis* in Chad, it was initially noted that most villages that had high Guinea worm transmission were fishing villages where small fish and fish guts were fed to or scavenged by dogs (Eberhard et al., 2014; Richards et al., 2020). Because fish consumed high numbers of copepods during this 24-hour trial period, it is likely that they would contain high numbers of copepods in their gastrointestinal tract after feeding (which could concentrate *Dracunculus* larvae inside the fish if those copepods were infected), thus posing as a potential infection risk to the dogs or other predators of fish.

No tadpole species consumed significantly more copepods than were lost in control trials, suggesting that copepod consumption by tadpoles is very low. However, previous studies have shown that many species of tadpoles can ingest sufficient numbers of *Dracunculus*-infected copepods to become infected with *D. medinensis*, *D. insignis*, and *D. ophidensis* during infection trials (Brackett, 1938; Eberhard et al., 2016a; in preparation). In a study by Eberhard et al. (2016a) it was noted that unlike fish, which consumed all copepods that were offered within 24 hours, tadpoles did not ingest all offered copepods even after three days. Another study found that early Gosner stage tadpoles (those without limb buds) had difficulty in ingesting copepods, while later Gosner stages would ingest copepods readily (Eberhard and Brandt, 1995). We, however, found no differences in copepod ingestion rates by different Gosner stages.

Dracunculus L3s from infected tadpoles have been shown to successfully infect definitive hosts in the laboratory and natural *D. medinensis* infections have been found in a low percentage (1.4% [4/276]) of wild frogs in Chad (Brackett, 1938; Eberhard et al., 1995; Eberhard et al., 2016a; Cleveland et al., 2019). In North America, *D. insignis* infections were reported from a higher percentage of wild frogs surveyed in Di-Lane Plantation, Waynesboro, GA, USA (16.2% [11/68]) Cleveland et al., 2020). Our findings indicate that tadpoles do not consume copepods readily under laboratory conditions within 24 hours. If this low copepod ingestion rate holds true in the wild, it may explain the low prevalence of *D. medinensis* infections in wild frogs (Cleveland et al., 2019). However, the higher prevalence of *D. insignis* infection found in wild frogs by Cleveland et al. (2020) suggests potential differences, regarding the role of paratenic hosts in transmission, between species of anurans or *Dracunculus* nematodes.

Adult African clawed frogs are fully aquatic at all life stages. Therefore, their copepod ingestion rates are unique to animals with this life history and would not be applicable to adult terrestrial frogs. It is also unlikely that adult African clawed frogs would lead to high numbers of infections in dogs or other terrestrial mammals, as aquatic African clawed frogs are not readily accessible prey to these animals. Although several *Xenopus* species are harvested and eaten by people in West Africa, it does not appear they are consumed in high numbers in Chad (Mallon et al., 2015; Cleveland et al., 2019). It is, however, possible that aquatic frog species could play a more substantial role in the transmission of *Dracunculus* spp. to aquatic definitive hosts (e.g., *D. lutrae* to North American river otters [*Lontra canadensis*]).

We found that adult Eastern red-spotted newts did not consume a significant number of copepods, although it is well known that larval Eastern red-spotted newts, as well as larval salamanders, can rely heavily on cyclopoid copepods as a food source and adult Eastern red-

spotted newts also can ingest copepods (Brophy, 1980; Jarroll, 1980). Newts are hosts for *Sprioxys* sp. nematodes and *Bothriocephalus rarus* cestodes, both of which use cyclopoid copepods as intermediate hosts. Therefore, at some point in the newt life cycle, sufficient numbers of copepods are ingested to allow for parasite transmission (Jarroll, 1980). Our use of adult newts, which do not consume as many copepods as larval newts, may have led to an incorrect assumption that newts are not involved in *Dracunculus* life cycles, in particular, that of *D. insignis* (Cleveland et al., 2018). Additional insight into the role these animals may play in *Dracunculus* transmission could be gained by comparing copepod consumption across different age or life-stage groups and other salamander species.

One potential limitation of this study is the copepod recounting method. We recorded an average loss of five out of 50 (11%) copepods in control trials. This loss may be attributed to the cannibalistic behavior of adult copepods or copepods that may have become stuck in the 100-micrometer filter used for removing copepods from water (Toscano et al., 2016). Another potential limitation is that our 24-hour trials may not have been long enough for the tadpoles to ingest a significant number of copepods, regardless of species or Gosner stage. It is also possible that the altered behavior of *Dracunculus*-infected copepods (infectious copepods may become more sluggish and remain lower in the water column) would make them easier for tadpoles to ingest compared to the uninfected copepods which were used in this study (Onabamiro, 1954). In the wild, other more effective predators of copepods (such as fish) may also eat these sluggish, infectious copepods before they could be consumed by the less voracious tadpoles, resulting in fish eating more of the infected copepods on the landscape.

We also only tested small fish (3-12 cm), and it is likely that copepod ingestion would vary between different sizes of fish. For example, researchers have found that Nile tilapia feed

on copepods as juveniles, but not as adults (Ibrahim et al., 2015). Therefore, our results only apply to the sizes and ages of the specific fish species that we included in our trials. Further research may be conducted to determine to what degree differences in size or age may impact copepod consumption by different species of fish. We also only tested a limited number of species and may have been able to draw broader conclusions if a greater diversity of animal species were tested. Despite these potential limitations, we believe this work provides relevant and essential information on the potential for *Dracunculus* transmission by paratenic or transport hosts.

In conclusion, we found that African clawed frogs and several species of small fish ingest copepods, with fish being the major consumer of copepods in the study. The significance of various species as paratenic and transport hosts in the transmission of *Dracunculus* spp. would vary depending on multiple factors (e.g., the diet of the paratenic, transport, and definitive host). For example, with *D. insignis*, some wildlife species (e.g., Virginia opossum [*Didelphis virginiana*]) are more likely to consume terrestrial prey (frogs), whereas others (e.g., North American river otters) may consume more aquatic prey (fish, tadpoles, and aquatic newts) (Schoonover and Marshall 1951; Hart et al., 2019). Even though fish and aquatic frogs may consume large numbers of copepods, if a species is not a viable paratenic or transport host (or even if it is a viable transport or paratenic host but is not a common prey item for definitive hosts of *Dracunculus* spp.), it is possible that ingestion of copepods could actually contribute to decreased transmission overall. Further efforts to understand the interactions between definitive hosts and potential paratenic or transport hosts may elucidate transmission routes of these parasite species to relevant definitive hosts. This research contributes to the understanding of *Dracunculus* transmission by highlighting that fish and aquatic frogs are voracious consumers of

copepods and that these feeding habits may have an impact, although that impact is yet unknown, on *Dracunculus* transmission.

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Table 2.1. Fixed-Effects ANOVA results for copepods consumed by animal type.

Predictor	Sum of Squares	*df*	Mean Square	*F*	*p*	partial η^2	partial η^2 90% CI [LL, UL]
(Intercept)	5268.01		5268.01	77.16	.000		
Animal type	40043.71	4	10010.93	146.62	.000	.68	[.63, .71]
Start temperature (°C)	717.39	1	717.39	10.51	.001	.04	[.01, .08]
Error	18981.31	278	68.28				

Note. LL and UL represent the lower-limit and upper-limit of the partial η^2 confidence interval, respectively.

Table 2.2. Fixed-Effects ANOVA results for copepods consumed by fish species.

Predictor	Sum of Squares	*df*	Mean Square	*F*	*p*	partial η^2	partial η^2 90% CI [LL, UL]
(Intercept)	1317.27	1	1317.27	11.81	.001		
Fish species	3617.89	4	904.47	8.11	.000	.34	[.15, .44]
Fish length (mm)	248.32	1	248.32	2.23	.141	.03	[.00, .13]
Start temperature (°C)	248.88	1	248.88	2.23	.140	.03	[.00, .13]
Error	6913.84	62	111.51				

Note. LL and UL represent the lower-limit and upper-limit of the partial η^2 confidence interval, respectively.

Table 2.3. Fixed-Effects ANOVA results for copepods consumed by tadpole species.

Predictor	Sum of Squares	*df*	Mean Square	*F*	*p*	partial η^2	partial η^2 90% CI [LL, UL]
(Intercept)	42.91	1	42.91	1.41	.237		
Tadpole species	147.68	6	24.61	0.81	.564	.04	[.00, .06]
Start temperature (°C)	4.38	1	4.38	0.14	.705	.00	[.00, .03]
Gosner stage	28.15	1	28.15	0.93	.338	.01	[.00, .05]
Error	3982.37	131	30.40				

Note. LL and UL represent the lower-limit and upper-limit of the partial η^2 confidence interval, respectively.

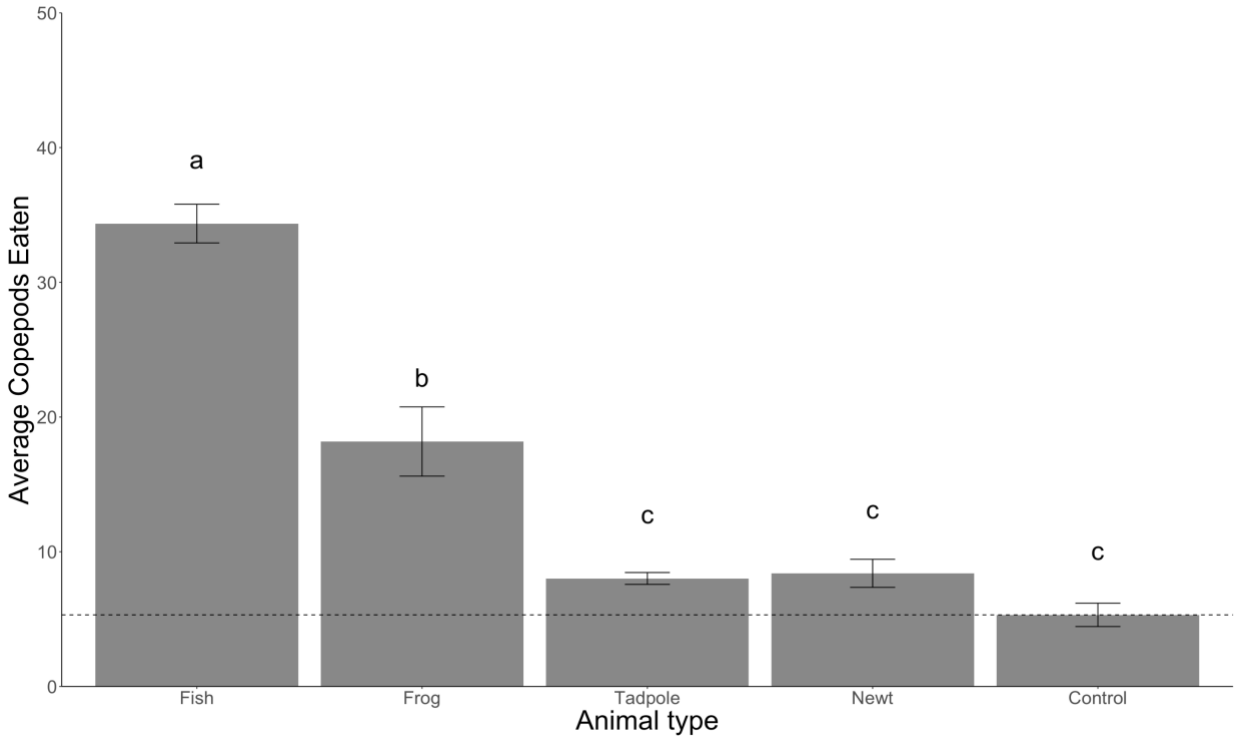

Figure 2.1. Average copepods eaten by animal type during the feeding trial. Bars represent average copepods consumed, error bars represent standard error, and dotted line shows average copepod loss in control trials. Significant differences determined by Tukey post-hoc contrasts are indicated by ‘a’, ‘b’, and ‘c’.

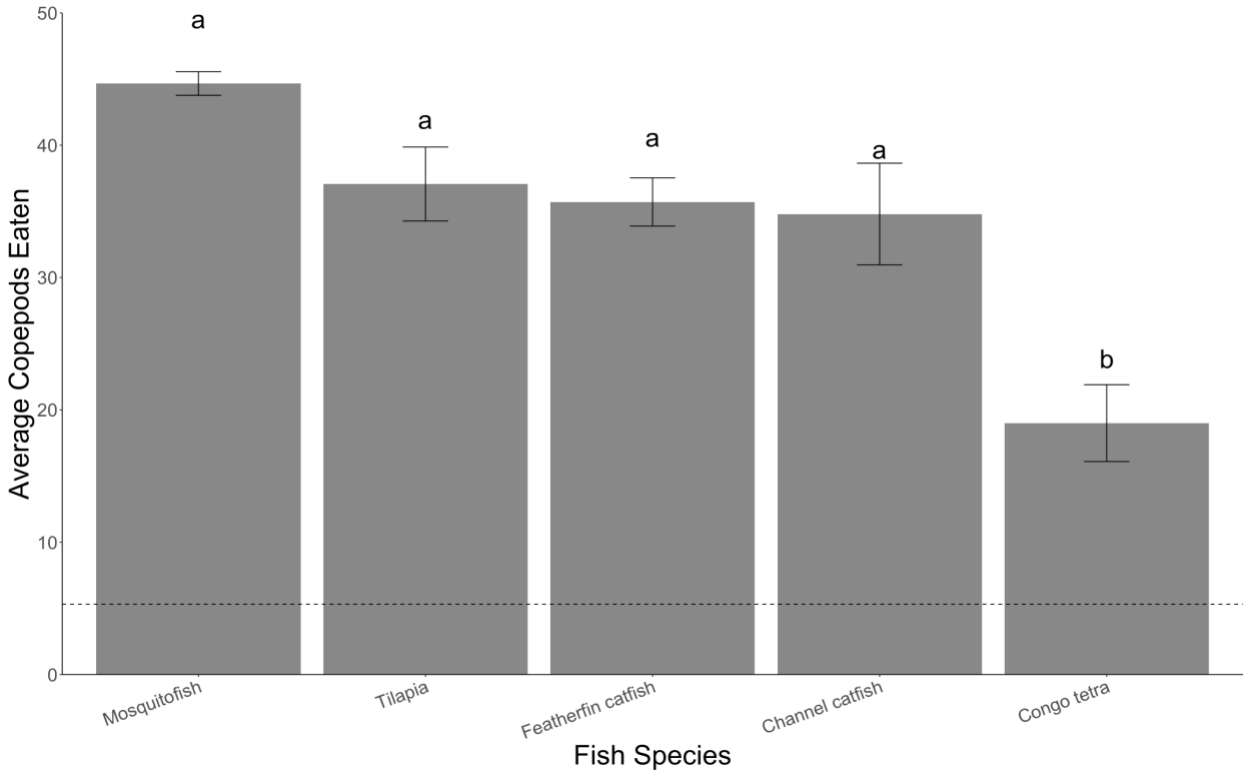

Figure 2.2. Average copepods eaten by fish species during the feeding trial. Bars represent average copepods consumed, error bars represent standard error, and dotted line shows average copepod loss in control trials. Significant differences determined by Tukey post-hoc contrasts are indicated by ‘a’ and ‘b’.

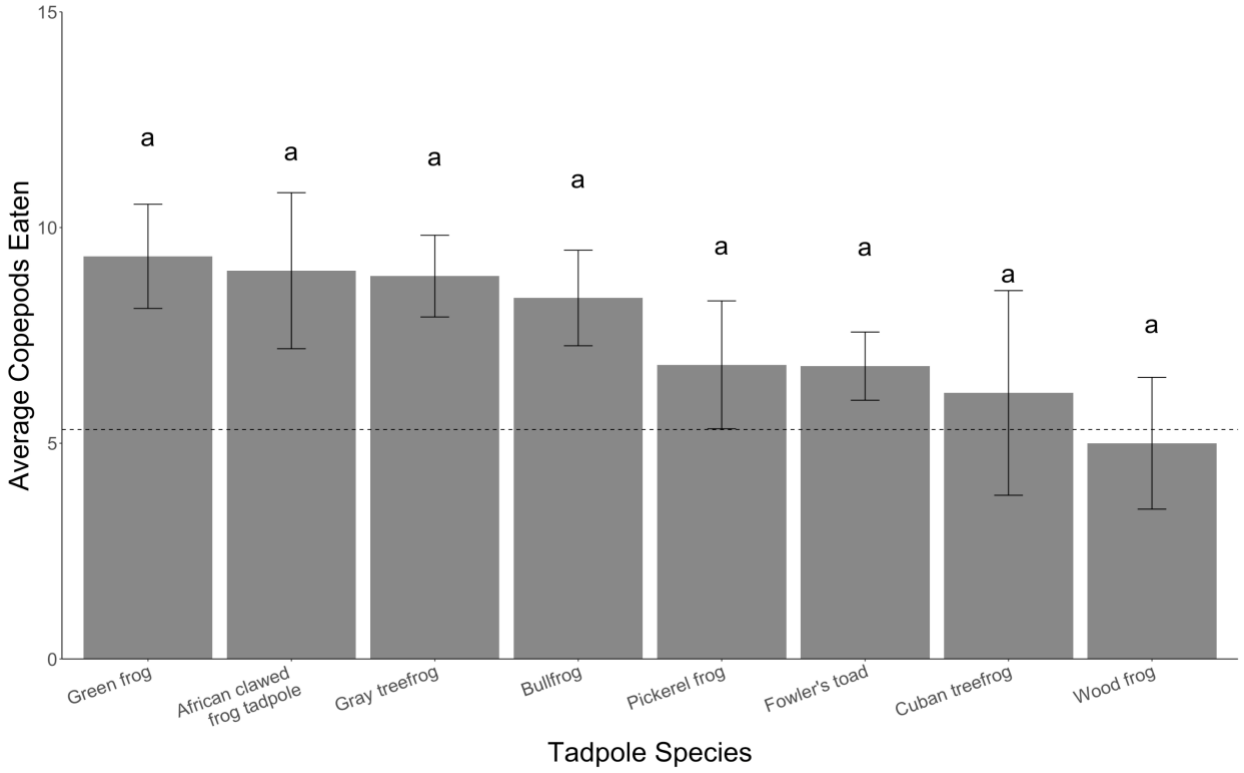

Figure 2.3. Average copepods eaten by tadpole species during the feeding trial. Bars represent average copepods consumed, error bars represent standard error, and dotted line shows average copepod loss in control trials. The lack of significant differences determined by Tukey post-hoc contrasts are indicated by ‘a’.

CHAPTER 3

SUSCEPTIBILITY OF AMPHIBIANS, LIZARDS, AND FISH TO INFECTION WITH *DRACUNCULUS* SPECIES LARVAE AND IMPLICATIONS FOR THEIR ROLES AS PARATENIC HOSTS.[1]

[1]Box, E.K., Cleveland, C.A., Garrett, K.B., Thompson, A.T., Wyckoff, S.T., Yabsley, M.J. To be submitted to the *International Journal of Parasitology: Parasites and Wildlife.*

ABSTRACT

Dracunculus species are parasitic nematodes that infect many species of mammals and reptiles on multiple continents. The most notable species is the human Guinea worm (*D. medinensis*), which is the focus of an international eradication campaign. The life cycles of *Dracunculus* species are complex, and unknowns remain regarding the role of paratenic and transport hosts in transmission. In this study, we had two primary objectives: to assess the susceptibility of various species of amphibians, lizards, and fish as paratenic hosts of *Dracunculus* species and to determine the long-term persistence of *Dracunculus* infections in African clawed frogs as paratenic hosts. To fulfill these objectives, animals were exposed to copepods containing infectious third-stage larvae (L3s) of either *D. insignis* or *D. medinensis*. After a number of days or months, animals were necropsied and examined for *Dracunculus* larvae. *Dracunculus* L3s were recovered from seven species (four amphibian, two reptiles, and one fish), highlighting an increase in species diversity that may become infected. In long-term persistence trials, *D. medinensis* L3s were recovered up to two months post infection, and *D. insignis* L3s were recovered up to eight months post-infection from African clawed frogs. Our findings regarding the susceptibility of novel species of frogs, lizards, and fish to infection with *Dracunculus* nematodes, and long-term persistence of L3s in paratenic hosts, address pressing knowledge gaps regarding *Dracunculus* life cycles and transmission and may guide future research regarding the transmission of *Dracunculus* to definitive hosts.

1. INTRODUCTION

Dracunculus species are parasitic nematodes that infect a diversity of mammal and reptile species on multiple continents (Cleveland et al., 2018). The most well-known species of this

genus is *Dracunculus medinensis*, or human Guinea worm (WHO, 2020). This parasite has been targeted for eradication by a global Guinea Worm Eradication Program (GWEP), which has succeeded in decreasing human case numbers by over 99% (Ruiz-Tiben and Hopkins, 2006). In 1986, there were an estimated 3.5 million cases in 21 countries in South Asia and sub-Saharan Africa (Ruiz-Tiben and Hopkins, 2006; Cleveland et al., 2018). In 2019, only five countries were still considered endemic, with an annual case total of 54 (WHO, 2020). In recent decades, an increasing number of *D. medinensis* infections have been reported from dogs (*Canis lupus familiaris*), particularly in Chad, Africa (Eberhard et al., 2014; WHO, 2020). This increase poses a serious challenge for the *D. medinensis* eradication effort (Eberhard et al., 2014).

In North America, another *Dracunculus* species, *D. insignis*, infects numerous mammalian hosts, including raccoons (*Procyon lotor)*, North American river otters (*Lontra canadensis*), Virginia opossums (*Didelphis virginianus*), and domestic dogs and cats (*Felis catus*) (Cleveland et al., 2018). Another North American *Dracunculus* species that only infects river otters, *D. lutrae*, appears to be a host specialist (Elsasser et al., 2009). There is an additional *Dracunculus* species that infects opossums in South America, sporadic reports of infected dogs in Argentina and a single report of an infected dog in Spain, however, the majority of *Dracunculus* species worldwide infect reptiles (Hoyos et al., 1995; Bono Battistoni et al., 2011; Cleveland et al., 2018; Diekmann et al., 2020). Sporadic reports of infected reptiles come from across the globe, and many of these *Dracunculus* species are described from only one or very few specimens (Cleveland et al., 2018). Interestingly, a new zoonotic species of *Dracunculus* was reported from Vietnam, although the typical host of this parasite is unknown (WHO, 2020).

In definitive hosts, the large, gravid female *Dracunculus* nematode typically migrates to the distal extremities of the infected host where a blister is formed at the eventual site of

emergence (Cairncross et al., 2002). The anterior end of the worm emerges from the blister and, if the worm is submerged in water, hundreds of thousands of first-stage larvae (L1s) will be released into the water (Cairncross et al., 2002). Cyclopoid copepods (the intermediate host) ingest these larvae, which then molt to infectious third-stage larvae (L3s) (Cairncross et al., 2002). In humans, infection classically occurs by drinking water contaminated with infected copepods (Muller, 1971).

While the life cycle of *D. medinensis* and its transmission to humans is relatively well studied and understood, there is still a gap in knowledge concerning the primary mode of natural infection for wildlife hosts. It seems unlikely that direct ingestion of copepods would explain patterns of *D. medinensis* transmission seen in dogs in Sub-Saharan Africa (Garrett et al., 2020). When drinking, many animals such as dogs and opossums lap water, while many snake species suck in water (McManus, 1970; Cundall, 2000; Crompton and Musinsky, 2011). When lapping, animals drink water from the top of the water column, while copepods are primarily found lower in the water column, especially when infected with *Dracunculus* sp. larvae (Onabamiro, 1954.; Eberhard and Brandt, 1995). The disturbance caused by the animal drinking from the water may also cause copepods to flee the area (Eberhard and Brandt, 1995). Because of these reasons, it has been suggested that *Dracunculus* transmission to wildlife hosts may be occurring through alternative infection routes (e.g., the use of a paratenic or transport host infected with L3s), not through direct ingestion of infected copepods. In reference to *Dracunculus* spp. nematodes, we use the term paratenic host to refer to a host that may become infected with and transmit L3s, but in which the larvae do not develop (Eberhard et al., 2016b). Paratenic hosts can be contrasted with short-term transport hosts, which, after ingesting *Dracunculus* L3 infected copepods, could

transmit the parasite if eaten while the larvae are still in the gastrointestinal tract (Cleveland et al., 2017).

Fish, amphibians, and reptiles have been investigated for their potential as paratenic hosts of *Dracunculus* (Brackett, 1938; Crichton and Beverley-Burton, 1977; Eberhard et al., 2016b; Cleveland et al., 2019). Previous work has demonstrated that laboratory infected amphibians can act as paratenic hosts for *D. ophidensis* infection in snakes (garter snakes and water snakes [*Nerodia sipedon*]), *D. insignis* infection in raccoons, and *D. medinensis* infection in ferrets (Brackett, 1938; Crichton and Beverley-Burton, 1977; Eberhard et al., 2016b). *Dracunculus insignis* larvae have been previously recovered from infected tadpoles up to 37 days post-exposure, at which time the animals were necropsied (Crichton and Beverley-Burton, 1977). *Dracunculus medinensis* L3s have been recovered from wild frogs (*Hoplobatrachus occipitalis* and *Phrynobatrachus francisci*) in Chad, Africa, and *D. insignis* L3s have been recovered from wild frogs (*Lithobates* [Rana] *catesbeiana* and *Lithobates* [*Rana*] *sphenocephalus*) in Di-Lane Plantation Wildlife Management Area (WMA), GA, USA (Eberhard et al., 2016a; Cleveland et al., 2019; Cleveland et al., 2020).

Wild-caught fish have been inspected for the presence of *Dracunculus* larvae, but no larvae have been recovered (Cleveland et al., 2019; Cleveland et al., 2020). Fish have been experimentally fed *Dracunculus* L3s and L3 infected copepods, and although L3s were recovered from some experimentally infected fish, larva recovery rates were low (0.6- 2.0%) (Chrichton and Beverly-Burton, 1977). Some fish species have been shown as capable short-term transport hosts for *Dracunculus* species, successfully transmitting *D. insignis* and *D. medinensis* to ferrets (*Mustela putorius furo*) (Cleveland et al., 2017).

Wild reptiles have also been investigated for the presence of *Dracunculus* nematodes in muscle and subcutaneous tissues, specifically Nile monitor lizards (*Varanus niloticus*); no *Dracunculus* nematodes have been recovered during these investigations (Cleveland et al., 2019). Nile monitor lizards were considered as potential paratenic hosts for *Dracunculus* as they live in aquatic habitats and eat fish and amphibians, which could potentially lead to their infection if the fish or amphibian were infected with *Dracunculus* larvae (Arbuckle, 2009). Nile monitor lizards have been reported to harbor infections with adult nematodes, some of which were called *Dracunculus* spp., although no reports have been molecularly confirmed and most failed to provide any morphologic criteria for identification (Mirza and Basir, 1937). Suspect subcutaneous nematodes found in Nile monitor lizards during a 2019 study were determined not to be *Dracunculus* species (Cleveland et al., 2019).

The objectives of this study were to investigate the susceptibility of several amphibian, lizard, and fish species to infection with *Dracunculus* spp. L3s and to determine the long-term persistence of *Dracunculus* larvae in amphibians. We hypothesized that amphibians would become most readily infected and that lizards and fish would develop few, if any, infections. We also hypothesized that larvae would persist at least several weeks, likely longer, within infected paratenic hosts. The insight gained from this work will help to better understand the role that these animals may play as paratenic hosts for *Dracunculus* transmission to domestic animals and wildlife hosts.

2. METHODS

2.1. Copepods and *Dracunculus* larvae

Dracunculus insignis larvae used in this study were obtained from raccoons from Di-Lane WMA, Georgia, USA in April and May of 2016 (Cleveland et al., 2020). *Dracunculus medinensis* larvae were obtained from infected dogs in Guinea worm endemic zones along the Chari River in Chad, Africa in May through July of 2016. Lab-raised copepods (from wild-caught stock from Athens, GA) of the genus *Macrocyclops* were used for *D. insignis* trials. Wild-caught African copepods (*Mesocyclops* spp.) were used for *D. medinensis* trials. Copepods were infected by adding *Dracunculus* L1s to their water and allowing copepods to feed for 72 hrs. After two weeks, the infection status of copepods was checked via microscopy to ensure *Dracunculus* larvae had molted to L3s. Copepod infection rate and *Dracunculus* maturation was assessed as previously described by Eberhard et al. (2016b).

2.2. Animals used in this study

Six species of amphibians were used. African clawed frogs (*Xenopus laevis*) were captive-bred (Xenopus Express, Brooksville, FL). All other amphibian species were wild-caught (American toad [*Anaxyrus americanus*], Cope's gray treefrog [*Hyla chrysoscelis*], Cuban treefrog [*Osteopilus septentrionalis*], and southern leopard frog [*Lithobates* (*Rana*) *sphenocephalus*]). Species identification was made using the book *Tadpoles of the southeastern United States coastal plain* (Gregoire, 2005). Tadpoles were categorized as Gosner stages <42, froglets were categorized as Gosner stages 42-45, and adults were Gosner stage 46. Lizards used were juvenile, captive-bred Nile monitor lizards (Backwater Reptiles, Rocklin, CA) and adult, wild-caught anoles (*Anolis carolinensis*) from Georgia (USA). Two species of captive-bred,

commercially sourced fish, bichir (*Polypterus* sp.) and featherfin catfish (*Synodontis eupterus*) were included in the study.

2.3. Infection methods

Animals were inoculated by one of two routes: group batch or oral inoculation (Table 3.1; Table 3.2; Table 3.3). Animals that were exposed via group batch were added to a 500ml beaker containing infected copepods and allowed to feed for a period of 72 hrs. For those species that did not readily consume copepods, or were too large to expose in a small beaker, oral inoculation was performed by concentrating infected copepods in a small volume of water and performing oral gavage with a pipette. The pipette was rinsed to ensure all copepods were ingested. If copepods were recovered during the rinse, oral gavage was repeated until all copepods were ingested.

2.3.1. Infection of potential paratenic hosts with Dracunculus*-infected copepods*

Seventy-four individual amphibians of five species were exposed to *D. insignis*-infected copepods via group batch (Table 3.1). Fifty-two amphibians were exposed in batches with 10 *D. insignis* infected copepods per individual; 22 amphibians were exposed in batches with 20 *Dracunculus* infected copepods per individual (Table 3.1). Seven amphibians were exposed to *D. medinensis* infected copepods; all were adults, orally inoculated with 20 *D. medinensis* infected copepods each (Table 3.1). Six individual reptiles were exposed to *D. insignis* via oral inoculation with infected copepods (Table 3.1). Eight individual fish (four bichir and four featherfin catfish) were exposed to *D. medinensis* larvae via oral inoculation or group batch with infected copepods (Table 3.1). Animals were euthanized after a varying number of days (6-140) and examined for the presence of *Dracunculus* larvae (Table 3.1).

Some *D. medinensis* L3s recovered from experimentally infected animals were used to orally inoculate two adult African clawed frogs (11 and 15 larvae were given to each animal). African clawed frogs were euthanized four months post-inoculation and examined for the presence of *Dracunculus* larvae.

2.3.2. Persistence of Dracunculus *in paratenic host*

Thirty-one adult African clawed frogs were exposed to 50 *D. insignis* or *D. medinensis* L3 infected copepods each via group batch methods (Table 3.3). Frogs were euthanized at approximately two, three, four, five, six, and eight months post-exposure (Table 3.3).

2.4. Recovery of *Dracunculus* larvae from paratenic hosts

Animals used in this study were humanely euthanized using a buffered MS-222 bath (amphibians and fish) or isoflurane (lizards) followed by pithing and decapitation (following American Veterinary Association guidelines). Gastrointestinal tract and muscle tissues were placed in separate Petri dishes, macerated and allowed to sit at room temperature in water. Petri dishes were observed under a dissecting microscope for movement of larvae immediately after necropsy and tissue preparation and then at four, eight, and 24 hours. For larger animals (i.e., fish, adult African clawed frogs, and lizards), tissues were divided into multiple dishes.

Ethical approval and informed consent

All animal procedures in this study were reviewed and approved by the University of Georgia Institutional Animal Care and Use Committee (A2018 01-010-Y3-A2).

3. RESULTS

3.1. Infection of potential paratenic hosts with *Dracunculus*-infected copepods

3.1.1. Amphibians

Among the amphibian species tested, four of six species tested developed infections with *Dracunculus*. *Dracunculus insignis* larvae were recovered from 10 of 22 (45.5%) amphibians that were exposed by group batch with 20 copepods offered per individual; all larvae were recovered from muscle tissue (Table 3.1). No larvae were recovered from amphibians that were exposed by group batch methods with only 10 copepods offered per individual (Table 3.1). *Dracunculus insignis* L3s were recovered from amphibians up to 99 days post-infection (Table 3.1).

Dracunculus medinensis larvae were recovered from three out of seven (42.9%) of the exposed amphibians included in Table 3.1. Larvae from *D. medinensis* infected amphibians were all recovered at six days post-inoculation (Table 3.1). All *D. medinensis* larvae were recovered from muscle, except one, which was recovered from the viscera of an American toad (Table 3.1).

3.1.2. Lizards

Dracunculus larvae were recovered from two of five (40%) Nile monitors and the single exposed green anole. Larvae were recovered from lizard tissue at a minimum of six days and a maximum of 14 days post-inoculation (Table 3.2). From one Nile monitor, a single *D. insignis* larva was recovered from gastrointestinal or visceral tissue. From another Nile monitor, a single *D. insignis* larva was recovered from the muscle. From the anole, four *Dracunculus* larvae were recovered (two from the abdomen, one from the tail or legs, and one from the viscera; Table 3.2).

3.1.3 Fish

Dracunculus medinensis larvae were recovered from the muscle of three of four (75%) featherfin catfish at 10 days post-inoculation. All four exposed bichir were negative (Table 3.2). All larvae recovered from amphibians, lizards, and fish were L3s.

3.1.4. Ability of Dracunculus *L3s from a paratenic host to infect another paratenic host*

No larvae were recovered from the African clawed frogs that were exposed to *D. medinensis* larvae recovered from previous paratenic hosts.

3.2. Persistence of *Dracunculus* in paratenic hosts

Dracunculus insignis larvae were recovered from adult African clawed frogs at three, four, six, and eight (maximum length of time tested) months post-infection. *Dracunculus medinensis* larvae were recovered from adult African clawed frogs at two months post-infection, but not beyond. All larvae remained L3s (Table 3.3).

4. DISCUSSION

This study demonstrated that a range of anuran genera (*Xenopus, Lithobates* [*Rana*], *Hyla*, and *Anaxyrus* [*Bufo*]), as well as Nile monitor lizards, anoles, and featherfin catfish, are susceptible to infection with *D. insignis* or *D. medinensis* L3s. We also found that *D. insignis* and *D. medinensis* larvae can persist in anuran tissues for at least eight and two months, respectively. The number of L3s recovered from each infected paratenic host was generally low; the maximum larvae recovered from one individual was 15, the average number of larvae recovered from all infected animals was 5.3, while the mode number of larvae recovered per infected individual was only one.

Tadpoles were infected with *D. insignis* by group batch method, which mimics how animals may naturally become infected, as the tadpoles autonomously ingest the copepods. The only adult amphibians that were exposed via group batch methods were African clawed frogs exposed to *D. insignis* infected copepods, as African clawed frogs are fully aquatic even as adults and were still able to ingest the copepods autonomously as adults. The American toads and Cope's Gray treefrogs that were exposed to *D. medinensis* infected copepods were orally inoculated, as they had metamorphosed into terrestrial adults before *D. medinensis* larvae became available for use and would not ingest copepods autonomously.

Six anurans that were inoculated as tadpoles underwent metamorphosis before being necropsied. *Dracunculus insignis* L3s were recovered from two of these animals, supporting previous findings that *D. insignis* larvae can persist in anuran tissues through metamorphosis (Eberhard and Brandt, 1995). The persistence of larvae in the tissues through metamorphosis may facilitate *Dracunculus* transmission from aquatic to terrestrial food chains. This could be an important factor in transmission, as the majority of definitive hosts of *Dracunculus* nematodes are terrestrial.

Previously, *D. insignis* L3s were found to persist in paratenic hosts for up to 37 days post-exposure, at which time the animals were necropsied (Crichton and Beverley-Burton, 1977). In this long-term infection trial, we found that *D. insignis* larvae persisted for 244 days (approximately 8 months), while *D. medinensis* larvae persisted 58 days (approximately two months) post-infection. These results demonstrate that infection of a paratenic host can extend the time that L3s may persist in the environment well beyond the lifespan of a copepod (Hopp et al., 1997). Our findings also suggest that *D. insignis* may persist longer in paratenic hosts than *D. medinensis*; this difference could contribute to the higher proportion of wild-caught adult frogs

found to be infected with *D. insignis* than with *D. medinensis* during field surveys (Eberhard et al., 2016a; Cleveland et al., 2019; Cleveland et al., 2020). However, in this study, the sample size of animals infected with *D. medinensis* was much lower than those which were exposed to *D. insignis*. Further testing would be required to determine whether the persistence of larvae actually differs between paratenic host species.

No *Dracunculus* larvae were recovered from the two adult African clawed frogs that were inoculated with *D. medinensis* L3s that had been recovered from other paratenic hosts. It is possible that our very small sample size (two animals) or the low number of larvae used to inoculate the secondary paratenic hosts (11 and 15 larvae each) could have led to this negative result. As these animals were necropsied four months after being inoculated with *D. medinensis* larvae, it is also possible that the frogs did become infected, but that the larvae did not persist until necropsy. It may be worth repeating paratenic to paratenic host transmission studies with an additional number of frogs that are necropsied at shorter time intervals or exploring this phenomenon with other predatory animals, such as Nile monitor lizards.

Lizards were included in this study because large, subcutaneous nematodes (believed to be *Dracunculus* sp.) were historically reported from Nile monitor lizards (Mirza and Basir, 1937). Recent work in Chad, Africa found similar large subcutaneous nematodes in wild Nile monitor lizards, and using molecular analysis determined that they were not *Dracunculus* sp., but were actually most similar to *Ochoterenella* species (Cleveland et al., 2019). While our current study confirms that Nile monitor and anole lizards could become infected with *Dracunculus* larvae, no maturation of larvae past L3s was observed. This suggests that, while lizards could play a role in *D. insignis* or *D. medinensis* transmission, it is unlikely that they are capable of performing as definitive hosts for these *Dracunculus* species as was historically suspected. Note

that, although Anoles were exposed to both *D. insignis* and *D. medinensis* larvae, it is most likely that the recovered larvae were *D. insignis*, as only two *D. medinensis* infected copepods were administered and, in this study, trials using fewer than 20 infected copepods did not yield infections. This cannot be confirmed, however, as *Dracunculus* larvae can only be identified to species using molecular diagnostic techniques, which would destroy the sample, and these larvae were used in other experimental infection trials after recovery.

Fish were investigated for their potential role in *Dracunculus* transmission, as many fish species consume copepods as part of a natural diet (Garcia-Berthou, 1999; Piasecki et al., 2004). Despite this, *Dracunculus* larvae have not been recovered during multiple studies screening wild-caught fish (Cleveland et al., 2019; Cleveland et al., 2020). *Dracunculus insignis* L3s have previously been recovered from experimentally infected fish (Crichton and Beverly-Burton, 1977; Eberhard et al., 2016b). Although, in even the most successful previous trial, larval recovery rates were very low (0.6- 2.0% recovery; 1-2 larvae per fish) and only 3/19 (15.8%) of the fish became infected (Crichton and Beverly-Burton, 1977). While previous trials testing fish as potential paratenic hosts of *Dracunculus* have not been successful, fish experimentally functioned as short-term transport hosts of *D. medinensis* and *D. insignis* to experimentally infect domestic ferrets (Cleveland et al., 2017). Our findings from this trial are interesting, as we recovered up to six *D. medinensis* L3s from the tissues of three out of eight (37.5%) exposed fish. Although our sample size was small, our current findings are evidence that some fish species may be more capable of serving as paratenic hosts for *Dracunculus* than those that have been previously tested. This finding further supports the continuation of the screening of wild fish muscle tissues for *Dracunculus* larvae. Of particular interest are featherfin catfish, from which *D. medinensis* larvae were successfully recovered during this study. This fish species is

common in the Chari River Basin area in Chad, Africa where high numbers of *D. medinensis* infections are reported in peridomestic dogs living in fishing villages (Cleveland et al., 2019; IUCN [International Union for Conservation of Nature], 2019; Guagliardo et al., 2020). Dogs in these villages often eat discarded small fish or fish viscera (Eberhard et al., 2014).

In all trials, infection occurred only in those animals that were offered or inoculated with at least 20 copepods per individual. This leads us to consider the role of parasite dose-dependent infection probability of *Dracunculus* nematodes. As copepod infection intensity was estimated for this trial, we do not know exactly how many L3s were ingested by each individual animal host. Also, because multiple animals were being exposed simultaneously using group batch methods, the exact number of copepods consumed by any specific individual could not be determined. Although the sample size of animals that were fed low numbers of copepods was relatively limited, parasite dose-dependent infection probability may merit further investigation, as it could aid in the effort to cease transmission of *D. medinensis* in Sub-Saharan Africa and help researchers to more effectively study transmission in the laboratory.

5. CONCLUSIONS

This study demonstrates that a wide range of animals (amphibians, fish, and lizards) are susceptible to infection with *D. insignis* and *D. medinensis* L3s. Fish, which have been thought primarily to be capable of performing as transport hosts, may also serve as paratenic hosts of *Dracunculus* species. Nile monitor lizards and anoles were successfully infected with L3s, although no larval maturation occurred within the lizards. Our findings support the conclusion that lizards do not perform as definitive hosts of *D. insignis or medinensis*, but suggests that they are capable of serving as paratenic hosts. *Dracunculus* larvae remained L3s in the tissues of

tested amphibians for up to 244 days, extending the temporal availability of infectious larvae in the environment. No larvae were recovered from frogs that were fed L3s recovered from other paratenic hosts. Further investigation is warranted into the roles that these animals may play as paratenic hosts in the natural transmission of *Dracunculus* nematodes to various definitive hosts. These findings contribute to a better understanding of *Dracunculus* transmission, which is valuable to understanding transmission to wildlife hosts and informing GWEP management decisions aiming to decrease and eventually eliminate human infections with *D. medinensis*.

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Table 3.1. Methods and results of amphibian paratenic host experimental infections with *Dracunculus insignis* or *D. medinensis* larvae via ingestion of infected copepods.

Host species	*Dracunculus* species	Age group	N hosts	N copepods per host	Exposure type	Days from exposure to necropsy	Infection status (N)	N Larvae recovered per host
AMPHIBIANS								
African clawed frog (*Xenopus laevis*)	*D. i.*	tadpole	5	10	GB	11	NEG	
	D. i.	tadpole	8	10	GB	29	NEG	
	D. i.	tadpole	6	10	GB	29	NEG	
	D. i.	adult	3	20	GB	99	**POS** (1)	2
	D. i.	adult	2	10	GB	140	NEG	
Southern leopard frog (*Lithobates sphenocephalus*)	*D. i.*	tadpole	2	10	GB	9	NEG	
	D. i.	tadpole	2	10	GB	11	NEG	
	D. i.	tadpole	3	10	GB	29	NEG	
	D. i.	tadpole	3	10	GB	31	NEG	
	D. i.	tp→froglet*	4	20	GB	44	**POS** (2)	1 & 1
	D. i.	tp→froglet*	2	20	GB	50	NEG	
	D. i.	tadpole	6	20	GB	58	**POS** (4)	3, 4, 8 & 8
	D. i.	tadpole	2	20	GB	70	**POS** (2)	1 & 1
	D. i.	tadpole	1	20	GB	76	NEG	
	D. i.	tadpole	1	20	GB	86	NEG	
	D. i.	tadpole	1	20	GB	97	**POS** (1)	14
Bullfrog tadpoles (*Lithobates catesbeianus*)	*D. i.*	tadpole	6	10	GB	16	NEG	
Cuban treefrogs (*Osteopilus septentrionalis*)	*D. i.*	tadpole	3	10	GB	16	NEG	
	D. i.	tadpole	10	10	GB	16	NEG	
	D. i.	tadpole	2	10	GB	22	NEG	
Cope's gray treefrog (*Hyla chrysoscelis*)	*D. m.*	adult	2	20	OI	6	**POS** (1)	15
	D. i.	tadpole	2	20	GB	51	NEG	
American toad (*Anaxyrus americanus*)	*D. m.*	adult	5	20	OI	6	**POS** (2)	1 & 1

D. i.= Dracunculus insignis; *D. m.= D. medinensis;* GB= group batch; OI= oral inoculation; tp= tadpole

*indicates metamorphosis occurred between infection and necropsy

Table 3.2. Methods and results of lizard and fish paratenic host experimental infections with *Dracunculus insignis* or *D. medinensis* larvae via ingestion of infected copepods.

Host species	*Dracunculus* species	Age group	N hosts	N copepods per host	Exposure type	Days from exposure to necropsy	Infection status (N)	N Larvae recovered per host
LIZARDS								
	D. i.	juvenile	1	25	OI	12	NEG	
Nile monitor	*D. i.*	juvenile	2	25	OI	13	**POS** (1)	1
(*Varanus niloticus*)	*D. i.*	juvenile	1	25	OI	14	**POS** (1	1
	D. i.	juvenile	1	25	OI	15	NEG	
Green anole (*Anolis carolinensis*)	*D. i. & D. m.*	adult	1	25 (23 *D. i/* 2 *D. m.*)	OI	6	**POS** (1)	4
FISH								
Bichir (*Polypterus* sp.)	*D. m.*	juvenile	4	20	OI (2)/ GB (2)	10	NEG	
Featherfin catfish (*Synodontis eupterus*)	*D. m.*	juvenile	4	20	GB	10	**POS** (3)	2, 3 & 6

D. i.= Dracunculus insignis; *D. m.= D. medinensis;* GB= group batch; OI= oral inoculation

Table 3.3. Results of experimental infection and long-term persistence trials of African clawed frogs (*Xenopus laevis*) exposed via group batch methods to 50 *Dracunculus insignis* or *D. medinensis* infected copepods each.

***Dracunculus* species**	**N hosts**	**Approx. months from exposure to necropsy**	**Days from exposure to necropsy**	**Infection status (N)**	**N Larvae recovered per host**
D. i.	10	3	99	POS (1)	1
D. i.	5	4	121	POS (1)	6
D. i.	5	5	141	NEG	
D. i.	3	6	188	POS (2)	1 & 1
D. i.	5	8	244	POS (1)	8
D. m.	1	2	58	POS (1)	1
D. m.	1	4	125	NEG	
D. m.	1	4	115	NEG	

D. i. = Dracunculus insignis

D. m. = Dracunculus medinensis

CHAPTER 4

CONCLUSIONS

Dracunculus nematodes infect a broad range of definitive hosts. *Dracunculus medinensis*, human Guinea worm, causes the debilitating disease dracunculiasis in people and is targeted by a Guinea Worm Eradication Program (GWEP). This eradication campaign has been successful in decreasing global case numbers >99.99% since 1986. In 2010 in Chad, Africa, after over a decade of no known dracunculiasis cases, a human case was reported. Soon after, in 2012, infections were reported in dogs. In the years since, the number of reported dog infections has continued to increase. In 2020, infected wild and domestic animals were reported from four of the five remaining *D. medinensis* endemic countries. Significant knowledge gaps exist concerning the transmission of *Dracunculus* nematodes to wild and domestic animal hosts. Filling these gaps is vital to the success of the GWEP, as well as to increasing the general knowledge of *Dracunculus* spp. transmission to wildlife and domestic animals. Aquatic paratenic and transport hosts are likely to serve some role in this transmission, although many unknowns remain regarding the specifics of these roles. These studies were intended to expand the general understanding of *Dracunculus* transmission via paratenic or transport hosts.

Cyclopoid copepods are the obligate intermediate host of *Dracunculus* species. Therefore, developing an understanding of copepod ingestion rates by potential paratenic or transport hosts would be beneficial in understanding the roles that these animals may themselves have in *Dracunculus* transmission. Copepod ingestion was directly compared between aquatic animal species which may serve as paratenic or transport hosts (fish, frogs [tadpoles and adults], and newts). I hypothesized that fish would consume more copepods than amphibians. The results

of this study confirm that African clawed frogs and fish readily consume copepods, but that fish ingest significantly higher numbers of copepods (34/50 [68%]) than adult African clawed frogs (18/50 [36%]) during the same 24-hour time period. Tadpoles (all species) and newts did not ingest a significant number of copepods during feeding trials. These findings suggest that amphibians and fish are consuming copepods, possibly even *Dracunculus* sp. infected copepods, in areas endemic areas to these parasites. Further research is required to determine whether, in the wild, fish or frogs are performing as paratenic or transport hosts, thus increasing *Dracunculus* transmission, or whether they may perform simply as dead-end hosts or means of copepod population control, thus decreasing *Dracunculus* transmission.

To further assess the capability of aquatic and semi-aquatic hosts to serve as paratenic hosts of *Dracunculus* species, experimental infection trials were performed. I had two primary objectives: to assess the susceptibility of various species of amphibians, lizards, and fish as paratenic hosts of *Dracunculus* species, as well as to determine the long-term persistence of *Dracunculus* infections within paratenic hosts. We hypothesized that many of the exposed amphibians would become infected, while few fish or reptiles would become infected. We also expected that larvae would persist in the tissues for at least several weeks, To test these hypotheses, hosts were exposed to copepods infected with third stage larvae (L3s) of either *Dracunculus insignis* or *D. medinensis*. Upon necropsy, *Dracunculus* L3s were recovered from seven species (four amphibian, two lizard, and one fish), highlighting the diversity of animals that may become infected and could potentially serve as paratenic hosts for these parasites. Findings from this study support the continued surveillance of wild fish in addition to amphibians. Surveillance of wild reptiles for *Dracunculus* larvae may be warranted as well. In long-term persistence trials, *D. medinensis* and *D. insignis* L3s were recovered from amphibians

up to two and eight months post-infection, respectively, extending the known persistence of *Dracunculus* larvae in paratenic hosts. Additional studies would be required to determine whether this variation in L3 persistence impacts the relative role of long-term paratenic hosts in the transmission of *D. medinensis* versus *D. insignis*. Overall, the results of this study illuminate many details which contribute to the growing knowledge base of *Dracunculus* infection and persistence in potential paratenic hosts.

Together, the results of these studies contribute to our knowledge of how aquatic animals may facilitate *Dracunculus* spp. transmission. Understanding how *Dracunculus* parasites are transmitted to animal hosts is vital to continue and advance the *D. medinensis* eradication effort. Once transmission of *D. medinensis* to animal hosts is understood, our hope is that it can be stopped, and that the end of dracunculiasis in humans will soon follow.