COMPARATIVE STUDY OF LIFE CYCLE ECOLOGY AND HOST-PARASITE INTERACTIONS OF HORSEHAIR WORMS (PHYLUM: NEMATOMORPHA)

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OF HORSEHAIR WORMS (PHYLUM:

NEMATOMORPHA)

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"If you can't fly then run, if you can't run then walk, if you can't walk then crawl, but whatever you do you have to keep moving forward."

—Dr. Martin Luther King, Jr.

Whether I was flying or crawling, the development of this document was not possible without the guidance and support of several people over the last several years. There is no way to pinpoint the exact moment when my life was placed on the trajectory to receiving my Ph.D. but I believe there were several key points in my academic career over the last 20 years that directed me toward the path. The success I have attained during the last five years has been unbelievable and while I am humbled but proud, it truly was a combination of serendipitous occasions, small gestures, an unrelenting advisor, and endless learning opportunities. What no one tells us, is that you must fail several times before you succeed once but through it all, you persevere and move forward as Dr. King has so eloquently preached.

It was the idea that I could become an astronomer that led me to Palomar Junior College where during a biology course, Dan Sourbeer fueled my interests in biology, turning me away from space. His course in Yellowstone National Park truly gave me a new perspective on the outdoors, life, and the world. During the first year of my master's

work, I was smoking a cigarette, as I did during stressful times. On that serendipitous occasion in the rain, I found a strange and beautiful worm swimming in a puddle in my backyard. While I never smoked again, the worm was the beginning of a science career that would lead to Oklahoma and Iceland. But first, the worm led me to Dr. Ben Hanelt, who, after declaring it was a new species, suggested I could study these for my master's project. During this first year, Dr. Hanelt, introduced me to the concept of life cycles and trained me in his lab where a Ph.D. seed was planted. In 2012, he introduced me to the American Society of Parasitologists and Dr. Matt Bolek, who would become a pivotal influence, not only in my academic career, but also on how I viewed the world as a story. Dr. Bolek has not only exposed me to the wonders of parasites and their life cycles, but that we can share these wonders with scientist, and non-scientist alike, through the art of storytelling. His gift of telling interesting and relevant science stories is unmatched and has allowed me to engage audiences by portraying my excitement through science and storytelling. Of course, this was only facilitated through hours and hours of driving to conferences where he literally held his student audience captive. But during these times, the car became a vessel of learning about parasites, people, and the stories behind them, often cued by landmarks such as bridge no. 0.000 at the Kansas-Oklahoma border.

Several people have entered my journey along the way and have served as instrumental components to a web of support and it is to all of them, that I dedicate this work. For nearly 12 years, Kari Martinez has served as my best friend and family

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Abstract: For hundreds of years, the phylum Nematomorpha, commonly known as hairworms, has been the subject of rich and colorful folklore across the world. Many of these stories originated from characteristics such as their serpent-like shape, locations where they are found, and their ability to drive theirs hosts to water. Although we have gained significant knowledge in this understudied group, we are still in the infancy of understanding the diversity of species and their life cycles. For example, a recent study revealed that what was commonly known as *Gordius robustus* across North America, was a suite of eight genetically distinct species all of which were found to emerge between late spring and early fall. One exception to this were specimens collected from Texas, Louisiana, and Oklahoma. In chapter II, I describe this *Gordius* sp. based on my first-year collections in Oklahoma. These initial observation showed that hairworms were emerging from soil and congregating on lawns, sidewalks, and streets but seem to disappear within one or two days of rain drying out. But the most interesting find was the egg of this species which contained a double membrane, a trait not been observed in any hairworm species to date. In chapter III, I examined the natural history of this new *Gordius* species through a series of field and laboratory observations and experiments over a five-year period. I documented information on the habitat, seasonal occurrence, and infections of soil invertebrates as biodiversity indicators. In chapter IV, I expanded the principles of biodiversity indicators using aquatic snails as indicator hosts of hairworms across a large geographic region, Iceland. Finally, in chapter V, my objective was to expand our knowledge of host-parasite interactions in the phylum by examining survivorship and fecundity reduction in laboratory reared and infected crickets. Data presented in this dissertation provides a species description, a new life cycle strategy, a new documented species in Iceland, and new information that will change the perception of hairworms as parasitoids.

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CHAPTER I

OVERVIEW

For hundreds of years, the phylum Nematomorpha, commonly known as hairworms, has been the subject of rich and colorful folklore across multiple continents (Annandale 1905). In Asia, hairworms were believed to be the intermediate stage of an earthworm transforming to a freshwater eel. In Ireland, hairworms were known as "sky worms" as they were thought to come down with the rain. In northeast Iceland, hairworms were believed to be formed from the tail of a stallion (Gregor 1878). Much of these stories come from the mystery that hairworms possess probably due to their serpent-like shape, peculiar behaviors, and ability to drive their hosts to water. Despite our modern methods including DNA sequencing methods, improved sampling methods, and elucidation of the life cycle, hairworms are just as much a mystery as they were 200 years ago.

Hairworms are often found entangled in masses of two to many individuals and are therefore known as Gordian worms or gordiids because of a Phrygian tale in which Alexander the Great disentangled an impossible knot (Hanelt et al. 2005). The phylum

Nematomorpha is one of only three completely parasitic metazoan animal phyla. As the sister group to the phylum Nematoda, hairworms consist of the freshwater Gordiida and the marine Nectonematida. There are approximately 350 described freshwater species within 18 extant and 2 extinct genera (Poinar and Buckley 2006; Bolek, et al. 2015). During their parasitic phase, freshwater gordiids infect terrestrial arthropods such as orthopterans (grasshoppers, crickets, etc.), mantises, roaches, beetles, and millipedes (Poinar and Weissman 2004; Bolek et al. 2015). Inside their arthropod host and depending on the gordiid species, hairworms can grow to 40 centimeters in length for some North American species and up to 2 meters for some species in tropical regions (Schmid-Rhaesa 2012). During development, hairworms manipulate the behavior of their arthropod host to seek water, where worms emerge as free-living adults (Bolek, et al. 2015). In the aquatic habitat, hairworms do not feed but instead find mates and reproduce during their short two to eight-week, free-living phase of the life cycle (Fig. 1). Adult free-living hairworms are often difficult to observe in their natural habitats due to their ephemeral life span and ability to blend into their environment (Bolek et al. 2015).

The life cycles of three species of gordiids have been elucidated and all appear to have a similar life cycle utilizing two types of hosts. These include an aquatic paratenic host (transport host) which harbors the parasitic cyst stage and a terrestrial arthropod, final host where the parasitic juvenile worm develops to an adult stage just before it emerges from the host as a free-living adult into an aquatic habitat (Hanelt et al. 2005; Bolek et al. 2015). Despite our current knowledge of the gordiid life cycle, there is a disparity of our understanding on how these life cycles operate in nature. For example, there is a clear lack of knowledge of the range of host use including the paratenic and final arthropod hosts used by

most gordiid species. In fact, arthropod final hosts have been reported for less than 30% of the 350 described gordiid species (Schmidt-Rhaesa 2012; Harkins et al. 2016). Many of these reports are based on a single observation that may not represent the full extent of host use by some gordiid species (Hanelt et al. 2005; Bolek et al. 2015). Additionally, field studies on gordiid paratenic hosts are almost nonexistent (White 1966; 1969; Bolek and Coggins 2002; Chiu et al. 2016).

Because of the complex life cycle of gordiids, studies on the distribution, life cycles, and host use of hairworms have been challenging for at least three reasons (Harkins et al. 2016). First, the detection of adult free-living worms in their natural habitat is difficult because of their cryptic coloration allowing free-living worms to blend in with their natural environment; often being obscured beneath the substrate such as rocks or leaf litter in aquatic habitats. Second, adult free-living worms are seasonal and have a short life span of two to eight weeks (Bolek and Coggins 2002; Hanelt et al. 2005). Due to these detection impediments, there has been a lack of effective sampling for gordiids because locating free-living adult worms over large geographic areas is difficult. For example, a study of 50 sites in Lancaster County, Nebraska yielded three adult worms at one site over a period of three years of sampling (Hanelt et al. 2001). Finally, field studies examining infection parameters for gordiids in arthropod final hosts indicate that prevalence (percent infection among arthropod populations) and intensity (number of hairworms within infected hosts) is relatively low. The few field studies that have examined arthropods for gordiid infections indicate that gordiid prevalence (percent infected) among arthropod populations, ranges from less than 1% to as high as 28% (White 1966; 1969; Bolek and Coggins 2002; Looney et al. 2012; Schmidt-Rhaesa 2012). As with prevalence, field studies or observations of worms

emerging from their final arthropod hosts, indicate that the intensity of worms is also relatively low (1 to 7 worms per host) with most hosts containing a single worm (Kollar 1857; Studier et al. 1991; Poulin 1995; Bolek and Coggins 2002; Poinar and Weissman 2004; Looney et al. 2012).

More recently, novel culturing, sampling, and collecting techniques have been developed for gordiids which allow future field and laboratory studies on the distribution, host use, ecology, and general life cycles of gordiids in nature (Hanelt and Janovy 1999; Hanelt et al. 2001; Bolek and Coggins 2002; Hanelt and Janovy 2004; Hanelt et al. 2012; Bolek et al. 2013a, 2013b, Szmygiel et al. 2014; Harkins, et al. 2016). Below I describe these advances which will allow me to examine the ecology and host use of two species of gordiids in Oklahoma.

One of the most important advances in the study of gordiids was the development of novel culturing techniques for hairworms in the laboratory (Hanelt and Janovy 1999; Hanelt and Janovy 2004b; Hanelt et al. 2012; Bolek et al. 2013a; Bolek et al. 2013b). This work has resulted in the culturing of three North American and African species of gordiids (*Paragordius varius*, *Paragordius obamai*, and *Chordodes kenyaensis*) in the laboratory (Hanelt and Janovy 2004b; Hanelt et al. 2012; Bolek et al. 2013a; Bolek et al. 2013b). The dioecious North American *P. varius* has been maintained in culture for over a decade (Hanelt and Janovy 2004a; Bolek et al. 2013b). More recently, the first parthenogenetic gordiid, *P. obamai*, was discovered in Kenya and is currently maintained in culture (Hanelt et al. 2012; Bolek et al. 2013b). Studies on laboratory-cultured hairworms indicate that life cycles of gordiids involve five distinct life stages (Fig. 1) including: (1) egg strings, (2) free-living larvae, (3) parasitic cysts, (4) parasitic juveniles, and (5) dioecious or parthenogenetic free-

living adults (Hanelt and Janovy 2004a, b; Hanelt et al. 2012; Bolek et al. 2013a, b). Juvenile gordiids are obligatory parasites of terrestrial arthropods, whereas a variety of aquatic animals serve as paratenic hosts for the cyst stage (Hanelt et al. 2001; Bolek and Coggins 2002; Hanelt and Janovy 2003, 2004b).

These laboratory life cycle studies and recent field studies on gordiids indicate that cyst stages may be the most commonly encountered gordiid life stage in the environment (Hanelt et al. 2001; Hanelt et al. 2012; Bolek et al. 2013a; Harkins et al. 2016). Cysts of gordiids have been reported to be long lived in some paratenic hosts and are found in a variety of aquatic invertebrate and vertebrate species (Hanelt and Janovy 2003). However, Harkins et al. (2016) argued that aquatic snails are the most suitable indicator hosts to sample for gordiid cysts over wide geographic areas. Three major reasons for this argument include the wide and common distribution of snails in aquatic environments, the lack of immunological response of snails to gordiid cysts, and the ease of processing snails for gordiid cysts compared to other aquatic invertebrates (Hanelt et al. 2001; Bolek and Coggins 2002; Bolek et al. 2010; Hanelt et al. 2012; Bolek et al. 2013a). This is particularly important because an ideal indicator host should maintain a parasitic infection for long periods of time, allowing investigators to track the occurrence of that parasite long after other stages have disappeared from a particular geographic location. Additionally, the feeding behavior of snails on the bottom of aquatic habitats makes them ideal hosts to encounter gordiid larvae, which reside in these microhabitats (Hanelt et al. 2001; Bolek et al. 2015). These snails are likely to encounter the microscopic and semi-sessile gordiid larvae more commonly than other invertebrates and vertebrates in aquatic habitats (Hanelt et al. 2005). We hypothesize

that researchers should be able to examine snails in large geographic areas outside of North America to determine the distribution of hairworms.

Host specificity is a major component of life cycle complexity. As defined by Poulin (2007), the widely accepted definition of host specificity includes the extent to which a parasite taxon is restricted in the number of host species used at a given stage in the life cycle. Parasites that use only one species as a host are regarded as highly host specific. This specificity decreases as the range of host species increases. The ability to expand the range of host use is adaptive and may lead to an increase in the chance of transmission. Therefore, one of the goals of host-parasite relationship studies is to discover the range in which parasites use hosts. This can ultimately answer questions about the evolution of life cycles and transmission strategies (Combes 2001; Poulin 2007).

There are several ways to estimate host specificity of a parasite, and these include 1) estimating host specificity by examining published records; 2) examining natural history collections for infected hosts; 3) testing experimental infections in the laboratory; and 4) examining host use in nature. Each of these methods has benefits and challenges. However, the gold standard is by examining hosts in nature and testing experimental infections in the laboratory. Although experimental infections under laboratory conditions can establish compatibility within host-parasite relationships, they do not always offer an ecological perspective on establishment of infection or elucidate compatibility in nature (Hanelt and Janovy 2003, 2004a; Little et al. 2006). Therefore, these methods should be combined with detailed field studies of host use in nature. Unfortunately, such data is currently lacking for all gordiid species (Bolek et al. 2015).

Although cysts can be used as indicators of hairworm reproduction at a location, and they appear to be the most commonly detected stage in nature, few studies have examined the prevalence and distribution of potential paratenic hosts in nature (Yamashita et al., 2017). In a study in Taiwan, Chiu et al. (2016) examined aquatic larval stages of midges in the family Chironimidae for prevalence and seasonal patterns of gordiid cysts in Yangmingshan National Park. They observed three cyst types (*Chordodes formanosus*, *Acutogordius* sp., and an unknown gordiid cyst type), infecting three species of non-bottom midges (Chironomidae) within three different subfamilies (Chironiminae, Tanypodinae, Orthocladiinae). However, midges in the subfamilies Tanypodinae and Orthocladiinae were less commonly infected with hairworm cysts. Conclusions from this study suggest that the infection patterns found among the different larval chironimid groups were driven by simultaneous presence of hairworm larvae (Chiu et al. 2016). Similarly, a recent seasonal study in Japan on the occurrence of gordiid cysts in mayfly paratenic hosts indicated that gordiid cysts were most commonly observed in mayfly groups which were filter-feeders in depositional habits associated with pools rather than riffles in streams (Yamashita 2016). Together, these limited studies on paratenic host use suggest that opportunity is the driving factor between infection of a paratenic host and a gordiid cyst. Additional studies are necessary to determine if this is similar for other species of gordiids and their paratenic hosts as well as the paratenic host range used by gordiids.

Payne County, Oklahoma is an appropriate study site for examining the life cycles of two species of hairworms that utilize two different habitats. Previous studies have created a foundation for gordiid species distributions and species presents (Hanelt et al. 2015; Harkins et al. 2016; Fig. 3). Using gordiid cyst occurrence data from snail paratenic hosts, Harkins et

al. (2016) documented that 70% of first and second order streams in Payne Co., Oklahoma were positive for gordiid cysts. Using cysts folding patterns, it was determined that 50% of the streams samples contained cysts of the genus *Paragordius*, whereas 26% and 10% of the streams contained cysts of *Gordius*, and *Chordodes/Neochordodes*, respectively (Fig. 3). Laboratory infections of cricket hosts with field collected *Paragordius* type cysts confirm the presence of *Paragordius varius* while field collections of adult worms confirmed the presence of *Gordius* cf. *robustus* and *Chordodes morgani*. All hairworm species were identified using scanning electron microscopy for morphological characters and using the CO1 barcoding gene (Harkins et al., 2016). In the process a new species of *Gordius* was discovered. Prior to this work, the only known species of gordiid reported for Oklahoma was *Gordius robustus* (Hanelt et al. 2015; Harkins et al. 2016). Although the discovery of this new species was exciting, little is currently known about the ecology or life history of this new species of gordiid.

Previous work on cyst data suggests that the two most common species of hairworms in Payne County, Oklahoma include *P. varius* and *G.* cf. *robustus* (Harkins et al., 2015). However, my preliminary data suggest that each species appears to utilize a different habitat. For example, specimens of *G.* cf. *robustus* are found to emerge in winter, during bouts of heavy rain. Surveys over the past 24 months have identified adult free-living *G.* cf. *robustus* in street gutters, on sidewalks, within debris in street gutters, and in pooled water in terrestrial systems with numerous specimens witnessed emerging from soil (Fig. 4).

My field observations of deposited spermatophores on free-living female worms indicate that the free-living adults find each other and mate on water-soaked lawns and other terrestrial habitats. In contrast, all reports of free-living adults of *P. varius* indicate that these

worms emerge in streams and ponds during the months of June-August (Hanelt and Janovy 2004; McAllister et al. 2012). These observations seem to suggest that *G.* cf. *robustus* and *P. varius* use different habitats and emerge at different times of the year with *G.* cf. *robustus* demonstrating a terrestrial/semi-terrestrial life cycle, a trait not found in any hairworm thus far (Bolek et al. 2015).

To evaluate if *Gordius* cf. *robustus* completes its life cycle in a semi-terrestrial environment, I examined terrestrial invertebrates (earthworms and land snails) for the presence of *Gordius* type cysts from locations where adult free living worms were observed mating. Examination of land snails and earthworms revealed that they were infected with *Gordius* type cysts (Fig. 5), strongly suggesting that *G.* cf. *robustus* lay egg strings in these habitats and terrestrial invertebrates come in contact with the hatched larvae, ingest them and become infected. Because only *Gordius* type cysts were observed in infected earthworms and land snails, these observations suggest that *P. varius* may be absent from these terrestrial habitats, it is incapable of reproducing in terrestrial habitats, or larvae of *P. varius* are incapable of infecting terrestrial invertebrates such as land snails or earthworms.

Because all previous field observations on adult free-living *P. varius* indicate that these worms emerge during June-August and are found in streams it is currently unclear if larvae of *P. varius* are capable of infecting terrestrial invertebrates (Hanelt and Janovy 2004; McAllister et al. 2012; Bolek et al. 2015). However, previous laboratory studies on paratenic host specificity of three species of hairworms including *P. varius* indicate that there is little host specificity of gordiids in paratenic hosts and larvae of *P. varius* are capable of infecting aquatic snails and oligochaetes (Hanelt and Janovy, 2003). Taken together, these observations suggest that unlike other gordiid species, *G.* cf. *robustus* from Oklahoma is

capable of completing its life cycle in a semi-terrestrial environment. However, currently it is unclear what adaptations if any this species of gordiid has for semi-terrestrial life cycle.

To investigate this further, I collected 200 adult free-living *G.* cf. *robustus* from terrestrial habitats and brought them into the laboratory. Female and male worms were paired in individual standard dishes filled with water and allowed to mate. Once worms mated, females were separated in individual standard dishes filled with water and allowed to release egg strings. Over a period of 1-2 months most females deposited eggs strings. Surprisingly when eggs were examined with a compound microscope, they contained a double membrane (Fig. 6). All previous descriptions of freshwater gordiids including other representatives of the genus *Gordius* and *P. varius* indicate that eggs contain a single membrane (Schmidt-Rhaesa 2012; Bolek et al. 2015; Fig. 6). These observations suggest that the eggs of *G.* cf. *robustus* in Oklahoma may have specific adaptations for a semi-terrestrial life cycle allowing for larvae to develop, hatch and overlap in their distribution with terrestrial paratenic hosts such as earthworms and land snails.

Despite recent advances on the life cycles, distribution, and host use of horsehair worms, few studies have examined the mechanical and physiological impacts of hairworm infections on their arthropod hosts. Previous studies indicate that infected cricket hosts are manipulated by hairworms to jump into water, where worms escape from the host, and in the process the arthropod host commits "suicide" (Thomas et al. 2002; Biron et al. 2005; Biron et al. 2006; Sanchez et al. 2008). Although these studies have clearly demonstrated that hairworms manipulate the behavior of their infected arthropod hosts to enter water and release worms, the host suicide aspect of these studies is difficult to interpret. One reason for this difficulty is that all observations on infected crickets jumping into water and committing

suicide were based on infected crickets jumping into a swimming pool and drowning after the worms were released. Therefore, it is unclear if infected crickets could actually climb out of the swimming pool after they released their worms. Because anecdotal observations on field collected infected arthropods indicate that the arthropods' fat body and gonads are consumed during hairworm infections with only the host gut remaining, it has been assumed that the host dies during worm release or shortly thereafter (Schmidt-Rhaesa 2012). However, other contradictory field observations suggest that some arthropod hosts may survive after releasing their hairworm parasites. A few field observations on infected arthropod hosts indicate that infected arthropods will congregate along the edges of streams and place their abdomen into the water to release worms. After worms emerge the arthropod host moves away from the water source (Schmidt-Rhaesa 2012; Bolek et al. 2015). As part of my PhD investigation on the transmission strategies, host use and host parasite interactions of gordiids, I will evaluate the effects of hairworm infections on their final arthropod hosts during and after worm emerge from their hosts by using the laboratory maintained hairworm model system *P. varius* and its cricket host *Acheta domesticus*.

Other than anecdotal observations on the damage caused by hairworms to their final arthropod hosts, few studies have documented the damage caused by hairworms to their arthropod hosts (Bolek et al. 2015). For example, in a field study Chiu et al. (2015) found that adult male mantids (*Hierodula formosanus*) infected with *Chordodes formosanus*, exhibited antennal sensilla that were feminized. Additionally, morphological changes in wing and leg size of infected male mantids as compared to uninfected mantids, suggested resource exploitation of these hosts by the parasite (Chiu et al. 2015). In my experimental laboratory study, I exposed female house crickets (*Acheta domesticus*) with *P. varius*, and examined

their growth, fat body content and egg production from the time of infection until worms were released. Female house crickets infected with *P. varius*, were significantly smaller in length and had a significantly shorter ovipositor length compared to controls (Fig. 7). Additionally, infected crickets had a significantly smaller amount of fat body and produced no eggs compared to the controls (Fig. 8). Combined, these data suggest hairworms exert morphological and physiological changes in their arthropod hosts although the mechanism by which this occurs is unclear. Additionally, it is unclear whether the host, parasite, or a case of host pathology mediates these impacts.

The following four chapters in this dissertation contribute to our knowledge of hairworm biodiversity and host parasite interactions. In chapter II, I present a description of a new species of hairworm identified from Oklahoma, Texas, and Louisiana and based on my field observations, hypothesize this is the first terrestrial species of hairworm identified for the phylum. In chapter III, I provide evidence for this hypothesis by providing additional field observations supplemented with laboratory experiments to further examine this new species. Additionally, I employ recent and novel sampling techniques to examine the distribution of this species in Stillwater, OK. In chapter IV, I expand these novel sampling techniques to a large geographic region, Iceland. Using these techniques, I contribute to increasing our knowledge of the biodiversity of hairworms by providing the first report of hairworms from Iceland. Additionally, through these sampling techniques, I was able to describe a paratenic and final hosts. Finally, in chapter V, I examine host-parasite interactions by examining the mortality and costs to reproduction of a laboratory-reared species and a known final host. Altogether, each chapter expands our knowledge within the phylum Nematomorpha.

LITERATURE CITED

Annandale N (1905) Supplement—Note on the hair-worms in Oriental and European folklore Journal of the Asiatic Society of Bengal LXXIII: 75-77

Biron, D.G. F. Ponton, C. Joly, A. Menigoz, B. Hanelt, and F. Thomas. 2005. Water-seeking behavior in insects harboring hairworms: should the host collaborate? Behavioral Ecology 16:656-660

Biron, D.G., F. Ponton, L. Marché, N. Galeotti, L.Renault, E. Demey-Thomas, J. Poncet, S.P. Brown, P. Jouin, and F. Thomas. 2006. 'Suicide' of crickets harbouring hairworms: a proteomics investigation. Insect Molecular Biology 15:731-742

Bolek, M.G., and J.R. Coggins. 2002. Seasonal occurrence, morphology, and observations on the life history of *Gordius difficilis* (Nematomorpha: Gordioidea) from southeastern Wisconsin, United States. Journal of Parasitology 88:28-294

Bolek, M.G., A.Schmidt-Rhaesa, B. Hanelt, and D.J. Richardson. 2010. Redescription of the African *Chordodes albibarbatus* Montgomery, 1898, and description of *Chordodes janovyi* n. sp. (Gordiida, Nematomorpha) and its non-adult stages from Cameroon, Africa. Zootaxa 2631:36-50

Bolek, M. G., C. Szmygiel, A. Kubat, A. Schmidt-Rhaesa, and B. Hanelt. 2013a. Novel techniques for biodiversity studies of gordiids and description of a new species of *Chordodes* (Gordiida, Nematomorpha) from Kenya, Africa. Zootaxa 3717: 023-038

Bolek, M. G., E. Rogers, C. Szmygiel, R. P. Shannon, W. E. Doerfert-Schrader, A. Schmidt-Rhaesa, and B. Hanelt. 2013b. Survival of larval and cyst stages of gordiids (Nematomorpha) after exposure to freezing. Journal of Parasitology 99:397-402

Bolek, M. G., Schmidt-Rhaesa, A., De Villalobos, L.C., and B. Hanelt. 2015. Phylum Nematomorpha. In*:* Thorp, J., and Rogers, D.C. (Eds.), Volume I: Ecology and General Biology: Thorp and Covich's Freshwater Invertebrates, 4th ed., Academic Press, 303–326

Chiu, M., C. Huang, W. Wu, and S. Shiao. 2015. Morphological allometry and intersexuality in horsehair-worm infected mantids, *Hierodula formosana* (Mantodea: Mantidae). Parasitology doi:10.1017/S0031182015000360

Chiu, M., C. Huang, W. Wu, and S. Shiao. 2016. Annual survey of horsehair worm cysts in northern Taiwan, with notes on a single seasonal infection peak in chironomid larvae (Diptera: Chironomidae). Journal of Parasitology. 102:319-326

Combes, C. 2001. Parasitism: the ecology and evolution of intimate interactions. University of Chicago Press, 728 pp

Gregor W. (1881) Notes on the Folk-lore of the North-East of Scotland. The Folk-Lore Society by Elliot Stock, 62, Paternoster Row, E.D.

Hanelt, B. and J. Janovy. 1999. The life cycle of a horsehair worm, *Gordius robustus* (Nematomorpha: Gordioidea) Journal of Parasitology 85:139-141

Hanelt, B., L.E. Grother, and J. Janovy, Jr. 2001. Physid snails as sentinels of freshwater Nematomorphs. Journal of Parasitology 87:1049-1053

Hanelt, B. and J. Janovy. 2003. Spanning the gap: experimental determination of paratenic host specificity of horsehair worms (Nematomorpha: Gordiida). Invertebrate Biology 122:12-18

Hanelt, B. and J. Janovy. 2004a. Life Cycle and Paratenesis of American Gordiids (Nematomorpha: Gordiida). Journal of Parasitology 90:240-244

Hanelt, B. and J. Janovy. 2004b. Untying a Gordian knot: the domestication and laboratory maintenance of a Gordian worm, *Paragordius varius* (Nematomorpha: Gordiida). Journal of Natural History 38:939-950

Hanelt, B., F. Thomas, and A. Schmidt-Rhaesa. 2005. Biology of the Phylum Nematomorpha. Advances in Parasitology 59:243-305

Hanelt, B., M. G. Bolek, and A. Schmidt-Rhaesa. 2012. Going Solo: discovery of the first parthenogenetic Gordiid (Nematomorpha: Gordiida). Plos One7, e34472. http://dx.doi.org/10.1371/journal.pone.0034472

Hanelt, B., A. Schmidt-Rhaesa, and M. G. Bolek. 2015. Cryptic species of hairworm parasites revealed by molecular data and crowdsourcing of specimen collections. Molecular Phylogenetics and Evolution 82:211-218

Harkins, C., R. Shannon, M. Papes, A. Schmidt-Rhaesa, B. Hanelt, and M. G. Bolek. 2016. Using Gordiid cysts to discover the hidden diversity, potential distribution, and new species of Gordiids (Phylum Nematomorpha). Zootaxa 4088:515-530

Kollar, V. 1857: Zur Naturgeschichte der Gattung *Gordius* und *Mermis*, Fadenwürmer der Insecten. Verh. Dtsch. Zool. Bot. Ver. Wien 7: 141

Little, T.J., K. Watt, and D. Ebert. 2006. Parasite-host specificity: experimental studies on the basis of parasite adaptation. Evolution 60:31-38

Looney, C., B. Hanelt, and R.S. Zack. 2012. New Records of Nematomorph parasites (Nematomorpha: Gordiida) of ground beetles (Coleoptera: Carabidae) and camel crickets (Orthoptera: Rhaphidophoridae) in Washington state. Journal of Parasitology 98:554-559

McAllister, C. T., M. G. Bolek, and B. Hanelt. 2012. Horsehair worm, *Paragordius varius* (Nematomorpha: Gordiida): New to the fauna of Oklahoma. The Southwestern Naturalist, 58: 249–250

Poinar, G.O., and D. B. Weissman. 2004. Hairworm and nematode infections of North American Jerusalem crickets, field crickets, and katydids (Orthoptera; Stenopelmatidae, Gryllidae and Tettigonidae). Journal of Orthopteran Research 13:143–147

Poinar, Jr., G. and R. Buckley. 2006. Nematode (Nematoda: Mermithidae) and hairworm (Nematomorpha: Chordodidae) parasites in early cretaceous amber. Journal of Invertebrate Pathology 93:36-41

Poulin, R. 1995. Hairworms (Nematomorpha: Gordioidea) infecting New Zealand short–horned grasshoppers (Orthoptera: Acrididae). Journal of Parasitology 81, 121–122.

Poulin, R. 2007. Evolutionary ecology of parasites. Princeton University Press 332 pp.

Sanchez, M. I., F. Ponton, A. Schmidt-Rhaesa, D.P. Hughes, D. Misse, and F. Thomas. 2008. Two steps to suicide in crickets harbouring hairworms. Animal Behaviour 76:1621-1624

Schmidt-Rhaesa, A., B. Hanelt, and W. K. Reeves. 2003. Redescription and compilation of Nearctic freshwater Nematomorpha (Gordiida), with the description of two new species. Proceedings of the Academy of Natural Sciences of Philadelphia 153:77-117

Schmidt-Rhaesa, A., 2012. Nematomorpha. In: Schmidt-Rhaesa, A. (Ed.), Handbook of Zoology. Gastrotricha, Cycloneuralia and Gnathifera. Nematomorpha, Priapulida, Kinorhyncha and Loricifera, vol. 1. De Gruyter, Berlin, pp. 29–145

Studier, E. H., K. H. Lavoie, and C. M. Chandler. 1991. Biology of cave crickets, *Hadenoecus subterraneus*, and camel crickets, *Ceuthophilus stygius* (Insecta: Orthoptera): parasitism by hairworms (Nematomorpha). Journal of Helminthological Society of Washington. 58:248-250

Szmygiel, C., A. Schmidt-Rhaesa, B. Hanelt, and M.G. Bolek. 2014. Comparative descriptions of non-adult stages of four genera of Gordiids (Phylum: Nematomorpha). Zootaxa 3768:101-118

Thomas, F., A. Schmidt-Rhaesa, G. Martin, C. Manu, P. Durand, and F. Renaud. 2002. Do hairworms (Nematomorpha) manipulate the water seeking behavior of their terrestrial hosts? Journal of Evolutionary Biology 15:356-361

White, D. A. 1966. A new host record for Paragordius varius (Nematomorpha). Transactions of the American Microscopical Society 85:579

White, D. A. 1969. The infection of immature aquatic insects by larval *Paragordius* (Nematomorpha) Western North American Naturalist 29:44

Yamashita J, Sato T, Watanabe K (2017) Hairworm infection and seasonal changes in paratenic hosts in a mountain stream in Japan. Journal of Parasitology 103:32-37

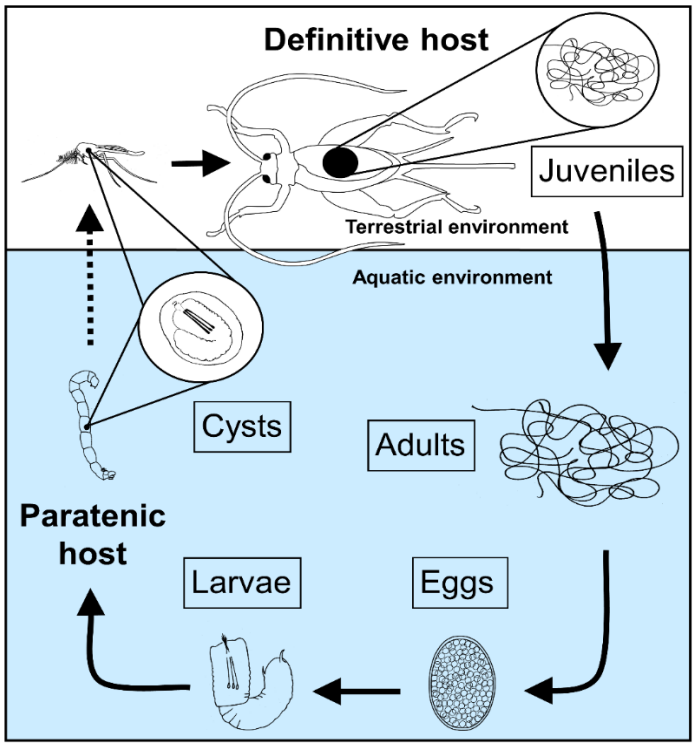

FIGURE 1. Typical life cycle of a freshwater gordiid, including parasitic juveniles within the terrestrial arthropod final host, free-living adults in an aquatic habitat, free-living larvae in an aquatic habitat, and cysts in an aquatic paratenic (transport) host (Hanelt et al., 2012).

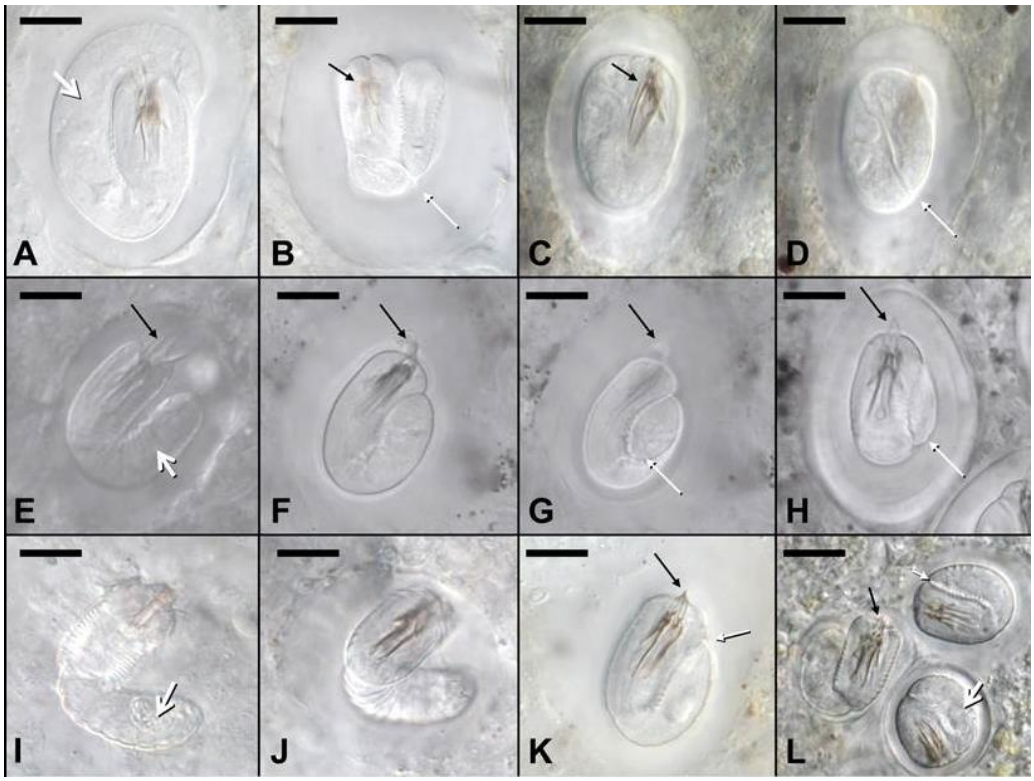

FIGURE 2. Larval folding pattern during cyst formation in four genera of gordiids. (A–D) Cyst characteristics of the genus Gordius. Note larvae fold twice and do not contain, no protruding spines (black arrow). (E–H) Cyst characteristics of the genus Paragordius. Note distinct spines on the preseptum (black arrows), and the position of the posterior end of the postseptum (white arrow). (I–L) Cysts of the genera Chordodes and Neochordodes. Note larvae only folded once (small white arrow) and the presence of relatively small protruding spines on the preseptum (black arrow). Scale bars = 15 µm.

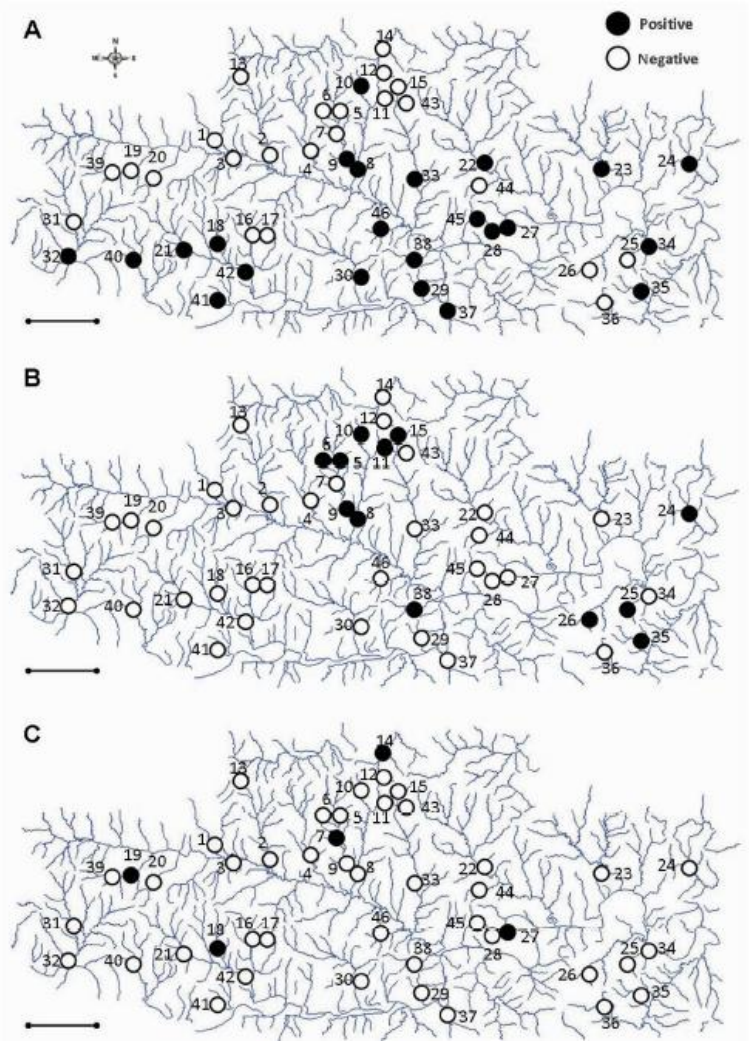

FIGURE 3. Positive (black circles) and negative (white circles) localities for (A) *Paragordius* spp., (B) *Gordius* spp., and (C) *Chordodes*/*Neochordodes* spp. in Payne Co., Oklahoma. Numbers represent names of each site. Scale bars = 6.9 km. From Harkins, et al. (2016).

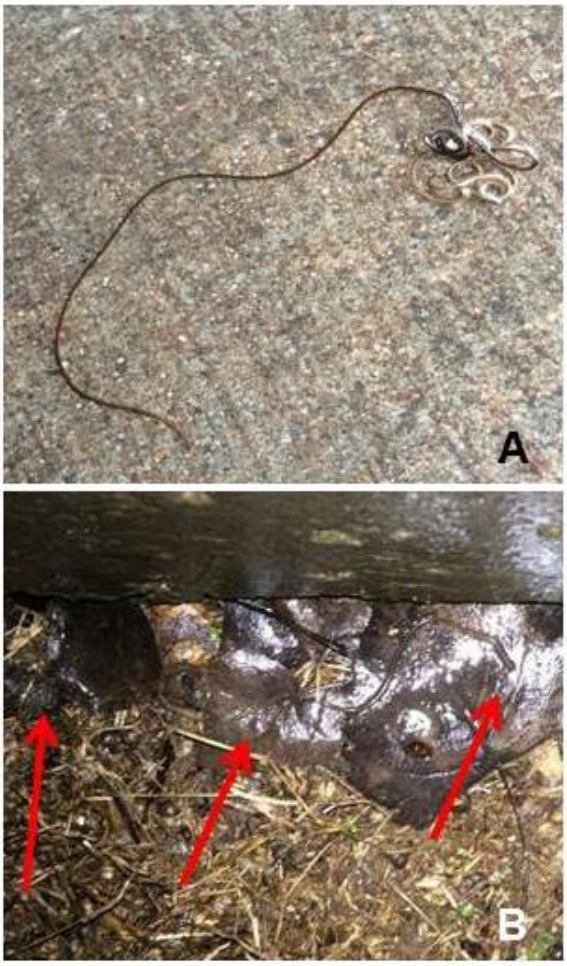

FIGURE 4. Typical habitats (A) concrete driveway and (B) moving along concrete foundation in yard where adult free-living Oklahoma *G*. cf. *robustus* are located and mate.

FIGURE 5. *Gordius* type cyst in an earthworm

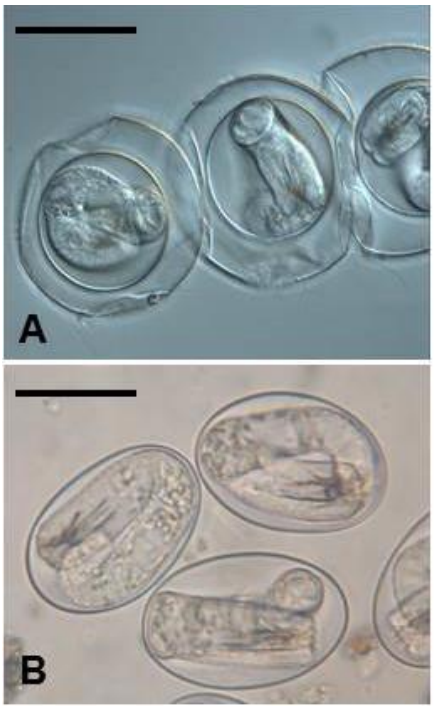

FIGURE 6. A. Double membrane eggs of *Gordius* cf. *robustus* collected from terrestrial habitats. B. Typical single membrane eggs of *Gordius difficilis* collected from a stream in southeastern Wisconsin. Scale bars = 20 µm.

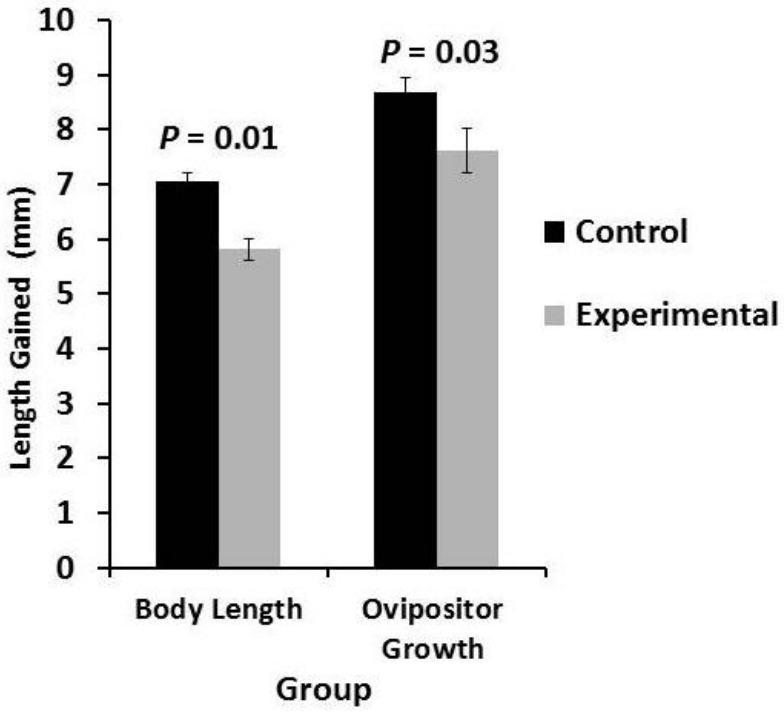

FIGURE 7. Comparison among body length and ovipositor growth of female *Acheta domesticus* infected with *P. varius* and non-infected controls. Error bars represent the standard error of the mean.

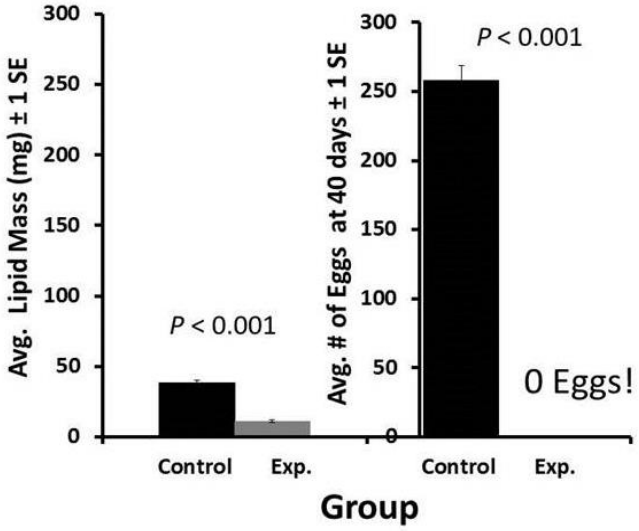

FIGURE 8. Comparison of lipid mass and number of eggs produced between infected female *Acheta domesticus* with *P. varius* and non-infected controls. Error bars represent the standard error of the mean.

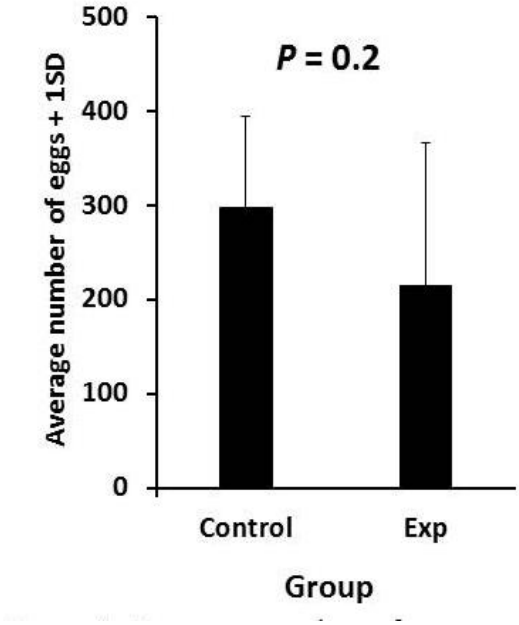

FIGURE 9. Average number of eggs produced by control uninfected group and experimental group of crickets which produced eggs.

CHAPTER II

A NEW SPECIES OF GORDIUS (PHYLUM NEMATOMORPHA) COLLECTED FROM TERRESTRIAL HABITATS IN NORTH AMERICA

ABSTRACT: Freshwater hairworms (class Gordiida) are members of the phylum Nematomorpha that use terrestrial arthropods as final hosts but reside as free-living adult worms in rivers, lakes, or streams. The genus *Gordius* consists of 90 described species, of which three species were described from freshwater habitats in North America. In this paper we describe a new species of *Gordius* collected from terrestrial habitats in Oklahoma, Texas and Louisiana, United States. Oddly, each year hundreds of adult free-living worms appear after bouts of heavy rain on streets, sidewalks, and lawns during the winter season, when terrestrial arthropod hosts are not active. The new species is described based on morphological characters of adults and non-adult stages including the egg strings, eggs, larvae, and cysts. Adult males have a unique row of bristles on the ventral inner side of each tail lobe and a circular pattern of bristles on the terminal end of each lobe, which distinguishes them from all other described North American species of *Gordius*. The egg string, larval and cyst morphology of this new species conform to previous descriptions of non-adult hairworm stages for the genus *Gordius*. However, the

eggs of this new species of hairworm are unique, as they contain a double membrane. The occurrence of this gordiid in terrestrial habitats, along with its distinct egg morphology, suggests that this new species of hairworm has a terrestrial life cycle.

INTRODUCTION

The Phylum Nematomorpha, commonly known as hairworms, or Gordian worms, or simply Gordiids, are parasites of terrestrial arthropods with a complex life cycle that includes a free-living and parasitic phase with multiple hosts (Carvalho 1942; Townsend 1970; Blair 1983; Poinar and Brockerhoff 2001; Hanelt et al. 2005). However, their short lifespan, coloration, and hiding behavior makes hairworms difficult to collect for biodiversity studies (May 1919; Hanelt et al. 2015). An analysis of all known life cycles indicate that juvenile Gordiids infect terrestrial arthropods from which free-living adults emerge into freshwater habitats, such as streams, rivers, and lakes (May 1919; Hanelt et al. 2005; Bolek et al. 2015). After emerging from their arthropod host, dioecious species mate and females deposit egg strings in aquatic habitats (Bolek et al. 2013). Within weeks, larvae develop, hatch, infect, and encyst indiscriminately within a variety of aquatic vertebrate and invertebrate animals (Hanelt and Janovy 2003). Some of these infected animals, such as aquatic insect larvae, act as paratenic (transport) hosts by carrying cysts to land where they are consumed by omnivorous or predatory final hosts including millipedes, crickets, beetles, cockroaches, and mantids (Bolek et al. 2015).

Although first described over 300 years ago, gordiids have been identified as one of the most understudied groups of parasites (Poulin 2006). Currently, it is hypothesized that only 18% of the estimated 2000 gordiid species have been described (Poinar 2008).

Because of their life cycle that includes an aquatic system where worms emerge as free-living adults from their arthropod host, sampling for hairworms and discovering their true biodiversity has been challenging (Hanelt et al. 2005). However, over the last 15 years, advances in sampling, culturing, and barcoding techniques for gordiids have resulted in the descriptions of more than 50 new species including a parthenogenetic species (Schmidt-Rhaesa and Prous 2010; Bolek et al., 2010; Hanelt et al. 2012; Bolek et al. 2013a; Chiu et al. 2017; Swanteson-Franz et al. 2018).

At present, approximately 350 Gordiid species have been described from across the world within 18 extant and two extinct genera (Schmidt-Rhaesa 2013; Bolek et al. 2015). Of those, the genus *Gordius* Linne 1758 is the second largest in terms of described species, with 90 valid species distributed across the world (Schmidt-Rhaesa 2010; Schmidt-Rhaesa 2013). The diagnostic characters for the genus *Gordius* are based on male characteristics and include a semicircular or parabolic cuticular fold posterior of the cloacal opening, known as the postcloacal crescent, and a bilobed posterior end with rounded posterior tips. The posterior end of females is rounded, with a terminal cloacal opening. The anterior end is distinctly tapering, with a white tip, known as a calotte, followed by a brown or black collar which is usually present in both sexes of most species. Additionally, various combinations of a dark ventral and/or dorsal line, and/or white spots on the cuticle are often present on free-living male and/or female worms of several species (Schmidt-Rhaesa 2010). However, compared to other gordiid genera, the genus *Gordius* contains few cuticular structures, such as areoles, that demonstrate intraspecific and interspecific variability making species identification difficult (Schmidt-Rhaesa 2013).

Currently three valid species of *Gordius* have been described from the Nearctic region and include *Gordius attoni* Redlich 1980, *Gordius difficilis* Smith 1994, and *Gordius robustus* Leidy 1851 (Schmidt-Rhaesa et al. 2003; Schmidt-Rhaesa 2010; Schmidt-Rhaesa 2013; Schmidt-Rhaesa et al. 2016). Of those, *G. robustus* is one of the most commonly reported and widely distributed hairworms throughout North America (Schmidt-Rhaesa 2010; Schmidt-Rhaesa et al. 2003). However, recent sampling efforts across North America for *G. robustus*, combined with molecular data indicate that *G. robustus* is a complex of at least 8 distinct species (Hanelt et al. 2015).

Based on genetic data, one of the eight species, identified as clade 7 by Hanelt et al. (2015), occurs in Oklahoma, Texas, and Louisiana. In this article, we describe free-living adults of this new species of *Gordius* collected from locations in Oklahoma and Texas using light and scanning electron microscopy. In addition, we describe the non-adult life stages, including the egg strings, eggs, larvae and cysts. Finally, based on morphological characteristics of non-adult stages, and the occurrence of adult free-living worms of this new species in terrestrial habitats, we provide evidence and suggest that this new species of gordiid has a terrestrial life cycle.

MATERIALS AND METHODS

Field Collections

A total of 39 female and 194 male free-living hairworms were collected from two suburban locations in the city of Stillwater, OK (36.12091, -97.03669; 36.13653, -97.04266). All free-living worms were collected after bouts of heavy rain from streets, sidewalks, or lawns during November through March of 2014 and 2015. In addition, each

location was searched for potential final arthropod hosts by visually scanning areas where worms were found and adjacent lawns. All specimens were placed in 950 ml glass jars containing aged tap water and transported to the laboratory at Oklahoma State University. A subsample of adult worms was processed for morphological characters; whereas the remaining worms were allowed to mate to obtain non-adult life stages (see below). Additionally, two male specimens from a single location in Montgomery, Texas (30.38988, -95.69552) and from Baton Rouge, Louisiana (30.40661, -91.18734 were collected by citizen scientists and sent to us per the instructions on our website (www.nematomorpha.net) and its Report-A-Worm feature.

Biological material and microscopy

Adults. Length, width, color, and color pattern (presence of a calotte, dark pigmented ring, and spots on the cuticle) were recorded for all male and female individuals collected from Stillwater, OK. Lengths of worms were obtained by placing individuals on a ruler without stretching the specimen and measured to the nearest 1 mm. The width of each worm was obtained using an Olympus SZ1145 Stereomicroscope and a calibrated ocular micrometer. Additionally, 10 male worms were fixed in 70% ethanol and a 5 mm section of the posterior end was removed with a razorblade. Posterior ends of males were then photographed with a Sony® Cybershot camera and the angle of postcloacal crescent was measured using ImageJ® software (Schneider et al. 2012).

For scanning electron microscopy (SEM), four female and six male worms collected from Oklahoma and two males collected from Texas were imaged as described by Harkins et al. (2016). Briefly, live worms were preserved in 70% ethanol at room temperature and 5–10 mm sections of the anterior, posterior, and mid-body regions of

each worm were cut with a razorblade. Specimens were then dehydrated in increasing concentrations of ethanol (70 %, 85 %, 95 %, 100 %), dried using hexamethyldisilazane (HDMS) according to Harkins et al. 2016, mounted on aluminum stubs, sputter coated with gold palladium, and examined with an FEI Quanta 600 field emission gun ESEM (ThermoFisher Scientific, Hillsboro, OR) with Evex EDS and HKL EBSD or a JEOL 5800LV SEM at 15 kV (JEOL Ltd., Tokyo, Japan). All terminology for adult worms follows Schmidt-Rhaesa (2010).

Obtaining non-adult stages of *Gordius terrestris* n. sp. A subset of single male and female worms from Stillwater, OK were paired and placed in 110 × 35 mm Stender dishes filled with filtered and aged tap-water (Szmygiel et al. 2014). Observations were made daily on the mating and oviposition behavior of worms. After males deposited a sperm drop on the posterior end of females, females were isolated and allowed to deposit eggs strings in individual Stender dishes filled with aged tap-water. Egg strings were rinsed in a solution of 1-part chlorine bleach to 250-parts water to prevent fungal growth and visually observed over a period of 2–5 weeks for larval maturation, indicated by a color change in egg strings from white to yellow in color. After hatching, a subset of larvae was pipetted into 0.2 mL microtubes and stored at -80°C for snail infections according to Bolek et al. (2013b). To obtain cysts, *Physa acuta* Say 1821 snails were reared in the laboratory according to Szmygiel et al. (2014). A subset of hatched larvae was thawed, collected with a Pasteur pipette and approximately 100–200 larvae were pipetted into 48, 1.5 ml well-plates filled with 1 mm of aged tap water. A single laboratory reared *Physa acuta* snail was then added to each well. Snails fed on the larvae mixture for 48 hours and snails were then maintained in 3.75 L jars filled with aerated

aged tap water and fed on a diet of frozen lettuce and Tetra Min® fish food for a period of four weeks. To evaluate cyst development, every week for a period of four weeks post infection (WPI), a subsample of snails was placed in labeled and capped 50 ml plastic centrifuged tubes, filled with approximately 35 ml of aged tap water, and frozen at -80 °C following the protocol of Bolek et al. (2013b).

Morphology of egg strings, eggs, and larvae. Photographs were taken of two-day old egg strings in Stender dishes and a plastic ruler as a reference using a Sony® Cybershot camera and the length and width of 20 egg strings was measured using ImageJ®. Individual developed eggs, and two-day old larvae after hatching were prepared as live wet mounts and observed using an Olympus BX–51 upright research microscope (Olympus, Tokyo, Japan) configured for bright field and Nomarski differential interference contrast (DIC) microscopy with plain fluorite objectives at 400× to 1000× total magnification. Measurements of developed eggs with larvae were taken from captured digital images using an Olympus 5–megapixel digital camera and ImageJ® software. Briefly, for developed eggs, 5 mm sections of egg strings were placed on microscope slides in a drop of water and they were covered with a coverslip without crushing and observed for their general morphology under an Olympus BX–51 upright research microscope and the length and width was recorded for 30 eggs. For larvae, the length and width of the preseptum, postseptum, pseudointestine and stylets was measured for 30 individuals following the protocols of Szmygiel et al. (2014).

Morphology of cysts. Laboratory infected and post-frozen snails were processed for gordiid cysts following Harkins et al. (2016). Freezing snails before processing prevents cracking of the shell which affects analyses. Briefly, all frozen snails were

thawed, each snail's body was removed from its shell under a dissection microscope using forceps and then pressed between two slides (Harkins et al. 2016). A wet mount was prepared by removing the top slide and adding a drop of water and covering the flattened tissue with a coverslip. Slides were then examined with an Olympus BX–51 microscope as described for eggs and larvae. Thirty cysts were digitally photographed at 1000× total magnification and the length and width of the cyst, cyst wall and encysted larvae were obtained using ImageJ® software. Finally, the folding pattern of all encysted larvae was recorded. Procedures and terminology for cyst stages of gordiids follows Hanelt and Janovy (2002), Szmygiel et al. (2014) and Harkins et al. (2016).

Larval preparation for SEM and larval characters. Pieces of egg strings with developed larvae and hatched larvae suspended in water, were pipetted onto Poly-L-Lysine coated coverslips placed in 1.5 ml plastic well plates and fixed in a solution of alcohol, formalin, and acetic acid. Fixed larvae were dehydrated in a graded series of ethanol in each plastic well with 0.5 ml of 30 %, 50 % and 70 % ethanol for 30 min each, followed by dripping 1 ml of 100% ethanol into the well over a period of an hour, 1 ml of ethanol was then removed from the well and the process repeated three additional times (Harkins et al. 2016). Finally, specimens were dried using HDMS, mounted on aluminum stubs, coated with gold palladium, and examined with an FEI Quanta 600 field emission gun ESEM with Evex EDS and HKL EBSD housed at Oklahoma State University. The following morphological surface characteristics were recorded for at least 30 individual larvae: number of terminal spines on the postseptum, the number and relative size of cuticular hooks on the preseptum, the proboscis orientation (dorso-ventrally or laterally compressed) and the number and orientation of spines on the proboscis. External

morphological characteristics for larvae examined with SEM followed terminology by Szmygiel et al. (2014). All measurements are reported as a mean ± 1 standard deviation followed by the range.

RESULTS

Taxonomy

Gordius terrestris sp. n.

Type Locality. The City of Stillwater, Payne County, Oklahoma; USA (36.12091, -97.03669; elevation: 276-296 m).

Holotype. Male collected on December 5, 2014, from type locality. Deposited into the Museum of Southwestern Biology (MSB) Parasite Division, University of New Mexico (UNM), New Mexico, USA with accession number MSB:Para:?????.

Paratypes. Allotype: female specimen collected on December 5, 2014, from the type locality. Deposited into the MSB Parasite Division, accession number MSB:Para:?????. Paratypes: two males collected January 14, 2003 in Montgomery, Texas (30.38988, -95.69552). Deposited into the MSB Parasite Division, accession numbers MSB:Para:19257 and MSB:Para:19258.

Other material deposited. Larvae fixed in 70% ethanol and on SEM stubs obtained from laboratory cultures from Oklahoma collected worms. Deposited into the Museum of Southwestern Biology (MSB) Parasite Division, University of New Mexico (UNM), New Mexico, USA with accession numbers MSB:Para:????? And MSB:Para:?????.

Host. Natural final host is unknown, and no arthropod hosts were found during worm collections.

Etymology. The new species is named after the terrestrial habitat all adult free-living individuals were collected from.

Distribution. Stillwater, Oklahoma (36.12091, -97.03669; 36.13653, -97.04266), Montgomery, Texas (30.38988, -95.69552) and Baton Rouge, Louisiana (30.40661, -91.18734).

Material examined. Adults (n=233), eggs, larvae, and cysts. Eight adult males, six from Oklahoma and two from Texas and Louisiana and four adult females from Oklahoma were imaged using SEM; and other male and female individuals were examined using DIC and bright field microscopy for color pattern. Additionally, egg, larvae, and cyst stages were imaged using SEM and/or DIC microscopy.

Description of male. Adult free-living males were creamy white to dark brown in color and contained distinct white spots throughout the length of the body (Fig 1D). A dark dorsal and ventral medial line was present along the length of the cuticle being most distinct in the mid-body region (Fig 1D). Males were 258 ± 73 (122–470) mm in length and 0.6 ± 0.1 (0.4–0.9) mm in width. The anterior end was tapered and contained a white calotte followed by a dark collar (Fig 1A, B). The cuticle was variable among individuals but contained one type of areole distributed on the anterior, midbody, and/or posterior regions of the body with various bristles distributed among the areoles (Figs 1C, F, I, 2E, F, G). Areoles were weakly developed, polygonal in shape, and 9–12 μm in diameter (Figs 1C, F, I, 2E, F, G). The posterior end of males contained two terminal tail lobes which were 0.50 ± 0.1 (0.4–0.7) mm long and 0.2 ± 0.04 (0.17–0.3 mm; Figs 1G, H, 2A,

B, C, D) wide. Each tail lobe contained a distinct row of bristles on the ventral inner side and distinct bristles distributed in a circular pattern on the terminal ends of each lobe (Fig 2D). Additionally, the inner-lobes were darkly pigmented compared to the lighter creamy white color of each lobe (Fig 1G). The circular cloacal opening was round and situated ventrally in a broad nonareolar field above the postcloacal crescent (Figs 1G, H, 2A, B, C, D). The postcloacal crescent was situated between the proximal ends of the two tail lobes and was dark brown in color (Fig 1G), and had an angle of 111 ± 9° (102–126°; Figs 1G, H, 2A, B, C, D).

Description of female. Adult free-living females were creamy white to dark brown in color, and contained dark dorsal and ventral lines along the length of the body. Females were 246 ± 41 (211–336) mm long by 1.0 ± 0.1 (0.7–1.3) mm wide. The anterior end was tapered and contained a white calotte followed by a dark collar (Fig 3A, B). Areoles were weakly developed, polygonal in shape, and 11–13 μm in diameter (Fig 3 B). The posterior end of females was round and cylindrical in shape and darkly pigmented on the terminal end (Fig 3G). The cloaca was round in shape and located on the terminal end.

Description of mating, oviposition, egg strings, and eggs. When placed together, male and female worms immediately formed Gordian knots. Males moved up and down the female's body with their coiled posterior end. Once the male's bifurcated tail was in proximity of the female's cloaca, the male deposited a mass of sperm on the female's posterior end. Egg strings were deposited within 7–30 days after copulation. Newly deposited egg strings were white in color and deposited in a continuous string that broke as it emerged from the female's cloaca into short segments (Fig 4A). Deposited

egg strings were 7 ± 4 (2–19) mm in length and 1.2 ± 0.3 (0.8–1.9) mm in width. Over two to three weeks the white eggs strings darkened to a tan color, and contained fully developed larvae within eggs (Figs 4C, D). Developed eggs were tightly aggregated together within egg strings and spherical to elliptical in shape (Figs 4B, C). Eggs were 55 ± 7 (42–72) µm long by 55 ± 7 (43–68) µm wide. Each egg contained an outer shell separated by distinct space from a thick inner membrane (Figs 4B, C, D). The distinct inner membrane was 38 ± 3 (29–42) µm long by 39 ± 4 (30–45) µm wide.

Description of larvae. Larvae of *G. terrestris* n. sp. possessed a cylindrical body divided by a septum into two regions, the preseptum and a postseptum (Figs 5A, B). The preseptum was 30 ± 6 (22–40) µm in length and 20 ± 2 (16–26) µm in width and contained an eversible proboscis supported with three internal stylets which were 17 ± 4 (10–25) µm in length and 5 ± 1.3 (2–8) µm in width (Fig 5B). The postseptum was 106 ± 12 (76–127) µm in length and 20 ± 18 (15–23) µm in width and contained a clearly visible pseudointestine. The pseudointestine was an elongated oval structure, subdivided into two portions (Fig 5A). The pseudointestine was 80 ± 10 (57–104) µm in length and 12 ± 2 (10–17) µm in width.

Externally, larvae were superficially annulated with a single spine located on the posterior region of the postseptum (Fig C). The preseptum had three sets of cuticular hooks (Fig 5D). The outer ring of hooks contained seven hooks, two of which were fused proximally and located on the ventral side (Fig 5D). The middle and inner rings contained six hooks each (Fig 5D). The eversible proboscis contained three pairs of spines and one terminal spine on the distal end of the left lateral, right lateral and dorsal sides (Figs 5E, F).

Cyst development and morphology. After being ingested by snails, larvae develop into cysts and became distributed throughout the snail tissues. During cyst formation the content of the larval pseudointestine was emptied and larvae folded their postseptum twice around the preseptum (Figs 6D, E, F). The posterior end of the postseptum always reached the posterior end of the preseptum and protruding spines were never visible on the anterior end of fully formed cysts (Figs 6A, B). Fully formed cysts of *G. terrestris* n. sp. were observed in laboratory exposed snails 2–3 WPI and possessed a clear cyst wall of unknown composition with a distinct inner layer surrounding the folded larva (Figs 6A, B). Cysts were 102 ±16.7 (68-131) µm in total length and 101 ± 13 (72-140) µm in total width (Fig. 6B). Folded larvae inside of the cyst were 29 ± 7 (17–39) µm in length and 31 ± 5 (18–43) µm in width.

Diagnosis and taxonomic comments. *Gordius terrestris* sp. n. has unique morphological features which warrant placing it as a new species and make it distinct from the other three described Nearctic species of *Gordius*. *Gordius terrestris* sp. n. differs morphologically from *G. difficilis* by lacking distinct pre-cloacal bristles which are present in males of *G. difficilis* (Bolek and Coggins 2002). Additionally, *G. terrestris* sp. n. has distinct polygonal areoles and therefore differs morphologically from the description of *G. robustus* which has a smooth cuticle (Schmidt-Rhaesa et al. 2003). Although, *G. attoni* and *G. terrestris* sp. n. both have polygonal shaped areoles and distinct white spots on the cuticle of males, *G. attoni* areoles contain microscopic processes which are absent on the areoles of *G. terrestris* sp. n. (Bolek and Coggins 2002; Schmidt-Rhaesa et al. 2003). In addition, male *G. terrestris* sp. n. contain an aggregation of bristles on the ventral inner side of each tale lobe posterior of the postcloacal crescent

and distinct bristles distributed in a circular pattern on the terminal ends of each lobe, which are not present in male *G. attoni*, *G. difficilis* or *G. robustus* (Redlich 1980; Smith 1994; Bolek and Coggins 2002; Schmidt-Rhaesa et al. 2003; Schmidt-Rhaesa 2010). Finally, published molecular data by our group on *G. terrestris* sp. n., *G. attoni* and seven other undescribed species of *Gordius* collected across the United States and one undescribed species from Mexico, indicate that *G. terrestris* sp. n. is genetically distinct from all other *Gordius* species for which genetic data are available (Hanelt et al. 2015). Mitochondrial CO1 genetic distances indicate that *G. terrestris* sp. n. differs by 8–21 % in the CO1 genetic distance from the other seven undescribed species of *Gordius* from the United States and one from Mexico and by 17% from *G. attoni*, but only differs by 1.5 % within individuals collected from Oklahoma, Texas, and Louisiana (Hanelt et al. 2015).

With one exception, the general morphology of the egg strings, larvae and larval folding pattern within the cysts of *G. terrestris* sp. n. conform to previous descriptions of these non-adult stages for hairworms in the genus *Gordius*, but these non-adult stages are morphologically distinct from other gordiid genera such as *Chordodes*, *Neochordodes* and *Paragordius* (Szmygiel et al. 2014; Swanteson-Franz et al. 2018). Although the larval morphology conformed to the typical *Gordius* larval type, the three pairs of left, right and dorsal spines on the distal end of the proboscis differed from the only other SEM imaged proboscis of an undescribed species of *Gordius* cf. *robustus* collected from streams in New Mexico (Clade 3 in Hanelt et al. 2015). Szmygiel et al. (2014) reported that the right and left lateral sides of the proboscis of the New Mexico *G.* cf. *robustus* contained four pairs of spines; whereas the dorsal side contained three pairs of spines, all

arranged in tandem. Finally, the egg morphology of *G. terrestris* sp. n. was unlike egg descriptions for any other hairworm species (Adrianov et al. 1998; Schmidt-Rhaesa 1997a; Marchiori et al. 2009; Szmygiel et al. 2014). Eggs of *G. terrestris* sp. n. contained an outer shell separated by distinct space from a thick inner membrane. All previous hairworm egg descriptions indicate that hairworm species collected from aquatic habitats, contain elliptical eggs with a distinct shell and thin inner membrane without a distinct space from the shell, and surrounding the developing larva (Bolek and Coggins 2002; Szmygiel et al. 2014; Bolek et al. 2015).

DISCUSSION

Gordius terrestris sp. n. represents the first hairworm species consistently collected from a terrestrial habitat. Hundreds of adult free-living worms always appeared after bouts of heavy rain on streets, sidewalks, and lawns during the winter season, where male and female worms were observed mating and some females were observed depositing egg strings (unpublished data). It is currently unclear what final host is used in the life cycle of *G. terrestris* sp. n. However, over a two-year sampling period, no arthropod hosts were observed in the areas when adult worms appeared on lawns and sidewalks during the winter months. More intriguing, free-living adult worms would disappear from these locations within days after the rains subsided.

Currently, there is only one other report of a gordiid depositing egg strings in a terrestrial habitat. Schmidt-Rhaesa (2013) reported that he observed male *Gordius aquaticus* in shallow forest streams and ponds in Zweischlingen, Bielefeld, Germany; however, females of this species were observed laying eggs under moist rotting leaves

directly adjacent to the water. In contrast to the *G. aquaticus* observations, all collections of adult *G. terrestris* sp. n. in this study and our previous collections of this species from Oklahoma, Texas and Louisiana in Hanelt et al. (2015) and Harkins et al. (2016) were from terrestrial habitats. Finally, field surveys by Harkins et al. (2016) for hairworm cysts in aquatic paratenic hosts from 46 streams in Payne Co. Oklahoma indicate that *Gordius*-type cysts accounted for 1.7 % (31/1,749) of the total cysts collected, compared to 98.3% of cysts being represented by aquatic hairworm species in the genera *Paragordius*, *Chordodes*, and *Neochordodes* where they commonly mate. This is particularly significant since adults of *G. terrestris* sp. n. is the most commonly encountered gordiid by the public in Texas and Oklahoma (MGB unpublished data) suggesting that *G. terrestris* sp. n. is commonly encountered in terrestrial habitats and nonadult stages are rarely found in aquatic habitats.

One significant observation is the unique egg morphology of *G. terrestris* sp. n. with a thick inner membrane surrounding the developing larval stage. Although few detailed descriptions of hairworm eggs or egg photographs exist in the literature, all indicate that the developing larval stage is surrounded by a thin inner membrane (Schmidt-Rhaesa 1997b; Bolek and Coggins 2002; Bolek et al. 2010; Bolek et al. 2013a; Bolek et al. 2015; Schmidt-Rhaesa 2013). Additionally, our unpublished observations on the egg morphology of three undescribed *Gordius* species collected from aquatic habitats in Nebraska, New Mexico, and California (Clades 2, 3 and 4 in Hanelt et al. 2015) also indicate that the eggs of these aquatic species lack this thick inner egg membrane. Considering the terrestrial habitat free-living adult *G. terrestris* sp. n. occur in, we hypothesize that this unique egg morphology may be an adaptation for terrestrial habitats.

In the future, we plan to publish our detailed observations on mating and oviposition by this species in terrestrial environments and the occurrence of cysts of *G. terrestris* sp. n. in terrestrial paratenic hosts.

LITERATURE CITED

Adrianov AV, Malakhov VV, Spiridonov SE (1998) Fine structure of the larva of *Gordius* sp. (Nematomorpha). Doklady Akademii Nauk 361: 558–561.

Blair D. (1983) Larval horsehair worms (Nematomorpha) from the tissues of native freshwater fish in New Zealand. New Zealand Journal of Zoology 10: 341–344.

Bolek MG, Coggins JR. (2002) Seasonal occurrence, morphology, and observations on the life history of *Gordius difficilis* (Nematomorpha: Gordioidea) from southeastern Wisconsin, United States. Journal of Parasitology 88: 287–294. http://dx.doi.org/10.2307/3285575

Bolek MG, Schmidt-Rhaesa A, Hanelt B. Richardson DJ. (2010) Redescription of the African *Chordodes albibarbatus* Montgomery 1898, and description of *Chordodes janovyi* n. sp. (Gordiida, Nematomorpha) and its non-adult stages from Cameroon, Africa. Zootaxa 2631: 36–54. DOI: 10.11646/zootaxa.2631.1.3

Bolek MG, Szmygiel C, Kubat A, Schmidt-Rhaesa A, Hanelt B. (2013a) Novel techniques for biodiversity studies of gordiids and description of a new species of *Chordodes* (Gordiida, Nematomorpha) from Kenya, Africa. Zootaxa 3717: 23–38. http://dx.doi.org/10.11646/zootaxa.3717.1.2

Bolek MG, Rogers E, Szmygiel C, Shannon RP, Doerfert-Schrader, WE, Schmidt-Rhaesa A, Hanelt B. (2013b) Survival of larval and cyst stages of gordiids (Nematomorpha) after exposure to freezing. Journal of Parasitology 99: 397–402. http://dx.doi.org/10.1645/12-62.1

Bolek MG, Schmidt-Rhaesa A, De Villalobos LC, Hanelt B. (2015) Phylum Nematomorpha. *In:* Thorp, J., and Rogers, D.C. (Eds.), Volume I: Ecology and General Biology: Thorp and Covich's Freshwater Invertebrates, 4th ed., Academic Press, 303–326. ISBN-10:0123850266

Carvalho JCM. (1942) Studies on some Gordiacea of North and South America. Journal of Parasitology 28: 213–222.

Chiu MC, Huang CG, Wu WJ, Shiao SF. (2017) A new orthopteran-parasitizing horsehair worm, *Acutogordius taiwanensis* sp. n., with a redescription of *Chordodes formosanus* and novel host records from Taiwan (Nematomorpha, Gordiida). Zookeys 683: 1–23. http://dx.doi.org/10.3897/zookeys.160.2290

Hanelt B, Janovy J Jr. (2002) Morphometric analysis of nonadult characters of common species of American gordiids (Nematomorpha: Gordioidea). Journal of Parasitology 88: 557–562. http://dx.doi.org/10.2307/3285448

Hanelt B, Janovy J Jr. (2003) Spanning the gap: experimental determination of paratenic host specificity of horsehair worms (Nematomorpha: Gordiida). Invertebrate Biology 122: 12-18. http://dx.doi.org/10.1111/j.1744-7410.2003.tb00068.x

Hanelt B, Thomas F, Schmidt-Rhaesa A. (2005) Biology of the phylum Nematomorpha. Advances in Parasitology 59: 243–305. http://dx.doi.org/10.1016/s0065-308x(05)59004-3

Hanelt B, Bolek MG, Schmidt-Rhaesa A. (2012) Going solo: discovery of the first parthenogenetic gordiid (Nematomorpha: Gordiida). PlosONE https://doi.org/10.1371/journal.pone.0034472.

Hanelt B, Schmidt-Rhaesa A., Bolek MG. (2015) Cryptic species of hairworm parasites revealed by molecular data and crowdsourcing of specimen collections. Molecular Phylogenetics and Evolution 82: 211–218. http://dx.doi.org/10.1371/journal.pone.0034472

Harkins C, Shannon R., Papeş M, Schmidt-Rhaesa A, Hanelt B, Bolek MG. (2016) Using Gordiid cysts to discover the hidden diversity, potential distribution, and new species of Gordiids (Phylum Nematomorpha). Zootaxa 4088:515–530. doi: 10.11646/zootaxa.4088.4.3.

Leidy J. (1851) On the Gordiaceae. Proceedings of the Academy of Natural Sciences of Philadelphia 5: 262- 263, 266 and 275.

Marchiori NC, Pereira JJr, Castro AS. (2009) Morphology of larval *Gordius dimorphus* (Nematomorpha: Gordiida). Journal of Parasitology 95: 1218–1220. doi: 10.1645/GE-2014.1.

May HG. (1919) Contributions to the life histories of *Gordius robustus* Leidy and *Paragordius varius* (Leidy). Illinois Biological Monographs. 5: 1–119.

Poinar GO, Brockerhoff AM. (2001) *Nectonema zealandica* n. sp. (Nematomorpha: Nectonematoidea) parasitizing the purple rock crab *Hemigrapsus edwardsi* (Brachyura: Decapoda) in New Zealand, with notes on the prevalence of infection and host defence reactions. Systematic. Parasitology 50: 149–157. DOI: 10.1023/A:1011961029290

Poinar GO. (2008) Global diversity of hairworms (Nematomorpha: Gordiaceae) in freshwater. Hydrobiologia 595: 79–83. http://dx.doi.org/10.1007/s10750-007-9112-3

Poulin R. (2006) Evolutionary ecology of parasites. Princeton University Press, New Jersey. 360 pp.

Redlich A. (1980). Description of *Gordius attoni* sp. n. (Nematomorpha, Gordiidae) from Northern Canada. Canadian Journal of Zoology 58: 382–385. https://doi.org/10.1139/z80-049

Schmidt-Rhaesa A. (1997a) Ultrastructural observations of the male reproductive system and spermatozoa of *Gordius aquaticus* L. 1758 (Nematomorpha). Invertebrate Reproduction and Development 32: 31–40. https://doi.org/10.1080/07924259.1997.9672602

Schmidt-Rhaesa A. (1997b) Nematomorpha. In: Schwoerbel, J. & Zwick, P. (Eds.), Süßwasserfauna Mitteleuropas. Gustav Fischer Verlag, Stuttgart, pp. 1–124.

Schmidt-Rhaesa A. (2010) Considerations on the genus *Gordius* (Nematomorpha, horsehair worms), with the description of seven new species. Zootaxa 2533: 1–35. DOI: 10.5281/zenodo.196586

Schmidt-Rhaesa A. (2013) Nematomorpha. In: Schmidt-Rhaesa, A. (Ed.), Handbook of Zoology. Gastrotricha, Cycloneuralia and Gnathifera. Nematomorpha, Priapulida, Kinorhyncha and Loricifera. Vol 1. De Gruyter, Berlin, pp. 29–145.

Schmidt-Rhaesa A, Hanelt B, Reeves WK. (2003) Redescription and compilation of Nearctic freshwater Nematomorpha (Gordiida), with the description of two new species. Proceedings of the Academy of Philadelphia 153: 77–117. http://dx.doi.org/10.1635/0097-3157(2003)153[0077:raconf]2.0.co;2

Schmidt-Rhaesa A, Prous, M. (2010) Records of horsehair worms (Nematomorpha) in Estonia, with description of three new species from the genus *Gordius*. Estonia Journal of Ecology 59: 39–51. doi: 10.3176/eco.2010.1.03

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9: 671–675. https://doi.org/10.1038/nmeth.2089

Smith, D.G. (1994): A reevaluation of *Gordius aquaticus difficilis* Montgomery, 1898 (Nematomorpha, Gordioidea, Gordiidae). Proc. Acad. Nat. Sci. Philadelphia 145: 29-34.

Swanteson-Franz RJ, Marquez DA, Goldstein CI, Schmidt-Rhaesa A, Bolek MG, Hanelt B. (2018): New hairworm (Nematomorpha, Gordiid) species described from the Arizona Madrean Sky Islands. ZooKeys 733: 131–145. doi: 10.3897/zookeys.733.22798

Szmygiel C, Schmidt-Rhaesa A, Hanelt B, Bolek MG. (2014) Comparative descriptions of non-adult stages of four genera of Gordiids (Phylum: Nematomorpha). Zootaxa 3768:101–118. http://dx.doi.org/10.11646/zootaxa.3768.2.1

Townsend JI. (1970) Records of gordian worms (Nematomorpha) from New Zealand Carabidae. New Zealand Entomology 4: 98–99. DOI: 10.1645/GE-2929.1

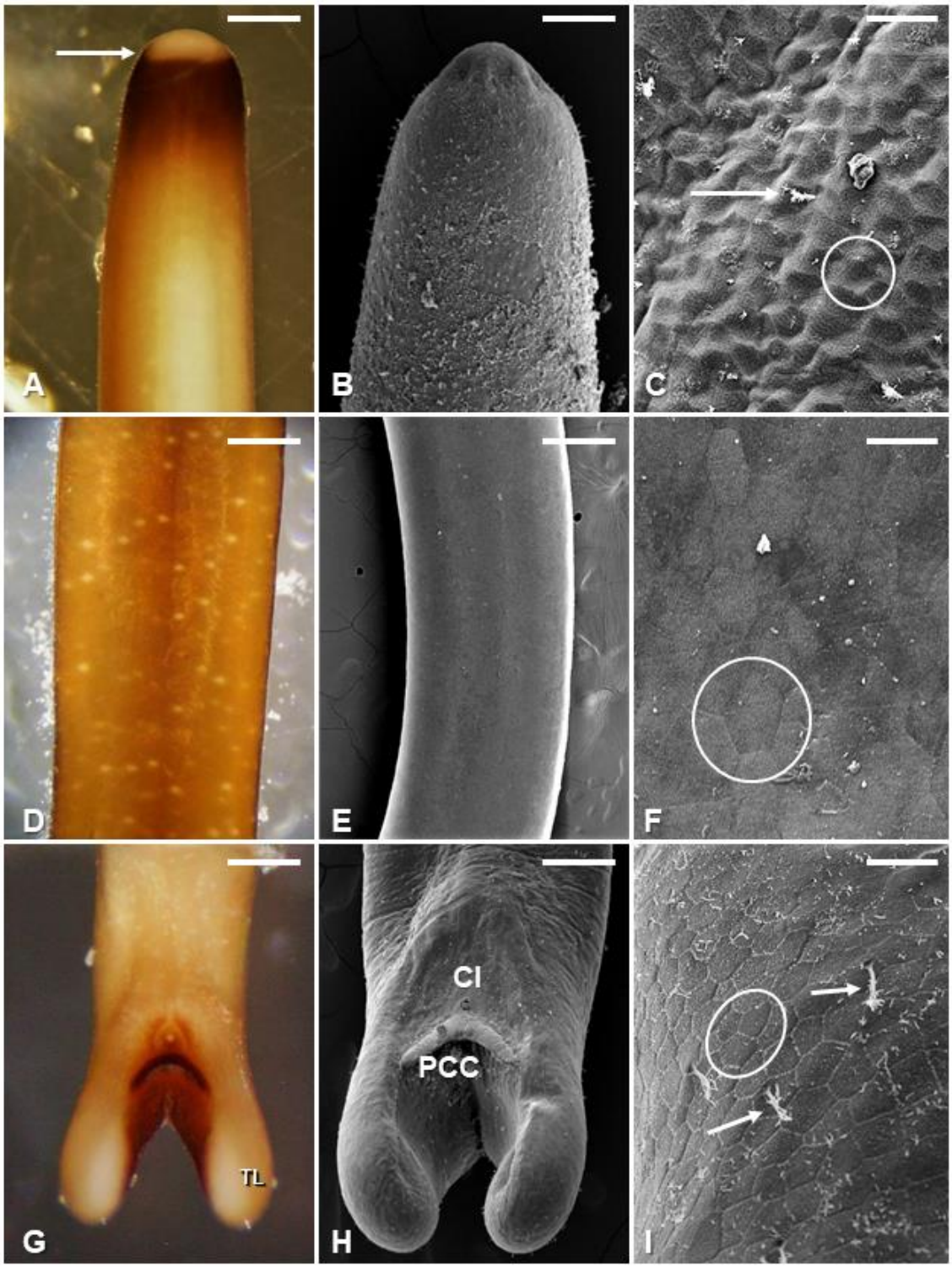

FIGURE 1**.** *Gordius terrestris* sp. n. adult male from Stillwater, Oklahoma. **A** Anterior body region showing typical color pattern, showing the distinct calotte (white arrow) and

the dark ring. Scale bar = 210 µm. (**B)** Typical cuticular pattern; dorsal view of the anterior end showing typical cuticular pattern. Scale bar = 130 µm (**C)** Areole pattern on the anterior body region. Note the weakly developed areoles (circle) and the presence of bristles (white arrows). Scale bar = 18 µm. (**D)** Dorsal view of the midbody region showing distinct white spots and medial line. Scale bar = 220 µm. (**E)** Dorsal view of the midbody region showing typical cuticular pattern. Scale bar = 290 µm. (**F)** Areole pattern on the midbody region. Note the weakly developed polygonal shaped areoles (circle). Scale bar = 10 µm. (**G)** Ventral view of the posterior body region showing distinct coloration. Note the darkly pigmented postcloacal crescent and dark pigmentation on inner sides of the tale lobes (TL). Scale bar = 220 µm. (**H)** Ventral view of the posterior region, showing the cloaca (Cl) and postcloacal crescent with (PCC). Scale bar = 175 µm. (**I)** Areole pattern on the posterior body region. Note the weakly developed polygonal shaped areoles (circle). Scale bar = 20 µm.

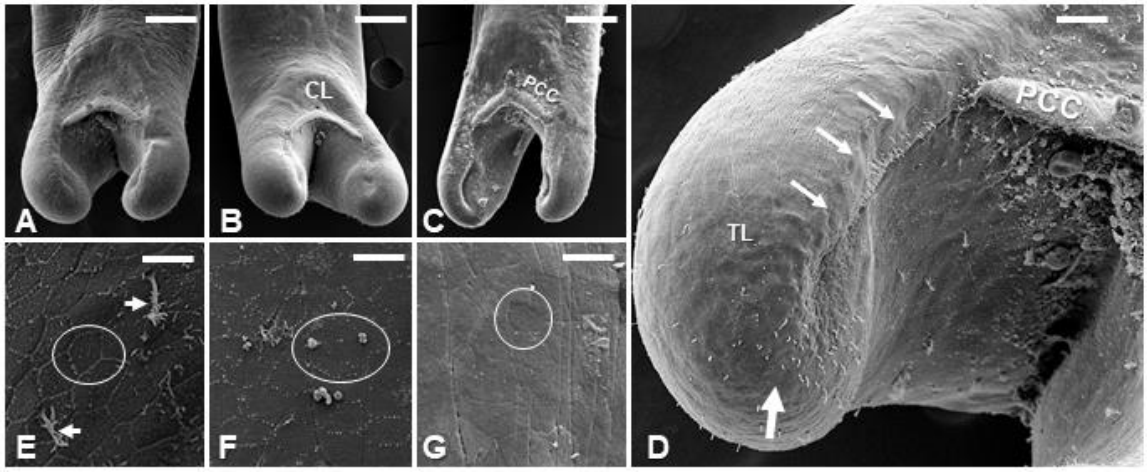

FIGURE 2**.** *Gordius terrestris* sp. n. adult males from Stillwater, Oklahoma. (**A-C)** Ventral view of the posterior body region showing variation in the shape of tail lobes and postcloacal crescents (PCC) below the cloaca (CL). Scale bars =175 µm. (**D)** Higher magnification of tail lobe showing the distinct row of bristles beginning below the postcloacal crescent (PCC) and progressing on the ventral inner side (small arrows) of the tail lobe (TL); and bristles distributed in a circular pattern on the terminal end (large arrow) of the tail lobe. Scale bar = 75 µm. (**E-F)** Variation in the weakly developed polygonal shaped areoles (circles) on the posterior body region of different male individuals. Note the branching bristles (arrows) in E. Scale bars = 8 µm.

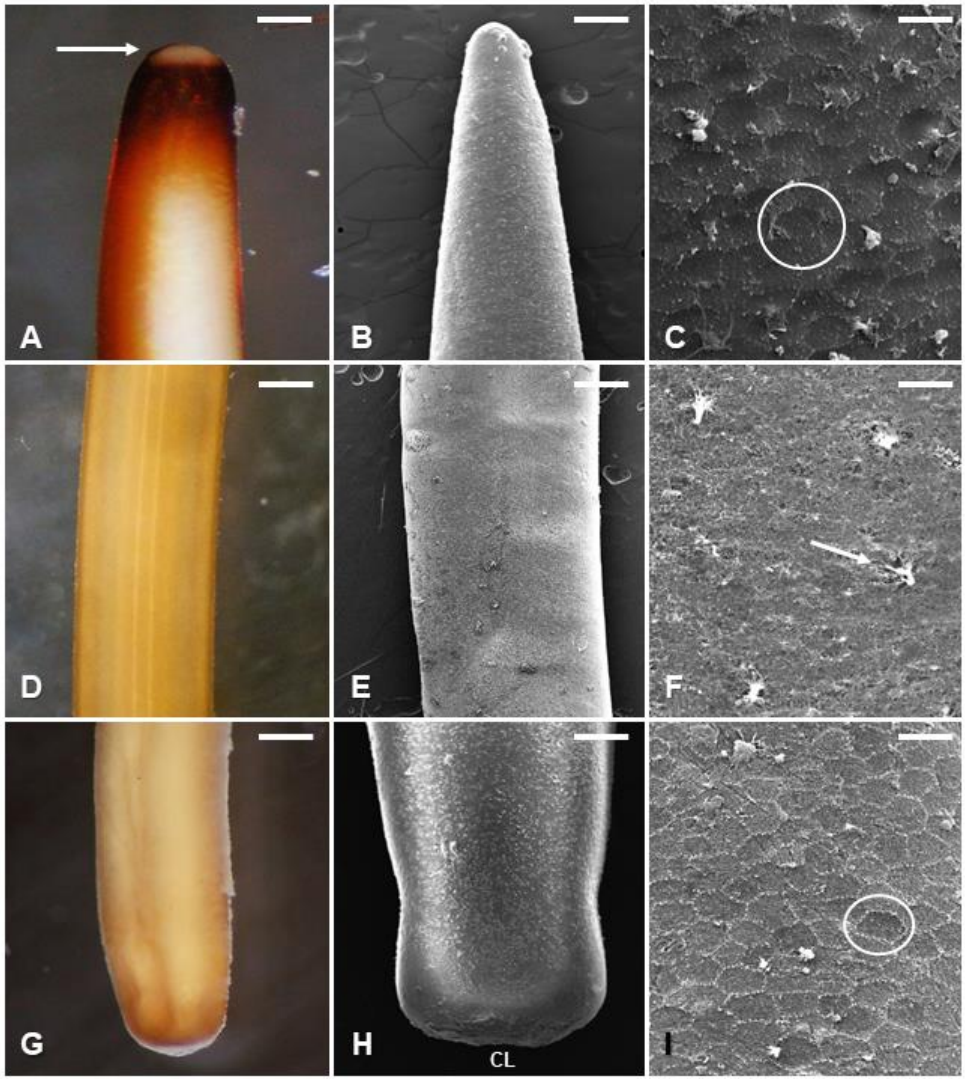

FIGURE 3**.** *Gordius terrestris* sp. n. adult female from Stillwater, Oklahoma. (**A**) Anterior body region showing typical color pattern, showing the distinct calotte (white arrow) followed by a dark ring. Scale bar = 160 µm. (**B**) Dorsal view of the anterior end showing typical cuticular pattern. Scale bar = 150 µm (**C**) Areole pattern on the anterior body region. Note the weakly developed polygonal shaped areoles (circle). Scale bar = 10 µm. (**D**) Lateral view of the midbody region showing typical color pattern. Scale bar = 440 µm. (**E**) Dorsal view of the midbody region showing typical cuticular pattern. Scale bar = 330 µm. (**F**) Higher magnification of the midbody body region. Note the branching

bristles (arrow). Scale bar = 15 µm. (**G**) Ventral view of the posterior body region showing typical coloration. Scale bar = 440 µm. (**H**) Ventral view of the posterior body region showing the location of the cloaca (CL). Scale bar = 190 µm. (**I**) Areole pattern on the posterior body region. Note the weakly developed polygonal shaped areoles (circle). Scale bar = 15 µm.

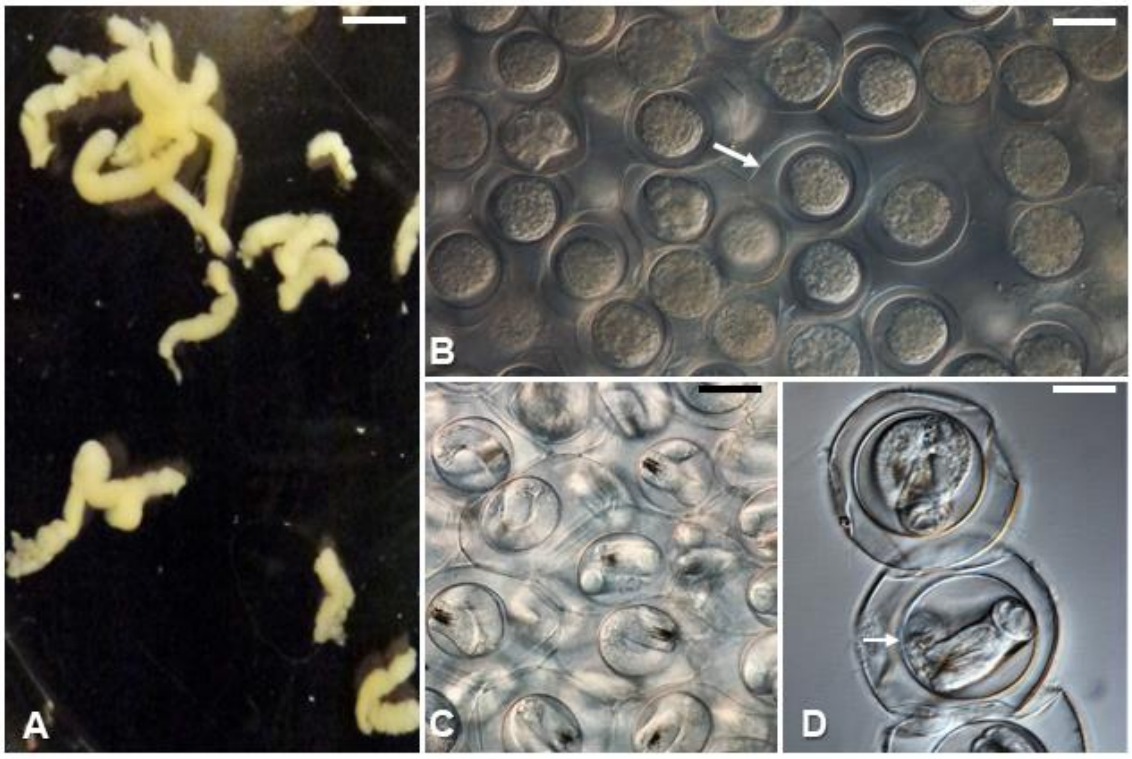

FIGURE 4. *Gordius terrestris* sp. n. eggs and egg strings (**A**) Newly deposited egg strings. Scale bar = 4 mm. (**B**) Higher magnification of an egg string segment showing tightly aggregated undeveloped eggs. Note the egg shell (arrow). Scale bar = 40 µm. (**C**) Segment of an egg string showing developing larvae within eggs. Scale bar = 25. µm (**D**) Higher magnification of eggs with fully developed larvae. Note the distinct space between the egg shell and the thick inner membrane. Scale bar = 20 µm.

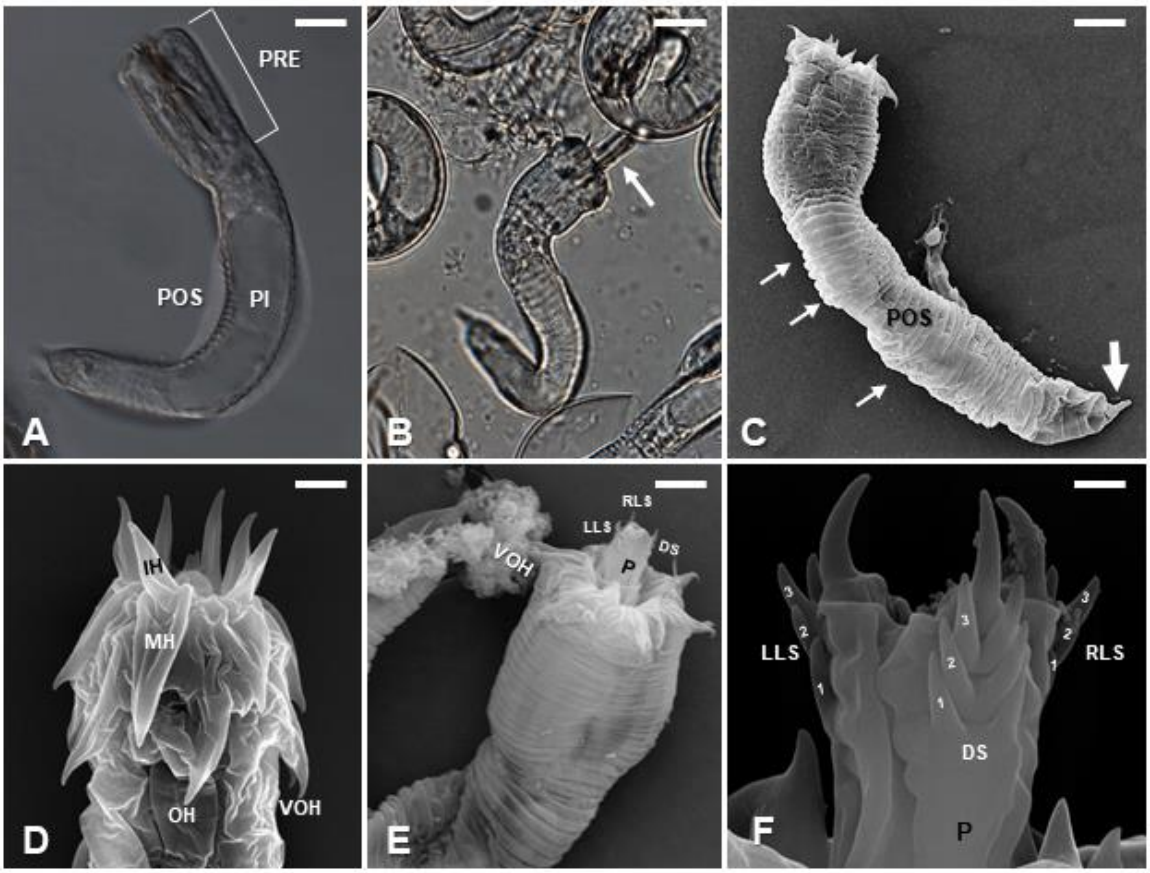

FIGURE 5. *Gordius terrestris* sp. n. larvae (**A**) Photomicrograph of a live larva, showing the preseptum (PRE), postseptum (POS) and pseudointestine (PI). Scale bar = 12 µm. (**B**) Recently hatched larvae showing everted proboscis (arrow). Scale bar = 13 µm. (**C**) SEM photomicrograph showing superficial annulations (small arrows) and a single terminal spine located (large arrow) on the posterior region of the postseptum (POS). Scale bar = 8 µm. (**D**) SEM photomicrograph of the preseptum showing the arrangement of three sets of cuticular hooks, including outer hooks (OH), middle hooks (MH) and inner hooks (IH). Note the fused ventral outer hooks (VOH). Scale bar = 2.5 µm. (**E**) SEM photomicrograph showing the position of the eversible proboscis. Note the distinct spines on the distal end of the left lateral side (LLS), right lateral side (RLS) and dorsal side (DS) in respect to the ventral outer hooks (VOH). Scale bar = 6 µm. (**F**) Higher

magnification of a partially everted proboscis (P) showing three pairs of spines (numbers) and one terminal spine on the distal end of the left lateral (LLS), right lateral (RLS) and dorsal sides (DS). Scale bar = 0.8 μm.

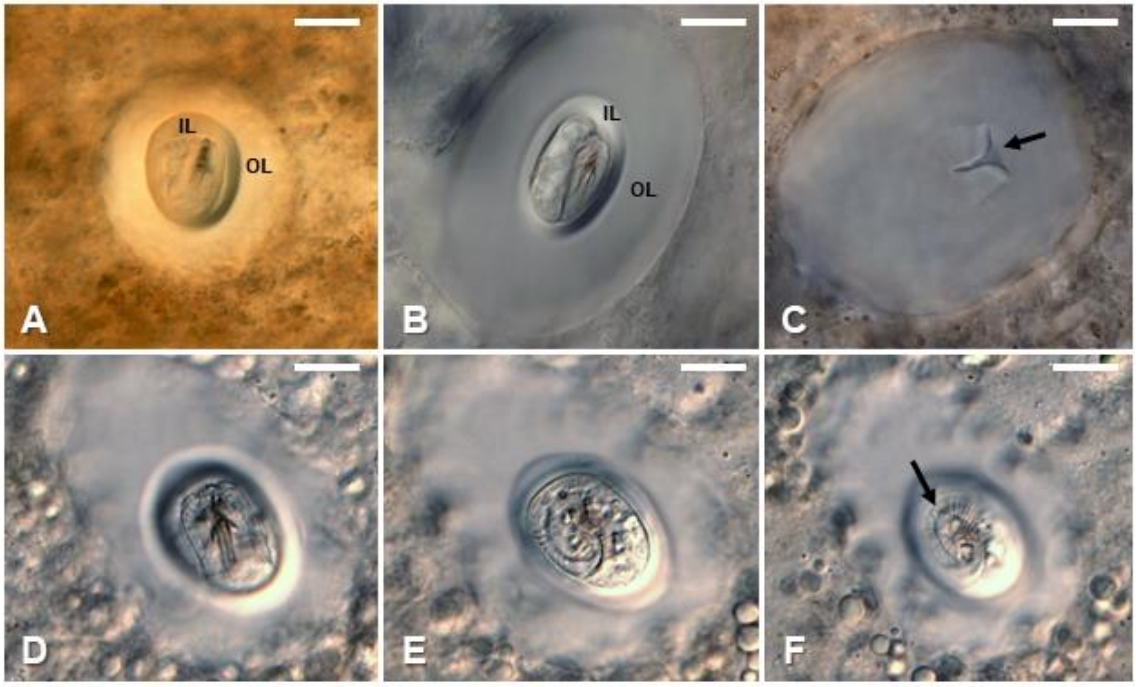

FIGURE 6. *Gordius terrestris* sp. n. cysts (**A**-**B**) Fully formed cysts in experimentally infected *Physa acuta* snails. Note the folded larva surrounded by a clear cyst wall of unknown composition with a distinct inner layer (IL) and outer layer (OL). Scale bars = 20 µm. (**C**) Remaining cyst wall after the folded larvae was extruded under coverslip pressure. Note the opening where the larvae emerged (arrow). Scale bar = 20 µm. (**D-F**) Different focal planes showing the distinct larvae folding pattern. Note the location of the terminal spine (arrow) in **F** and that the larva folds twice within the fully formed cyst. Scale bars = 20 µm.

CHAPTER III

FIELD AND LABORATORY OBSERVATIONS OF *GORDIUS TERRESTRIS* (PHYLUM NEMATOMORPHA), COLLECTED FROM TERRESTRIAL HABITATS IN NORTH AMERICA

ABSTRACT To date all free-living adult hairworms have been reported from aquatic habitats. However, in Oklahoma a recently described gordiid, *Gordius terrestris*, is consistently encountered in terrestrial habitats. More importantly, this gordiid species has a unique egg, unlike that of any other hairworm species, with an outer shell separated by distinct space from a thick inner membrane surrounding the developing larva. Because of this unique egg morphology and the occurrence of free-living hairworms in terrestrial habitats, it was hypothesized that *G. terrestris* represents the first report of a hairworm species with a terrestrial life cycle. In this study I provide information on the habitat, seasonal occurrence, and sex ratio for adult free-living stages of *G. terrestris* collected over a 5-year period from terrestrial habitats. In addition, I document the mating, burrowing and oviposition behavior of free-living adult worms in soil using field and laboratory observations. Finally, I document the cyst stages of this gordiid species in terrestrial earthworms in the field and those observations by infecting earthworms in the laboratory. Taken together these observations strongly support the notion that *G.*

terrestris has a terrestrial life cycle.

INTRODUCTION

The phylum Nematomorpha consists of freshwater and marine species, and represents one of three entirely parasitic animal phyla (Hanelt et al. 2005). Freshwater nematomorphs, commonly known as hairworms or gordiids, parasitize terrestrial arthropods as juveniles and use aquatic arthropods as paratenic (transport) hosts. As parasitic juveniles, hairworms manipulate their hosts to seek water where worms emerge as free-living adults (Meissner 1856). In aquatic habitats, females deposit millions of eggs that develop and hatch as microscopic-sized larvae. Larvae indiscriminately infect and encyst in a variety of aquatic animals (Hanelt and Janovy 2003; Torres et al. 2017). However, the only hairworms that complete their life cycles are those that are transported from aquatic habitats as cysts in metamorphosing aquatic insect paratenic hosts to a terrestrial enviorment where they are ingested by the appropriate terrestrial arthropod final hosts (Carvalho 1942; Townsend 1970; Blair 1983; Poinar and Brockerhoff 2001; Hanelt et al. 2005). Unfortunately, of the approximately 350 described species of freshwater gordiids, the life cycles of only five harirworm species have been elucidated in the laboratory (May 1919; Hanelt and Janovy 1999; Hanelt and Janovy 2004; Hanelt et al. 2012; Bolek et al. 2013a; Swanteson-Franz et al. 2018). As a result, we know less than 2% of the transmission strategies of freshwater gordiids.

Due to their complex life cycle, where adult gordiids exit their terrestrial arthropod hosts as free-living adults in aquatic habitats, studies on the biodiversity, distribution, host use and habittat of hairworms have been problematic (Poinar and Weissman 2004; Hanelt et

al. 2015; Harkins et al. 2016). The short lifespan (2 weeks-2 months), and the ability of free-living hairworms to blend into their environment, has been one of the major challenges in deciphering the biology of gordiids (Bolek and Coggins 2002; Bolek et al. 2015; Harkins et al. 2016). For example, both Hanelt et al. (2001) and Harkins et al. (2016) sampled for free-living adult and cyst stages of gordiids in 50 and 46 streams in a single county in Nebraska and Oklahoma U.S.A., respectively. Free-living adult gordiids were only found in one stream in Nebraska, and no free-living adults were observed in Oklahoma streams. In contrast, cysts of gordiids were found in aquatic snails in 70% of the streams sampled from Nebraska and Oklahoma. These studies indicate that cysts are the most commonly encountered gordiid stages in nature and may be useful for sampling large geographical areas for nematomorph biodiversity studies (Hanelt et al. 2001; Hanelt et al. 2012; Harkins et al. 2016 Bolek et al. 2013a). Using these sampling techniques over the last 10 years has resulted in elucidating three of the five known life cycles of freshwater hairworms in the laboratory and the discovery of the first parthenogenetic species of hairworm for the phylum (Hanelt et al. 2012; Bolek et al. 2013a; Bolek et al. 2013b; Swanteson-Franz et al. 2018).

In addition to their complex life cycles, the identification of adult free-living hairworms has been the other major impediment in our understanding of gordiid diversity and general biology. Most hairworm species were described based on one or a few free-living individuals which were collected haphazardly or as accidental discoveries (Bolek et al. 2015). However, more recently with novel collecting techniques, the standardization of scanning electron microscopy (SEM) and DNA barcoding techniques for species descriptions of gordiids, many novel discoveries have been made on the diversity and general biology of hairworms across the world (Schmidt-Rhaesa and Prous 2010; Bolek et al. 2010; Bolek et al.

2013a; Chiu et al. 2017; Hanelt et al. 2012; Hanelt et al. 2015). For example, *Gordius robustus* Leidy 1851, is one of the most commonly reported hairworms species across North America (Schmidt-Rhaesa et al. 2003; Schmidt-Rhaesa 2010). The major characteristics for the identification of *G. robustus* include color of adult worms, the presence of two tail lobes and a post cloacal cresent in males, and a smooth cuticle with no distinct areoles in both sexes (Schmidt-Rhaesa et al. 2003; Schmidt-Rhaesa 2010; Hanelt et al. 2015). However, most individuals identified as *G. robustus* have never been evaluated using SEM (Hanelt et al. 2015). In addition, observations of several North American populations of *G. robustus* by Hanelt et al. (2015) indicated differences in host use, numerous behaviors, and life cycle timing (seasonality) which suggested that this widely distributed species was comprised of multiple species. As a result, Hanelt et al. (2015) recruited citizen scientists to submit samples of hairworms identified as *G.* cf. *robustus* from across the USA and Canada and in combination with their collections of *G.* cf. *robustus* and other *Gordius* spp. from the USA, Mexico, and Nicaragua, for molecular barcoding. Their molecular evidence from mitochondrial (CO1 and cytB) and nuclear (partial 28S, ITS1, 5.8S and ITS2) DNA sequences indicated that eight clades comprising distinct species were present in the *G.* cf. *robustus* group.

As a result of the above impediments, almost no natural history observations exist for free-living hairworms in the literature (May 1919; Bolek and Coggins 2002). However, more recently one of the *Gordius* species assumed to be *G. robustus* (clade 7 in Hanelt et al. 2015), was described as a new species based on morphological characteristics of adult free-living adult worms and their non-adult stages by Anaya (Chapter II). The results of that work clearly demonstrate that this new species (*G. terrestris*) is morphologically distinct from

other *Gordius* species occuring across North America. More intriguing, the occurrence of this gordiid in terrestrial habitats during the winter months, along with its distinct egg morphology, suggests that this species has a terrestrial life cycle. In this chapter, I provide information on the habitat, seasonal occurrence and other observations on the life history of adult free-living stages of *G. terrestris* collected over a 5 year period. In addition, I document the mating, burrowing and oviposition behavior of free-living adult worms in soil using field and laboratory observations. Finally, I document the cyst stages of this species in terrestrial paratenic hosts (earthworms) in the field and supplement those observations by infecting earthworms in soil with the larvae of *G. terrestris* in the laboratory. Taken together these observations strongly support the notion that *G. terrestris* has a terrestrial life cycle.

MATERIALS AND METHODS

Field Observations

Initially, large numbers of free-living adult hairworms were discovered on lawns and sidewalks during November of 2014 in a 0.28 km^2 area of a suburban location in the City of Stillwater, OK (36.12091, -97.03669; Fig. 1). This area was visually searched for adult free-living worms by walking various streets for a total distance of 3.2-8 km each week during October 2014-August 2017. Because all free-living worms appeared during rain events during October-March, an additional 21 locations in the City of Stillwater were visited during rain events throughout October-March of 2015-2017 and 2018-2019 and visually searched for 45-60 minutes at each location for adult free-living worms and any live or dead arthropod final hosts (Fig. 1). To determine if there was a relationship with monthly precipitation and occurrence of free-living adult worms average monthly rainfall data for the City of Stillwater

was calculated by averaging the monthly rainfall for all months between 2014-2017 and 2018-2019 from data obtained from mesonet.org, and correlated with the number of free-living worms observed during each month.

Because hundreds of free-living worms were present at some of the locations sampled during 2015-2019, when possible, all free-living worms were sexed in the field based on size and the presence or absence of two posterior tail lobes (Fig. 2). Additionally, all female worms were inspected for the presence of a sperm drop on the posterior end to evaluate if they had mated (Fig. 2). All collected specimens were then placed in 946 mL polypropylene plastic containers with lids containing aged tap water and transported to the laboratory. To evaluate size differences among the sexes, the length of male and female worms was obtained in the laboratory by placing individuals on a ruler without stretching the specimen and measuring each worm to the nearest 1 mm. A Student's t-test was used to compare differences in mean length between male and female adult free-living worms; whereas the exact binomial test was calculated to evaluate differences in sex ratios of adult free-living worms collected during different months. Subsamples of collected worms were then processed for morphological analyses, behavioral experiments, or allowed to mate to obtain non-adult stages for experimental infections (see below).

To evaluate if hairworms were ovipositing in terrestrial habitats, and if their eggs developed and hatched as larvae, a common species of terrestrial earthworm (*Diplocardia* sp.), was sampled from 21 of the 22 locations and examined for the presence of hairworm cysts. Earthworms were collected from the surface of waterlogged lawns and sidewalks during the winter months, placed in 946 mL polypropylene plastic containers with lids, and transported to the laboratory. A subsample of earthworms was identified based on earthworm

distribution records for Oklahoma and species/genera identification referenced in Reynolds and Damoff (2010) and Damoff and Reynolds (2019). To process earthworms for hairworm cyst infections, groups of earthworms from each locality were initially placed together in 236 mL polypropylene containers filled with 100 mL of water and allowed to release their gut contents. Over 24 hours, earthworms died, and individual earthworms were transferred to 1.5 ml well-plates filled with 1 mm of aged tap water and allowed to decompose for 48 hours, making their tissue malleable for microscopy. A wet mount was then prepared by placing the partially decomposed earthworm body on a microscope slide with a drop of water, and gently pressing a 22 x 50 mm glass coverslip over the carcass. Wet mounts were examined using an Olympus BX–51 upright research microscope (Olympus, Tokyo, Japan) configured for bright field and Nomarski differential interference contrast (DIC) microscopy with plain fluorite objectives at 100× to 1000× total magnification. The number of cysts and the folding pattern of larvae within cysts was recorded for each earthworm according to terminology in Chapter II, Szmygiel et al. (2014) and Harkins et al. (2016). Digital images of cysts and/or larvae within the tissue of earthworms were obtained using an Olympus 5–megapixel digital camera. Infection parameters are reported as prevalence and mean intensity followed by the range according to Bush et al. (1997).

Finally, because adult free-living hairworms seemed to appear from nowhere during rain events and then disappear after the rains subsided, attempts were made to discover where adult free-living hairworms emerged from and congregated after they mated. All observations were conducted at site 1 during 2017 and 2019. On January 15, 2017, a light sprinkling rain began at approximately 6:00 p.m. and intensified as the evening progressed. A 120 m^2 area of lawn was visually searched every hour until 2:30 am for the appearance of

adult free-living hairworms. As hairworms appeared on the grass, a shovel was used to remove dirt (45 cm in diameter by 38 cm in depth) from a single location where free-living hairworms appeared. The soil was placed on the adjacent concrete, manually separated by hand and searched for free-living adult hairworms and/or any potential arthropod final hosts. Additionally, on December 28, 2018 and after the rain subsided, sod was lifted along the edges of streets and sidewalks and the roots were visually inspected for free-living hairworms and hairworm egg strings. Any free-living adult hairworms observed in the roots were photographed.

Laboratory observations

Because free-living adult *G. terrestris* seemed to disappear from lawns and sidewalks within days after the rains subsided, I evaluated the longevity of free-living and ovipositing female worms in the laboratory. In addition, estimates were obtained for the number of eggs oviposited by free-living adult females. To accomplish this, males and unmated females (without sperm drops) were collected during the first appearance of free-living worms. To prevent mating, worms were grouped based on sex and placed in 946 mL capped polypropylene plastic containers filled with aged tap water and transported to the laboratory. In the laboratory, the length of each female was measured as previously described and each female was paired with one or two males in a 110 × 35 mm Stender dish filled with filtered and aged tap-water. Once males deposited a sperm drop on the posterior end of the females, each female was isolated in a new Stender dish filled with filtered and aged tap-water. Females were monitored for oviposition and survival every three days. Because laboratory observations indicate that hairworms often stop moving for long periods of time, mortality was evaluated by placing females under the light of a dissecting microscope and the anterior

and posterior ends of each female were placed together. If the female did not move from this unnatural position, she was considered to have died. Every three days, a new male was placed with a female and allowed to deposit a sperm drop and the process was repeated until all the females that oviposited died. Any egg strings deposited by females over the three-day period were photographed in the Stender dish. A ruler placed next to the Stender dish was used for calibration and egg string length and width were measured from digital images using ImageJ® software (Schneider et al. 2012). Pearson's correlation was used to determine relationships among hairworm length and total egg string length. Estimates of the number of eggs deposited by each female was conducted by calculating the total volume of egg strings oviposited by each female during her lifespan and dividing the egg string volume by the volume of a single egg according to Hanelt (2009).

To evaluate if adult free-living *G. terrestris* burrowed, oviposited and/or survived in soil, a comparative study was conducted on the burrowing and oviposition behavior and survival of *G. terrestris* and two aquatic species of hairworms, *Gordius difficilis* and *Paragordius varius*. For these trials male and female adult free-living *G. terrestris* were collected in February of 2017 throughout Stillwater, OK and brought back to the laboratory as previously described. To obtain individuals of *P. varius* to be used for concurrent burrowing, oviposition and survival trials, a laboratory strain of *P. varius* was used. Briefly, infected and frozen laboratory reared *Physa acuta* snails with cysts of *P. varius* were thawed at room temperature, removed from their shells and the snail tissue was macerated with a razor blade. A small portion of the soft tissue from a single snail containing approximately 10-15 *P. varius* cysts was fed to a 24-hour-starved commercially reared cricket, *Acheta domesticus*. All crickets were maintained for 24 hours in exposure cages until they ingested

their snail tissue. Post-exposed crickets were maintained in groups of 15 in covered plastic shoeboxes (35 x 25 x 15 cm) with a paper-towel substrate and a 4-cm^2 egg carton for a hiding place. Crickets were provided water in a 50-mm plastic centrifuge tube, filled with aged tap water and plugged with a cotton wick at the end and a supply of Purina Puppy Chow® dog food was provided, ad libitum. Food and water were replaced as needed to avoid mold growth. After 30 days post-exposure (DPE), all crickets exposed to *P. varius* were placed in 110 x 35–mm Stender dishes partially filled with aged tap water and allowed to release worms. Finally, to evaluate if other *Gordius* species could burrow and/or oviposit in soil, males and females of *G. difficilis* were collected from well tanks and their overflow stream in Waukesha County, Wisconsin (42°11.78N, 88°21.65'W) on June 18, 2017, and used for burrowing and oviposition behavior trials.

For burrowing and oviposition trials, Earthgro Topsoils was placed in 236 mL polypropylene cups and moistened. One worm of each species was then placed at the center of each cup and observed for burrowing behavior. Worms were considered to burrow when they actively moved down into the soil as opposed to falling into a crevice around the edge of the containers. For *G. terrestris* only, if female worms burrowed into the soil, the soil was checked for egg strings every 48 hours for 14 days. Additionally, to observe female worm oviposition in the soil, approximately eight cm of soil was placed into an Uncle Milton's Ant Farm® to which individual female *G. terrestris* worms were added, and worms were observed for oviposition behavior every 48 hours. The chi-square test for independence was calculated to compare differences in burrowing behavior between male and female worms, when sample sizes were appropriate.

Finally, to evaluate if *G. terrestris* larvae can survive and infect terrestrial paratenic hosts, commercially available earthworms, *Eisenia fatida*, were exposed to *G. terrestris* larvae in the laboratory. Briefly, *G. terrestris* egg strings were obtained from field collected females as previously described. Egg strings were rinsed in a solution of 1:250 chlorine bleach water to prevent fungal growth and visually observed over a period of 2-5 weeks for larval development following the protocols in Hanelt and Janovy (2002) and Szmygiel et al. (2014). Hatched larvae were stored in 0.2 mL thin wall PCR tubes filled with aged tap water at -80°C following the protocol of Bolek et al. (2013b) and used as needed for earthworm exposures. Earthworms were housed in 29-liter polystyrene plastic storage boxes (L 46 cm x H 30 x W 25 cm) filled halfway with Earthgro Topsoils and stored at 21 °C on a 12:12 hour light: dark cycle. Earthworms were provided a diet of fresh lettuce and miscellaneous vegetable scraps, and the soil was misted with filtered water using a spray bottle as needed. For infections, 10 earthworms were first dissected to insure they were not infected with hairworm cysts. Next approximately 500-600 thawed larvae of *G. terrestris* were pipetted onto the soil surface into a container containing 12 earthworms. Earthworms were processed for cysts of *G. terrestris* 40 DPE as previously described for field collected earthworms.

RESULTS

Field observations

A total of 3,423 free-living adult worms were collected between 2014-2017 and 2018-2019, and included 468 individuals during 2014-2015, 568 individuals during 2015-2016, 1,389 individuals during 2016-2017, and 998 individuals during 2018-2019. Additionally, approximately 1,500 worms were also observed but not collected during the

four year survey. Morphologically, all worms conformed to the discription of *G. terrestris* (Chapter II). Males were 223 ± 74 mm (14-474, n = 1213) in length, creamy white to dark brown in color, with distinct dorsal and ventral lines along the length of the body, and contained white spots on the cuticle. The anterior end was tapered, and a white calotte followed by a dark collar was present. The posterior end of males contained two terminal tail lobes and a distinct post cloacal crescent. Females were 206 ± 59 mm (23-406, n = 240) in length, creamy white to dark brown in color, and contained dark dorsal and ventral lines along the length of the body. The anterior end was tapered, and a white calotte followed by a dark collar was present. The posterior end of females was round and darkly pigmented on the terminal end surrounding the cloaca. Significant differences existed in overall body length between the sexes, where males were significantly longer than females (2-tailed *t*-test, $t = 3.76$, $P < 0.001$; Fig. 3).

Although site 1 was surveyed throughout the year, all free-living adult worms were only encountered during late fall and throughout the winter months. Field observations revealed that free-living worms began appearing in late October, with most worms (62%) being observed during the months of December and January, and the last worms were observed during the month of March.

The occurence of free-living adults always coincided with rain events, even though average monthly percipitation was lowest during those months (Fig. 4). Sex ratio of free-living worms was variable depending on the month and year, but was always male biased (Table I). All adult free-living worms were encountered in terrestrial habitats on wet lawns, and where water pooled on streets and sidwalks (Fig. 5). During a rainy night survey on January 15, 2017, the first male hairworm was observed at 11:30 pm, and over the next three

hours 14 female and eight additional males were collected. Male and female worms were commonly observed mating on wet lawns, sidewalks and streets, and 73% of female worms contained sperm drops on their posterior end (Table I; Fig. 5). No live or dead arthropod hosts were encountered in any of the locations where free-living adult hairworms were observed, or in the soil that was excavated during hairworm emergence on January 15, 2017. However, sampling in February of 2019 revealed that after the rain subsided, one to hundreds of free-living hairworms were discovered underneath five of 20 sod pallets unrooted. Free-living worms were entangled in the roots of grasses in the soil and one female was observed depositing egg strings (Fig. 5).

Of the 22 locations surveyed, adult free-living worms were observed at 18 (82%) of the sites (Table II). Surveys of 946 earthworms from 21 of those locations indicated that hairworm cysts were present in at least one earthworm at 20 of those locations. Depending on the location, prevalence and mean intensity of cysts in earthworms ranged from 0 to 100% and 1 to 68, respectively (Table II). Cysts and larvae in the process of cyst formation were located throughout the musculature of earthworms. All cysts and larvae in the process of forming cysts were of the *Gordius* type. Unencysted larvae contained a postseptum that was three times or more the length of the preseptum; whereas fully formed cysts contained larvae that were always folded twice and without any protruding spines on the preseptum (Fig. 6).

Laboratory observations

A total of 45 *G. terrestris* female worms were observed in the laboratory for longevity and oviposition. Of those, 20 females did not oviposit egg strings despite receiving a sperm drop from a male. Of the remaining 25 females that released egg strings, two escaped from their Stender dishes and thus, observations were recorded for 23 females. On

average females survived 40 ± 12 (21–56) days from the time they were initially collected and 18 ± 13 (0–40) days after laying their last egg strings. Deposited egg strings were white in color and 13.3 ± 7.6 (6.0–33.2) mm in length and 1.2 ± 0.4 (0.3–1.8) mm in width. The average total egg string length oviposited by each female was 407 ± 303 mm (41–1219); whereas the average female body length was 206 ± 48 mm (122–312). As a result, the average total egg string length produced by females exceeded the average body length of females by 2.2 ± 1.6 (0.7–5.3) times. There was no significant correlations between female body length and egg string length (r = 0.072, P = 0.743). Based on the average volume of total egg strings 624 ± 694 (18–2,405) mm^3 oviposited by a female and the average volume of an egg, 8.71 x 10^{-5} mm^3, it was estimated that a female produced 7,171,540 ± 7,974,459 (209,784–27,616,648) eggs during her lifetime.

For concurrent laboratory observations on hairworm burrowing and ovipositing behavior in the soil, a total of 78 *G. terrestris* (57 females and 21 males) and 20 laboratory reared females of *P. varius* were evaluated. In addition, 3 females and 7 males of *G. difficilis* collected from Wisconsin were evaluated on a subsequent date. For *G. terrestris*, 65% (51/78) of worms burrowed into the soil when placed on its surface. However, there were differences in the burrowing behavior among male and female worms. Significantly more females (81%, 46/57) exhibited burrowing behaviors than males (24%, 5/21: χ^2 = 21.9463, P = 0.000003). Of the worms that burrowed into the soil, all did so within three hours of being placed on the soil surface (Fig. 7); and all females were completely covered by the soil with some individuals burrowing to the maximum depth (25 mm) of containers. In contrast, to *G. terrestris*, none (0/20) of the female *P. varius* burrowed into the soil, and all individuals died within 1 day of being placed on the soil surface due to drying (Fig. 7). Finally, all female

(3/3) and 71% (5/7) male worms of *G. difficilis* burrowed into the soil. *Gordius difficilis* remained in the soil until they were removed from the experiment three days later.

Of the 46 *G. terrestris* females that burrowed into the soil, 44 individuals were monitored for oviposition behavior. Of those, four females began laying egg strings within four days of entering the soil and continued to oviposit for an additional 14 days (Fig. 7). Unfortunately, all egg strings and all adult free-living females that burrowed into the soil died within one to two weeks because of a fungal infection. However, 72% (8/11) of earthworms, *E. fatida* that were exposed to *G. terrestris* larvae in the soil became infected. Earthworms contained a total of 97 cysts with a mean intensity of 8.8 ± 12 (2-37). All cysts were of the *Gordius* type and contained larvae that were folded twice without any protruding spines from the proseptum, and morphologically conformed to the cysts observed in field collected earthworms (Fig. 6). None of the 10 *E. fatida* earthworms dissected before being exposed were infected with any gordiid cysts or larvae.

DISCUSSION

Gordius terrestris represents the first gordiid species consistently encountered in terrestrial habitats (Bolek et al. 2015). Previous morphological work on the free-living adult and non-adult stages of *G. terrestris* indicates that this species has a unique egg, unlike that of any other hairworm species, with an outer shell separated by distinct space from a thick inner membrane surrounding the developing larva (Chapter II). Because of this unique egg morphology and the occurrence of free-living hairworms in terrestrial habitats during the winter months when no terrestrial arthropod hosts were active, Anaya (Chapter II), hypothesized that *G. terrestris* represents the first report of a hairworm species with a

terrestrial life cycle. Below I discuss my field and laboratory observations on the habitat, seasonal occurrence, sex ratio, mating, and terrestrial paratenic hosts of *G. terrestris*.

During 2014-2019, all free-living adult hairworms were collected from lawns, streets and sidewalks from 18 locations throughout late fall and winter months. Although a reference site was visually searched for adult free-living worms on a weekly basis over a three-year period, free-living worms only appeared during October through March on any given year. Understanding the seasonal occurrence of hairworms has been challenging because few studies have conducted surveys on free-living hairworms throughout the year (Bolek and Coggins 2002; Salas et al. 2011). However, the few studies that exist on multiple collections of adult free-living worms from a single location indicate that the occurrence of adult worms is seasonal (May 1919; Poulin 1996; Bolek and Coggins 2002, Salas et al. 2015; Daoust et al. 2012; Robison et al. 2012; Salas et al. 2011). For example, Bolek and Coggins (2002) reported the occurrence of free-living adults of *G. difficilis* from Wisconsin, United States, during June through October; whereas Inoue (1958) reported free-living adults of *Chordodes japonensis* from Japan during September and October. Additionally, over a period of three years, Schmidt-Rhaesa et al. (2005) captured hosts and recently emerged adults of two species of hairworms, (*Paragordius tricuspidatus* and *Spinochordodes tellinii*) around a swimming pool in Southern France. Their observations indicate that most adults of *P. tricuspidatus* emerged from their hosts during June through August, whereas most adults of *S. tellinii* emerged from their hosts during August through September. They hypothesized that differences in the seasonal occurrence of these two gordiid species were dependent on the occurrence of their arthropod final hosts. At their study site, both gordiid species infected

different species of final hosts and their occurrence was correlated with the abundance of these hosts.

In contrast to the observations of Schmidt-Rhaesa et al. (2005), and over a four-year collection period a total of 3,423 adult free-living worms were collected during this study. However, no live or dead potential arthropod final hosts were ever observed at any of the 18 locations during the times adult free-living worms were encountered. Although, it is not that surprising that ectothermic arthropod hosts would not be active during the winter months in Oklahoma, what is surprising is the sheer numbers of worms that would appear during rain events. One explanation for the lack of arthropod hosts during this study is that the potential final host for *G. terrestris* might be a fossorial arthropod that rarely, if ever, emerges on to the soil surface. Although this may imply that the manipulation that occurs on the terrestrial final host to seek water infected with aquatic species of hairworms may not be used in a fossorial host. Additional studies including the determination of the final host however, is needed to explore this hypothesis.

Although the emergence of free-living adult hairworms coincided with sporadic rainfall events during the late fall and throughout the winter, no adult free-living worms were ever observed during the spring and summer rains when precipitation was highest. Two explanations for this odd seasonality may involve transmission time and/or developmental time of *G. terrestris* in its final hosts. For example, either the final hosts of *G. terrestris* becomes infected later in the season or the developmental time of *G. terrestris* takes a considerable amount of time in its host during the year. Laboratory studies on the maturation of gordiids within their final host, indicate that this can take a considerable amount of time, ranging from as short as one month for *P. varius*, two to three months for *Chordodes*

kenyaensis and *P. obamai*, and eight months for *Gordius tolosanus* (Svábeník 1925; Hanelt and Janovy 2004; Hanelt et al. 2012; Bolek et al. 2013a).

Once free-living worms appeared during winter rains, they commonly congregated on the surface of lawns and sidewalks in pools of water, where they were observed mating, with 73% of females collected during 2015-2019 containing sperm drops on their posterior ends. Although in the laboratory adult free-living worms survived for an average of 39 days, unpublished observations on escaped laboratory individuals of *G. terrestris* indicate that these worms will desiccate and die within hours if not maintained in a moist environment, suggesting that winter rain events may allow males and females to find each other and mate. Currently, the mechanism by which hairworms find each other to mate is not known. However, their close association with water indicates that water may play some role in finding mates (Bolek et al. 2015). Additionally, during hairworm mating behavior, males move their posterior end up and down the female bodies before placing a sperm drop on the female's cloaca, as a result this mating behavior may be difficult to accomplish in the soil. (Bolek and Coggins 2002; Chapter II).

As in this study, field observations on the sex ratio of free-living gordiids rarely show equal sex ratios and are typically male biased (Poulin 1996; Bolek and Coggins 2002; Bolek et al. 2015). A number of hypotheses have been proposed for this strongly skewed sex ratio, including differences in the development times between male and female worms in their final hosts, and/or behavior differences among free-living males and females (Poulin 1996; Bolek and Coggins 2002). However, this study is the first to document behavioral differences among male and female hairworms. In the laboratory when male and female worms were placed on the surface of the soil, significantly more females burrowed into the soil than

males. This observation is important and provides a plausible explanation for the extremely male biased sex ratio (5.4:1.0) observed for *G. terrestris* in the field and suggests that females may be moving under the soil to oviposit after mating with males.

Although little is known about the burrowing behavior of gordiids, other aquatic North American and European hairworm species from the genus *Gordius* have been occasionally collected from moist soil or from the sediments of streams (May 1919; Thorne 1940; Bolek and Coggins 2002; Schmidt-Rhaesa 2013). For example, Bolek and Coggins (2002) observed that females of *G. difficilis* were often found underneath stream sediments where they deposited egg strings. Similarly, Thorne (1940) reported finding large aggregations of adult free-living *G. robustus* females entangled in roots of plants away from water, and suggested these worms were hibernating during the winter months. These anecdotal observations suggest that gordiids in the genus *Gordius* may commonly burrow into the sediments. Observations from this study on aquatic gordiids support these observations as eight of 10 *G. difficilis* but none of the 20 *P. varius* burrowed into the soil.

Unfortunately, during laboratory trials, only four *G. terrestris* females that burrowed in the soil deposited egg strings, all of which were destroyed by fungal infections. However, examination of earthworms from 21 locations across the study area, revealed that earthworms were commonly infected with *Gordius* type cysts. These observations were complimented by laboratory infections of earthworms with *G. terrestris* larvae, indicating that oviposition, development, and transmission of larvae must be occurring in these terrestrial habitats. The relatively high prevalence and mean intensity of *Gordius*-type cysts in earthworms across the study area is particularly significant when considering previous work on gordiid cysts in aquatic paratenic hosts by Harkins et al. (2016). In their study, they examined aquatic

paratenic hosts from 46 streams in Payne Co. Oklahoma for hairworm cysts. Their findings indicate that *Gordius*-type cysts accounted for only 1.7 % (31/1,749) of the total cysts collected, compared to 98.3% of cysts being represented by aquatic hairworm species in the genera, *Paragordius*, *Chordodes*, and *Neochordodes*. Taken together these observations strongly support the notion that *G. terrestris* has a terrestrial life cycle.

Although biologists and the public have been fascinated with hairworms for hundreds of years, it is remarkable there are so few reports of gordiids from terrestrial habitats. This lack of observations is most likely due to the cryptic habits of free-living adults. However, the discovery of earthworms as paratenic hosts for gordiid during this study, provides a novel tool for evaluating the presence of gordiids in terrestrial habitats across large geographical areas. It is hoped that using this technique can reveal how commonly gordiids occur in terrestrial habitats.

LITERATURE CITED

Annandale N. (1904) Supplement: Note on the hair-worms in Oriental and European folklore. Journal of the Asiatic Society of Bengal. 73:75-77.

Blair D. (1983) Larval horsehair worms (Nematomorpha) from the tissues of native freshwater fish in New Zealand. New Zealand Journal of Zoology 10: 341–344.

Bolek MG, Coggins JR. (2002) Seasonal occurrence, morphology, and observations on the life history of *Gordius difficilis* (Nematomorpha: Gordioidea) from southeastern Wisconsin, United States. Journal of Parasitology 88: 287–294. http://dx.doi.org/10.2307/3285575

Bolek MG, Schmidt-Rhaesa A, Hanelt B. Richardson DJ. (2010) Redescription of the African *Chordodes albibarbatus* Montgomery 1898, and description of *Chordodes janovyi* n. sp. (Gordiida, Nematomorpha) and its non-adult stages from Cameroon, Africa. Zootaxa 2631: 36–54. DOI: 10.11646/zootaxa.2631.1.3

Bolek MG, Szmygiel C, Kubat A, Schmidt-Rhaesa A, Hanelt B. (2013a) Novel techniques for biodiversity studies of gordiids and description of a new species of *Chordodes* (Gordiida, Nematomorpha) from Kenya, Africa. Zootaxa 3717: 23–38. http://dx.doi.org/10.11646/zootaxa.3717.1.2

Bolek MG, Rogers E, Szmygiel C, Shannon RP, Doerfert-Schrader, WE, Schmidt-Rhaesa A, Hanelt B. (2013b) Survival of larval and cyst stages of gordiids (Nematomorpha) after exposure to freezing. Journal of Parasitology 99: 397–402. http://dx.doi.org/10.1645/12-62.1

Bolek MG, Schmidt-Rhaesa A, De Villalobos LC, Hanelt B. (2015) Phylum Nematomorpha. *In:* Thorp, J., and Rogers, D.C. (Eds.), Volume I: Ecology and General Biology: Thorp and Covich's Freshwater Invertebrates, 4th ed., Academic Press, 303–326. ISBN-10:0123850266

Bush AO, Lafferty KD, Lotz JM, Shostak AW. (1997) Parasitology meets ecology on its own terms: Margolis et al. revisited. Journal of Parasitology 83:575-583.

Carvalho JCM. (1942) Studies on some Gordiacea of North and South America. Journal of Parasitology 28: 213–222.

Chandler CM. 1985. Horsehair worms (Nematomorpha, gordioidea) from Tennessee, with a review of taxonomy and distribution in the United states. Journal of the Tennessee Academy of Science. 60:59-62.

Chandler CMChiu MC, Huang CG, Wu WJ, Shiao SF. (2017) A new orthopteran-parasitizing horsehair worm, *Acutogordius taiwanensis* sp. n., with a redescription of *Chordodes formosanus* and novel host records from Taiwan (Nematomorpha, Gordiida). Zookeys 683: 1–23. http://dx.doi.org/10.3897/zookeys.160.2290.

Cochran, P.A., Newton, A.K. & Korte, C. (2004) Great gordian knots: sex ratio and sexual size dimorphism in aggregations of horsehair worms (*Gordius difficilis*). Invert. Biol. 123: 78-82.

Cotton S, Small J, Pomiankowski A. 2006. Sexual selection and condition-dependent mate preferences. Current Biology 16:R755-R765.

Daoust SP, Schatz B, Missé D, Thomas F. (2012) Ecology of Gordian knots in natural conditions. Invertebrate Biology. 131 :1-7. DOI: 10.1111/ivb.12000

Degrange, C. & Martinot, J.-P. (1995) Gordiacés parasites d´orthoptères du Parc National de la Vanoise. Trav. Sci. Parc Nat. Vanoise 19: 161-182.

Dong X, Donoghue PHC, Cunningham JA, Liu J, Cheng H. (2005) The anatomy, affinity, and phylogenetic significance of Markuelia. Evolution and Development 7:468-482.

Dorier, A. (1928) Quelques stations de Gordiacés en Dauphiné. Trav. Lab. Hydrobiol. Piscicult. Univ. Grenoble 20: 43-47.

Dujardin, F. (1842): Mémoire sur la structure anatomique des *Gordius* et d´un autre Helminthe, le *Mermis*, qu´on a confondu avec eux. Ann. Sci. Nat. Zool. 18: 129-151.

Hamid S, Aslam M (1985) Parasitism of grasshoppers by *Mermis nigrescens* Dujardin (Mermithidae: nematoda) in Pakistan. Pakistan Journal of Zoology 17:71-75.

Hanelt, B. and J. Janovy. 1999. The life cycle of a horsehair worm, *Gordius robustus* (Nematomorpha: Gordioidea) Journal of Parasitology 85:139-141.

Hanelt, B., L.E. Grother, and J. Janovy, Jr. 2001. Physid snails as sentinels of freshwater Nematomorphs. Journal of Parasitology 87:1049-1053. DOI: 10.2307/3285230

Hanelt B, Janovy J Jr. (2003) Spanning the gap: experimental determination of paratenic host specificity of horsehair worms (Nematomorpha: Gordiida). Invertebrate Biology 122: 12-18. http://dx.doi.org/10.1111/j.1744-7410.2003.tb00068.x

Hanelt B, Thomas F, Schmidt-Rhaesa A. (2005) Biology of the phylum Nematomorpha. Advances in Parasitology 59: 243–305. http://dx.doi.org/10.1016/s0065-308x(05)59004-3

Hanelt B, Bolek MG, Schmidt-Rhaesa A. (2012) Going solo: discovery of the first parthenogenetic gordiid (Nematomorpha: Gordiida). PlosONE https://doi.org/10.1371/journal.pone.0034472.

Hanelt B, Schmidt-Rhaesa A., Bolek MG. (2015) Cryptic species of hairworm parasites revealed by molecular data and crowdsourcing of specimen collections. Molecular Phylogenetics and Evolution 82: 211–218. http://dx.doi.org/10.1371/journal.pone.0034472

Harkins C, Shannon R., Papeş M, Schmidt-Rhaesa A, Hanelt B, Bolek MG. (2016) Using Gordiid cysts to discover the hidden diversity, potential distribution, and new species of Gordiids (Phylum Nematomorpha). Zootaxa 4088:515–530. doi: 10.11646/zootaxa.4088.4.3.

Heinze, K. (1937) Die Saitenwürmer (Gordioidea) Deutschlands. Eine systematisch-faunistische Studie über Insektenparasiten aus der Gruppe der Nematomorpha. Z. Parasitenkd. 9: 263-344.

Leidy J. (1851) On the Gordiaceae. Proceedings of the Academy of Natural Sciences of Philadelphia 5: 262- 263, 266 and 275.

May HG. (1919) Contributions to the life histories of *Gordius robustus* Leidy and *Paragordius varius* (Leidy). Illinois Biological Monographs. 5: 1–119.

Mesonet.org Norman, OK. https://www.mesonet.org/index.php

Morgulis A., Coulouris G., Raytselis Y., Madden T.L., Agarwala R., & Schäffer A.A. (2008) Database indexing for production MegaBLAST searches. Bioinformatics 15:1757-1764.

Mühldorf, A. (1914) Beiträge zur Entwicklungsgeschichte und zu den phylogenetischen Beziehungen der Gordiuslarve. Z. Wiss. Zool. 111: 1-75.

Poinar, GO (1991) Hairworm (Nematomorpha: Gordioidea) parasites of New Zealand wetas (Orthoptera: Stenopelmatidae). Can. J. Zool. 69: 1592-1599.

Poinar GO, Brockerhoff AM. (2001) *Nectonema zealandica* n. sp. (Nematomorpha: Nectonematoidea) parasitizing the purple rock crab *Hemigrapsus edwardsi* (Brachyura: Decapoda) in New Zealand, with notes on the prevalence of infection and host defence reactions. Systematic. Parasitology 50: 149–157. DOI: 10.1023/A:1011961029290

Poinar GO, Chandler CM (2004) synopsis and identification of North American hairworms (Gordioidea: Nematomorpha) Journal of the Tennessee Academy of Science 79:1-7

Poinar, G.O., and D. B. Weissman (2004) Hairworm and nematode infections of North American Jerusalem crickets, field crickets, and katydids (Orthoptera; Stenopelmatidae, Gryllidae and Tettigonidae). Journal of Orthopteran Research 13:143–147.

Poinar GO. (2008) Global diversity of hairworms (Nematomorpha: Gordiaceae) in freshwater. Hydrobiologia 595: 79–83. http://dx.doi.org/10.1007/s10750-007-9112-3

Poulin R. (1996) Observations on the free-living adult stage of *Gordius dimorphus* (Nematomorpha: Gordioidea). J. Parasitol. 82: 845-846.

Poulin R. (1997) Covariation of sexual size dimorphism and adult sex ratio in parasitic nematodes. Biological Journal of the Linnean Society 62:567-580. https://doi.org/10.1111/j.1095-8312.1997.tb00322.x

Robison HW, McAllister CT, Hanelt B. (2012). New geographic distribution records for horsehair worms (Nematomorpha: Gordiida) in Arkansas, including new state records for *Chordodes morgani* and *Paragordius varius*. Journal of the Arkansas Academy of Science 66:197-199.

Salas L, De Villalobos C, Zanca F. (2011) Sexual size dimorphism, sex ratio and the relationship between seasonality and water quality in four species of Gordiida (Nematomorpha) from Catamarca, Argentina. Journal of Helminthology 85: 319–324. https://doi.org/10.1017/S0022149X1000057X

Sato T. (2010) Adult hairworms face the risk of ingestion by stream salmonids via predation on their cricket hosts. Limnology 12:83-88.

Schmidt-Rhaesa A. (2013) Nematomorpha. In: Schmidt-Rhaesa, A. (Ed.), Handbook of Zoology. Gastrotricha, Cycloneuralia and Gnathifera. Nematomorpha, Priapulida, Kinorhyncha and Loricifera. Vol 1. De Gruyter, Berlin, pp. 29–145

Schmidt-Rhaesa A., Biron D.G., Joly Cécile, Thomas F. (2005) Host-parasite relations and seasonal occurrence of *Paragordius tricuspidatus* and *Spinochordodes tellinii* (Nematomorpha) in southern France. Journal of Comarative Zoology 244:51-57

Schmidt-Rhaesa A, Kristensen P. (2006). Horsehair worms (Nematomorpha) from the Baltic island Bornholm (Denmark), with notes on the biology of *Gordius albopunctatus*. Journal of Natural History 40:495-502.

Schmidt-Rhaesa A, Prous, M. (2010) Records of horsehair worms (Nematomorpha) in Estonia, with description of three new species from the genus *Gordius*. Estonia Journal of Ecology 59: 39–51. doi: 10.3176/eco.2010.1.03

Spiridonov, SE, Pikula ZP, Drljevic ET 1992. Redescription of *Dacochordodes bacescui* Capuse, 1966 (Nematomorpha: Chordodidae). Helminthologica 29, 193–196.

Švábeník, J. (1925) Parasitism and metamorphosis of the species *Gordius tolosanus* Duj. (Parasitismus a metamorfosa druhu *Gordius tolosanus* Duj.). Publ. Fac. Sci. Univ. Masaryk 58: 1-48 (in Czech with English summary).

Swanteson-Franz RJ, Marquez DA, Goldstein CI, Schmidt-Rhaesa A, Bolek MG, Hanelt B. (2018): New hairworm (Nematomorpha, Gordiid) species described from the Arizona Madrean Sky Islands. ZooKeys 733: 131–145. doi: 10.3897/zookeys.733.22798

Szmygiel C, Schmidt-Rhaesa A, Hanelt B, Bolek MG. (2014) Comparative descriptions of non-adult stages of four genera of Gordiids (Phylum: Nematomorpha). Zootaxa 3768:101–118. http://dx.doi.org/10.11646/zootaxa.3768.2.1

Thomas, F., A. Schmidt-Rhaesa, G. Martin, C. Manu, P. Durand, and F. Renaud (2002) Do hairworms (Nematomorpha) manipulate the water seeking behavior of their terrestrial hosts? Journal of Evolutionary Biology 15:356-361

Thorne G. (1940). The hairworm, *Gordius robustus* Leidy, as a parasite of the Mormon cricket, *Anabrus simplex* Haldeman. Journal of the Washington Academy of Sciences 30:219-231

Torres, P. Leyan, V., Lamilla, J. (2017) Cyst stages of gordiids (nematomorphs) and other eukaryotic parasites from the inanga, *Galaxias maculatus* (Osmeriformes: Galaxiidae), in the lingue river. Southern Chile Comp. Parasitol. 84:72-79

Townsend JI. (1970) Records of gordian worms (Nematomorpha) from New Zealand Carabidae. New Zealand Entomology 4: 98–99. DOI: 10.1645/GE-2929.1

Von Linstow, O. (1891) Weitere Beobachtungen an *Gordius tolosanus* und *Mermis*. Arch. Mikrosk. Anat. 37: 239-249

Weißbecker, M. & Schmidt-Rhaesa, A. (2010) Beobachtungen zur Lebensweise von Saitenwürmern mit Schwerpunkt in West- und Mittelhessen im Zeitraum 2005 – 2008. Hessische Faunistische Briefe 29: 21-45.

Table 1. Number of male and female *Gordius terrestris*, their sex ratio, and percent of female worms with a sperm drop collected during October-March of 2014-2017 and 2018-2019 from 22 locations in Stillwater, Payne Co., Oklahoma.

Month and year		Males	Females	Sex ratio of males to females	Exact binomial test	*P*-value	Percent of females with sperm drop
November	2014	4	0	4.0:0.0	1.00	0.13	ND
December	2014	22	1	22.0:1.0	0.96	<0.005	ND
February	2015	56	16	3.5:1.0	0.78	<0.005	ND
March	2015	306	63	4.9:1.0	0.83	<0.005	ND
October	2015	2	0	2.0:0.0	1.00	0.500	ND
November	2015	335	26	8.4:1.0	0.89	<0.005	85 (22/26)
December	2015	6	0	3.0:0.0	1.00	0.25	NA
January	2016	106	11	11.5:1.0	0.91	<0.005	73 (8/11)
February	2016	47	9	5.2:1.0	0.84	<0.005	78 (7/9)
March	2016	5	1	5.0:1.0	0.83	0.22	100 (1/1)
December	2016	105	33	3.2:1.0	0.76	<0.005	85 (28/33)
January	2017	833	164	5.1:1.0	0.84	<0.005	85 (139/164)
February	2017	220	22	22.0:1.0	0.91	<0.005	86 (19/22)
March	2017	12	0	12.0:0.0	1.00	<0.005	NA
October	2018	21	2	10.5:1.0	0.91	<0.005	50 (1/2)
November	2018	8	0	8.0:0.0	1.00	<0.005	NA
December	2018	697	159	4.4:1.0	0.81	<0.005	56 (89/159)
January	2019	14	11	1.3:1.0	0.56	0.69	36 (4/11)
February	2019	11	0	11.0:0.0	1.00	<0.005	NA
March	2019	94	1	94.0:1.0	0.99	<0.005	100 (1/1)
Total		2,904	519	5.4:1.0	0.84	<0.005	73 (319/439)

ND = Females were not evaluated for sperm drops during the first sampling year (fall 2014 and winter 2015); NA = Not applicable, no females found.

Table 2. Number of adult free-living *Gordius terrestris* collected, prevalence, and mean intensity (range) of *Gordius* type cysts in earthworms, *Diplocardia* sp., collected from 22 locations throughout Stillwater, Payne Co., Oklahoma.

Location	Latitude	Longitude	No. of hairworms collected	No. infected/no. examined	Prevalence (%)	Mean intensity	Total number of cysts
1	36.121083	-97.036857	1,194	47/70	67	29 (1-120)	1,372
2	36.12107	-97.030581	128	8/15	53	68 (1-233)	541
3	36.122109	-97.029975	51	6/16	38	11 (1-53)	65
4	36.120463	-97.035259	975	44/59	75	19 (1-660)	3474
5	36.144577	-97.045291	39	51/100	51	25 (1-121)	1,288
6	36.107269	-97.044048	109	53/101	52	4 (1-19)	209
7	36.098309	-97.125904	2	30/41	73	32 (1-134)	972
8	36.166412	-97.056281	108	54/105	53	54 (1-575)	2,903
9	36.096749	-97.087756	0	3/28	11	9 (1-7)	10
10	36.09715	-97.088892	74	53/79	67	39 (1-135)	2,082
11	36.104782	-97.096685	0	15/15	100	20 (2-166)	300
12	36.12205	-97.04577	20	1/20	5	2 (2)	2
13	36.10196	-97.111287	30	16/27	59	7 (1-46)	116
14	36.132525	-96.990082	148	1/11	9	3 (3)	3
15	36.150496	-97.037762	53	6/67	9	1 (1)	6
16	36.102145	-97.087360	22	6/39	15	2 (1-3)	13
17	36.168323	-97.059061	0	0/4	0	0	0
18	36.123236	-97.040286	0	1/15	7	1 (1)	1
19	36.145237	-97.067971	19	14/86	16	16 (1-85)	223
20	36.138083	-97.038376	132	8/22	36	6 (1-21)	48
21	36.14127	-97.038522	0	1/30	3	2 (2)	2
22	36.14339	-97.0669	319	NA	NA	NA	NA
Total			3,423	418/946	38	33 (1-660)	13,630

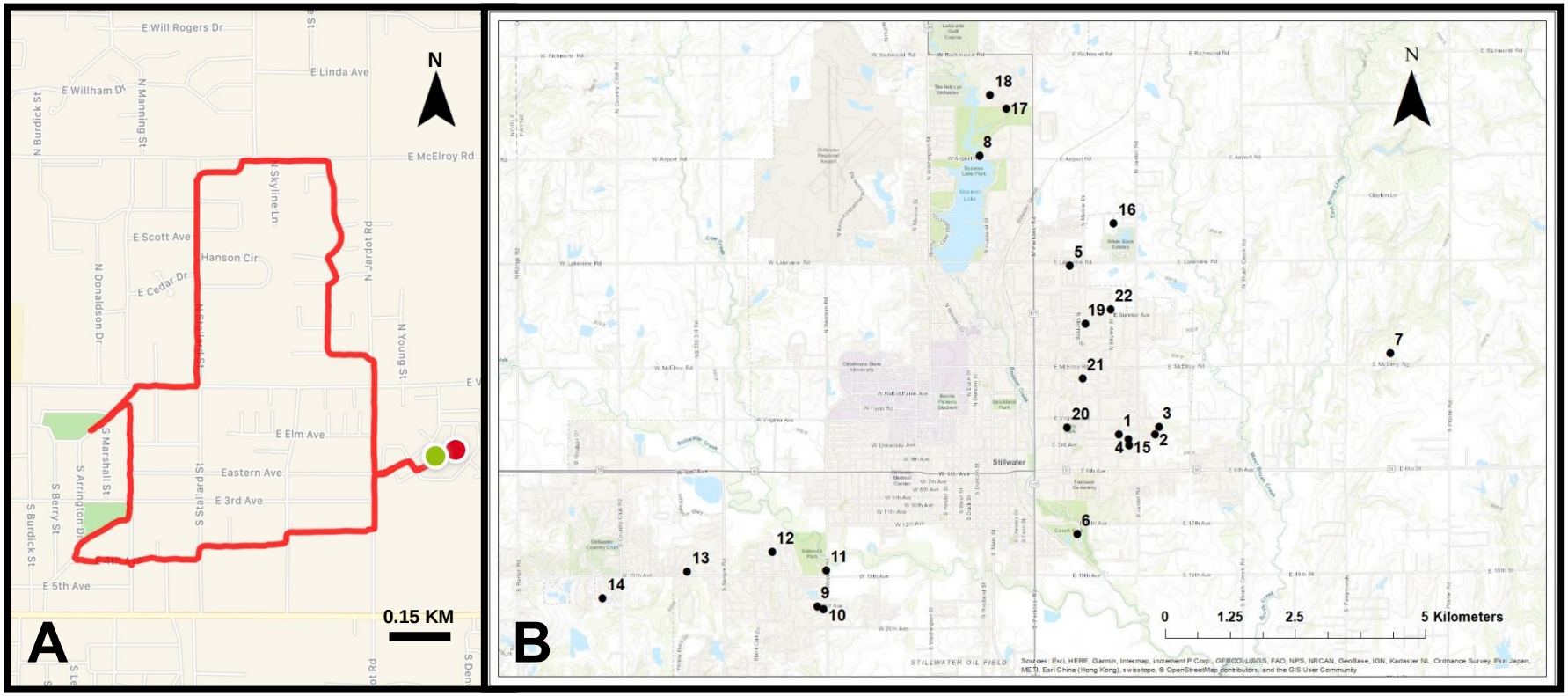

FIGURE 1. (**A**) Walking route where hairworms were surveyed over 4 years (2014-2017; 2018-2019). (**B**) Collection sites for adult free-living worms and earthworms.

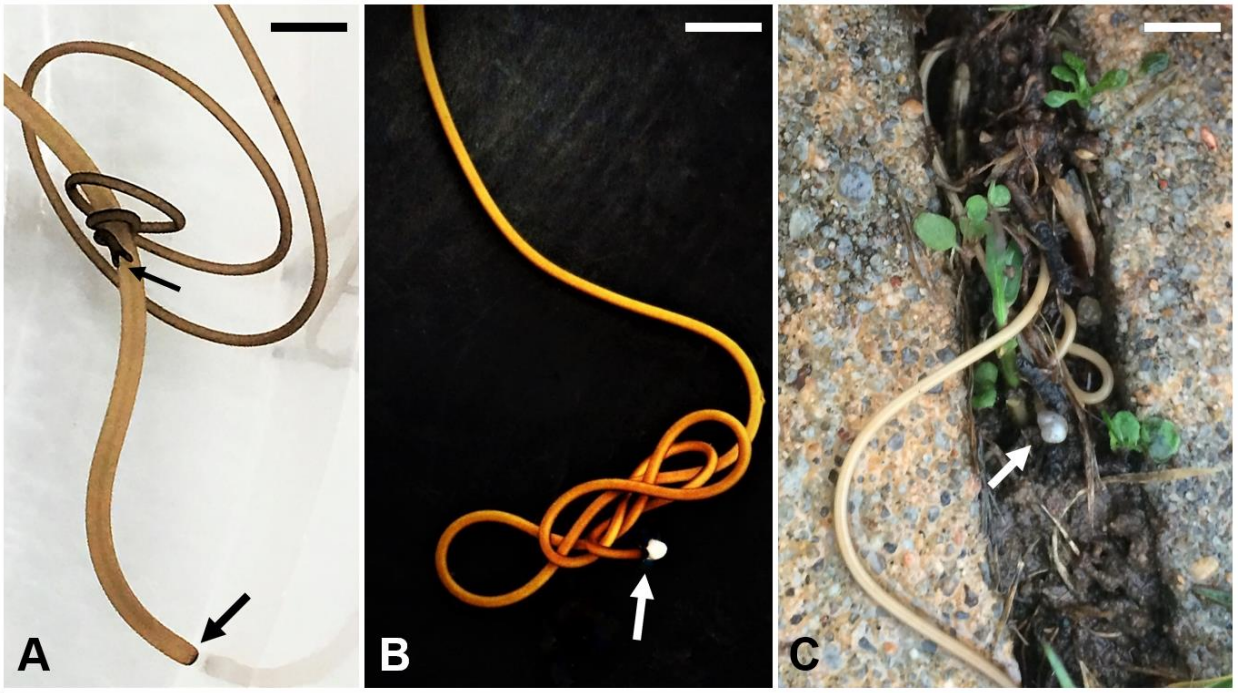

FIGURE 2. Female posterior ends before and after mating. (**A**) A male posterior end (small black arrow) coiled around female body (large black arrow). Scale bar 5 mm. (**B**) Female posterior end after male deposited a sperm drop (white arrow) on the cloaca. Scale bar 10 mm. (**C**) A female with a sperm drop (white arrow) after mating in a terrestrial system. Scale bar = 8 mm.

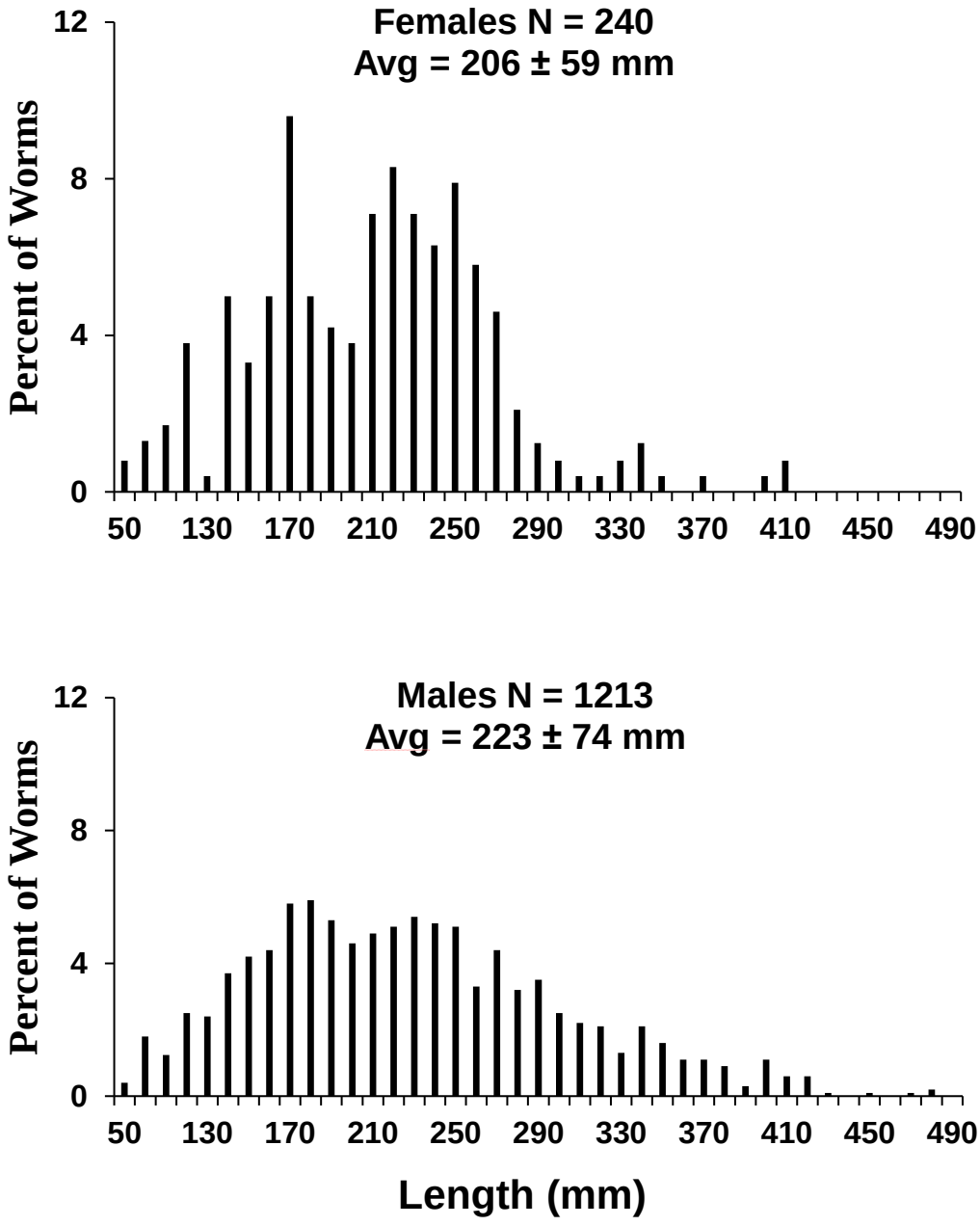

FIGURE 3. Frequency distribution of lengths of adult male and female *Gordius terrestris* collected from northcentral Oklahoma.

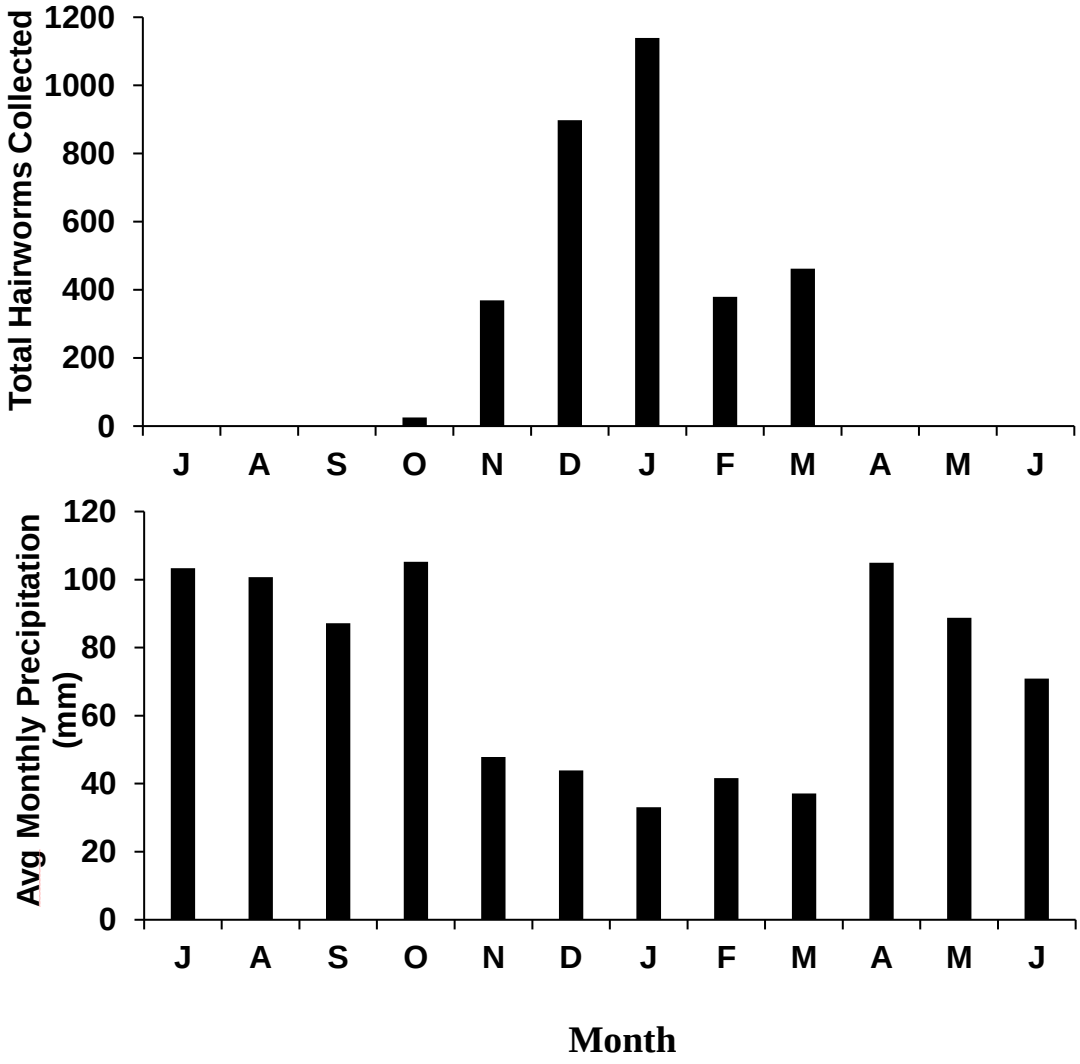

FIGURE 4. Total hairworm collections by months over a four year period 2014-2017, and 2018-2019 in relation to the average monthly rainfall totals in Stillwater, OK.

FIGURE 5. Locations where hairworms were commonly found after rain events. (**A**) Site 1 where water accumulates in a grassy area worms were often found in gordian knots. Scale bar = 10 mm. (**B**) Female with a sperm drop (white arrow) located near the sidewalk's edge, indicating mating had occurred. Note color variation of male in the vicinity and the coloration of female. Scale bar = 5 mm. (**C**) Group of male worms located below the grass's edge. Scale bar = 10mm. (**D**) Female with sperm drop located beneath grasses edge but partially submerged below concrete. Scale bar = 10 mm.

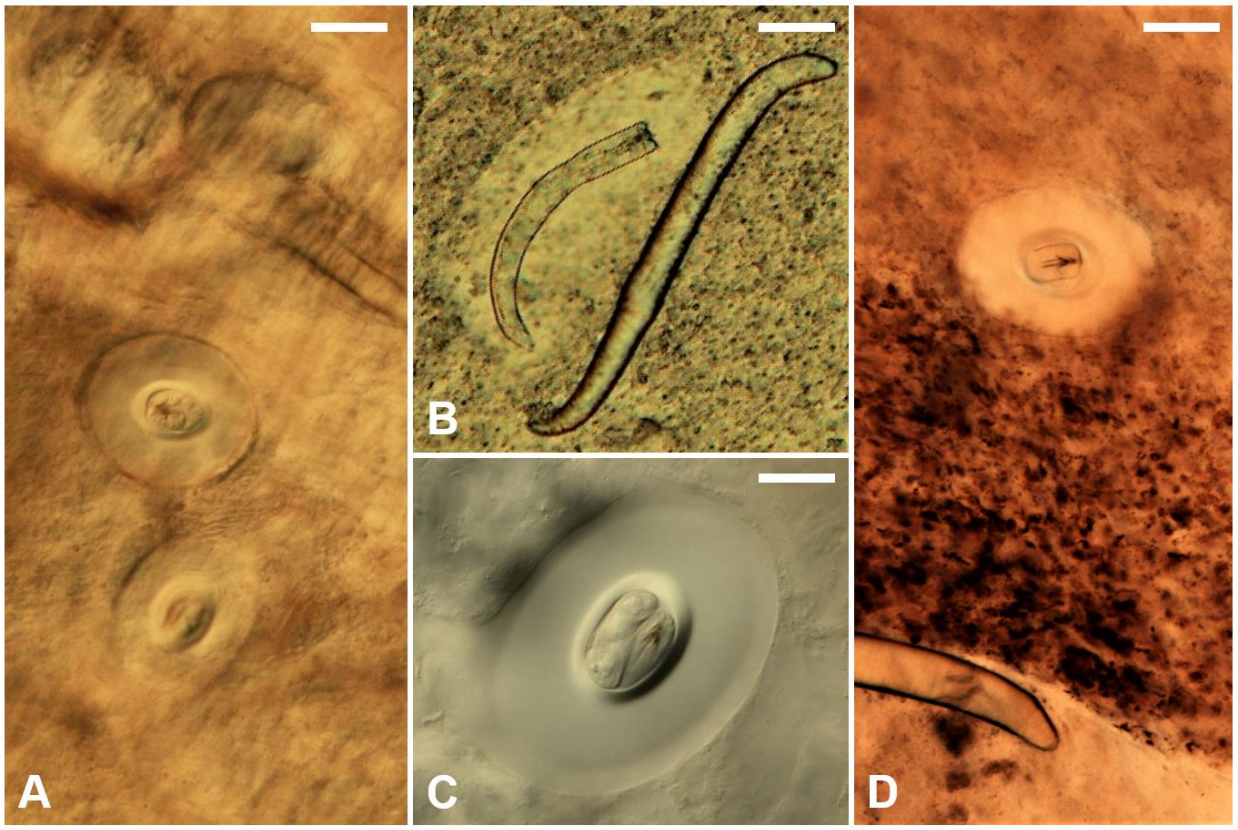

FIGURE 6. *Gordius terrestris* cysts in earthworms. (**A**) Naturally infected earthworms (*Diplocardia* sp.) recovered from Site 1 containing cysts of *G. terrestris*. 400× magnification Scale bar = 25 µm. (**B**) Hairworm larvae of *G. terrestris* next to the spicule of the earthworm cuticle in the process of folding to a cyst. Note presence of halo indicates recent infection. Scale bar = 25 µm. (**C**) Contrast phase microscopy of *G. terrestris* cyst. Note distinct halo surrounding folded larvae.Scale bar = 25 µm. (**D**) Laboratory experimental infection of *G. terrestris* in a redworm (*Eisenia fatida*) 400× magnification. Scale bar = 28 µm.

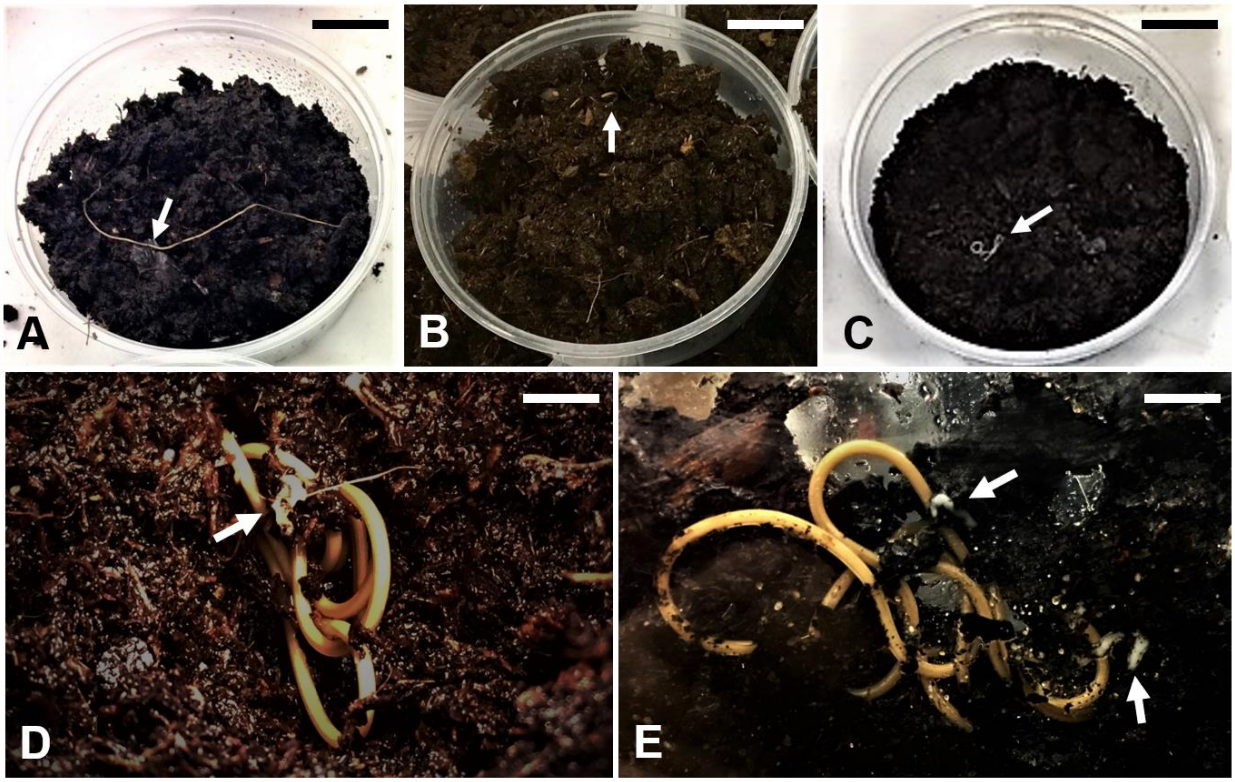

FIGURE 7. Burrowing behavior of female hairworms in soil. (**A**) *Paragordius varius* female two hours after being placed on top of soil. Scale bar = 20 mm. (**B**) *Gordius terrestris* adult female two hours after being placed on soil. Scale bar = 20 mm (**C**) *G. difficilis* female two hours after being placed on soil. Scale bar = 20 mm (**D**) *G. terrestris* female with newly deposited eggs. Scale bar = 10 mm (**E**) *G. terrestris* female with new and previously deposited eggs in soil (white arrows). Note that female has moved in soil after releasing eggs. Scale bar = 10 mm.

CHAPTER IV

FIRST REPORT OF ADULT AND FREE-LIVING HAIRWORMS, THEIR HOST AND NON-ADULT STAGES FROM ICELAND

ABSTRACT Twenty-two adult free-living hairworms were collected from seven locations across Iceland and represent the first credible documentation for gordiids from Iceland. In addition, non-adult stages of gordiids including cysts in snail paratenic hosts and juvenile worms in ground beetle final hosts were recorded from three locations from Iceland. Finally, I provide the first descriptions of the egg strings and eggs for the genus *Gordionus*. Scanning electron microscopy examination of adult free-living worms indicated that they all belonged to the genus *Gordionus*. However, due to cuticular variation among individuals examined it was not possible to determine if these specimens represent a new species of *Gordionus*. I discuss some of the issues in species descriptions of *Gordionus* species.

INTRODUCTION

The freshwater nematomorphs or gordiids (Nematomorpha: Gordiida) are macroparasites of arthropods that span aquatic and terrestrial environments during their life cycle (May 1919; Hanelt and Janovy 2004a). Currently, there are 350 described freshwater species

within 19 extant and 2 extinct genera from all continents except Antarctica (Bayliss 1944; Poinar 1991; deVillalobos and Voglino 2000; Schmidt-Rhaesa et al. 2009; Bolek et al. 2010; Hanelt et al. 2015). However, biodiversity studies on nematomorphs are relatively few compared to other parasite taxa because hairworm sampling is challenging for several reasons. Detection of adult free-living worms in their natural habitat is difficult because their coloration allows free-living worms to blend in with their natural environment; often being obscured beneath the substrate such as rocks or leaf litter in aquatic habitats (Bolek and Coggins 2002; Schmidt-Rhaesa 2013). Second, adult free-living worms are seasonal and have a short life span of two to eight weeks depending on the species (Bolek and Coggins 2002; Hanelt et al. 2005). It has been suggested that we are only aware of 18% of the hypothesized 2,000 plus species that may exist suggesting that detection impediments and their complex life cycle prevents us from revealing their true diversity (Poinar 2008).

All known life cycles indicate that juvenile gordiids infect terrestrial arthropods from which free-living adults emerge into freshwater habitats, such as streams, rivers, and lakes (May 1919; Hanelt et al. 2005; Bolek et al. 2015). After emerging from their arthropod host, dioecious species mate and females deposit egg strings in aquatic habitats (Bolek et al. 2013). Within weeks, larvae develop in eggs, hatch, infect, and encyst indiscriminately within a variety of aquatic vertebrate and invertebrate animals (Hanelt and Janovy 2003). Some of these infected animals, such as aquatic insect larvae, act as paratenic (transport) hosts by carrying cysts to land where they are consumed by omnivorous or predatory final hosts including millipedes, crickets, beetles, cockroaches, and mantids (Bolek et al. 2015).

Unfortunately, of 350 species, the life cycles of five species of gordiids have been elucidated (May 1919; Hanelt and Janovy 1999; Hanelt and Janovy 2004b; Hanelt et al. 2012; Bolek et al. 2013a; Swanteson-Franz et al. 2018). Despite our current knowledge of the gordiid life cycle, there is a disparity of our understanding on how these life cycles operate in nature. While there are a few studies that have surveyed potential paratenic hosts, few have verified infectivity to final arthropod hosts (White 1969; Poinar 1991). There is a clear lack of knowledge of the range of host use including the paratenic and final arthropod hosts used by most gordiid species. In fact, arthropod final hosts have been reported for less than 30% of the 350 described gordiid species (Schmidt-Rhaesa 2013; Harkins et al. 2016). Many of these reports are based on a single observation that may not represent the full extent of host use by some gordiid species (Hanelt et al. 2005; Bolek et al. 2015). Additionally, field studies on gordiid paratenic hosts are almost nonexistent (White 1966; 1969; Bolek and Coggins 2002; Chiu et al. 2016). One reason for our lack of life cycle understanding is the complexity of life cycles and little sampling effort. Because the life cycle utilizes both a paratenic and final host, it is important to consider the time of year sampling occurs for hosts.

For several years, the transmission from the aquatic free-living larvae to infection by final hosts was formed by conjecture beginning in 1856 when Meissner observed hairworm larvae develop into cysts (Meissner 1856). However, it would be several years later before this transmission mystery would be solved. Hairworm larvae are nonmobile and fall to the benthic portions of the habitat unless they are swept away by a stream current. Over the years, several hypotheses of transmission routes were formed and tested including direct ingestion by final arthropod hosts or through a paratenic host (Villot

1872; Dorier 1930,1935; Thorne 1940; Hanelt and Janovy 1999). It was determined that direct infection resulted in few successful infections compared to transmission when a paratenic host is ingested by a final host (Ochiai and Inoue 1970; Poinar and Doelman 1974; Hanelt and Janovy 1999; de Villalobos and Ronderos 2003). Therefore, if final hosts require a paratenic host in the life cycle, the filter concept, coined by Combes (1991) would apply and must be considered when determining host use among gordiids. Combes states two filters constrain parasite establishment within their hosts. First, hosts must encounter the parasite to become infected and second, the host must not mount an immune response that would result in fighting off infection (Combes 1991).

Recent work indicates that the cyst stage of Gordiid worms is the most commonly encountered gordiid life stage in the environment because hairworm larvae appear to readily encyst in several aquatic invertebrates including freshwater snails (Hanelt et al. 2001; Bolek et al. 2013; Szmygiel et al. 2014; Harkins et al. 2016). Cysts of gordiids have been reported to be long lived in some paratenic hosts and are found in a variety of aquatic invertebrate and vertebrate species (Hanelt and Janovy 2003). However, Harkins et al. (2016) argued that aquatic snails are the most suitable indicator hosts to sample for gordiid cysts over wide geographic areas. They had three major reasons for their argument, including the wide and common distribution of snails in aquatic environments, the lack of immunological response of snails to gordiid cysts, and the ease of processing snails for gordiid cysts compared to other aquatic invertebrates (Hanelt et al. 2001; Bolek and Coggins 2002; Bolek et al. 2010; Hanelt et al. 2012; Bolek et al. 2013). This is particularly important because an ideal indicator host should maintain a parasitic infection for long periods of time, allowing investigators to track the occurrence of that

parasite long after other stages have disappeared from a particular geographic location. Additionally, the feeding behavior of snails on the bottom of aquatic habitats makes them ideal hosts to encounter gordiid larvae, which reside in these microhabitats (Hanelt et al. 2001; Bolek et al. 2015). These snails are likely to encounter the microscopic and semi-sessile gordiid larvae more commonly than other invertebrates and vertebrates in aquatic habitats (Hanelt et al. 2005). However, it is not clear if all species of aquatic snails can become infected. In addition, it cannot be ruled out that aquatic snails themselves may serve as paratenic hosts. Often living in ephemeral ponds and water catchments, snails are often predated upon by beetles in these habitats when the water has receded (Schmidt-Rhaesa 2013).

The purpose of this study was to use modern hairworm sampling techniques and apply them to a region of the world where hairworm have not been described from and for which only anecdotal evidence of their presence exists. In Iceland, my goals were to sample aquatic snails for cyst stages of gordiids and examine specimens at a natural history repository. In this study, I provide the first description of free-living adult hairworms from Iceland using light and scanning electron microscopy. In addition, I describe the non-adult life stages, including eggs, cysts and larvae within cysts. Finally, I provide a new host record for the species including paratenic and final hosts.

METHODS

Field collections and samples

The freshwater snail, *Radix peregra*, was sampled from 34 streams and ponds across Iceland during September of 2017- July of 2018 and examined for hairworm cysts (Table

I). Snails were collected by hand or with a sieve, placed in 473 mL polystyrene capped containers with water, brought back to the laboratory and frozen to be examined later. All snails were identified according to Eversham (2013) and processed for gordiid cysts (see below). Additionally, any adult free-living hairworms and/or final terrestrial arthropod hosts that were observed at streams and ponds were collected. Live worms were placed in 473 mL polystyrene containers filled with stream water from the location they were collected from; whereas live arthropod hosts were placed in dry 473 mL polystyrene containers and returned to the laboratory. Hairworms were placed in 110 × 35 mm Stender dishes filled with natural spring water, stored at room temperature and observed for mating and oviposition behavior, and then fixed and processed for light microscopy and scanning electron microscopy observations (see below). Potential arthropod hosts were frozen, identified to genus and dissected for immature hairworms using a stereomicroscope.

Finally, 16 additional adult free-living hairworms were obtained from previous collections and evaluated for morphology (see below). Twelve worms were obtained from the Department of Aquaculture and Fish Biology at Hólar University College in Sauðárkrókur, Skagafjördur, Iceland. These specimens were collected by Krystal Mannion in July of 2017 from the River Grimsa (65.78793, -19.82824) in Skagafjördur as part of a fish diet study and stored in 95% ETOH. Four additional samples of adult free-living worms with locality data were obtained from the Icelandic Institute of Natural History.

Biological material and microscopy

Adults. Worms were sexed under a stereomicroscope and the length and width measured to the nearest 1 mm. Length, width, and color were recorded in the laboratory for all specimens collected. Length of worms was obtained by placing individuals on a ruler without stretching the specimen and measured to the nearest 1 mm. The width of each worm was obtained using an Olympus SZ1145 Stereomicroscope and a calibrated ocular micrometer. Adult worms were rinsed in a solution of 1-part chlorine bleach to 250-parts water to prevent fungal growth. After observations were completed, worms were killed and preserved in 95% ethanol.

One adult female and one adult male, collected from Uxahryggjavegur road, and 12 adult male worms collected from the River Grimsa were processed for scanning electron microscopy (SEM) as described by Harkins et al. (2014). Briefly, live worms were preserved in 70% ethanol at room temperature and 5–10 mm sections of the anterior, posterior, and mid-body regions of each worm was cut with a razorblade. Specimens were then dehydrated in increasing concentrations of ethanol (70 %, 85 %, 95 %, 100 %), dried using hexamethyldisilazane (HMDS), mounted on aluminum stubs coated with gold palladium and examined with an FEI Quanta 600 field emission gun ESEM (ThermoFisher Scientific, Hillsboro, OR) with Evex EDS and HKL EBSD. All terminology for adult worms follows Schmidt-Rhaesa (2001), and de Villalobos et al. (2001).

Obtaining non-adult stages. To obtain non-adult stages of gordiids, single male and female worms from Uxahryggjavegur road were paired and placed in 110 × 35 mm Stender dishes filled with tap water. Observations were made daily on the mating and oviposition behavior of worms. After males deposited a sperm drop on the posterior end

of females, females were isolated and allowed to deposit egg strings in individual Stender filled with tap water. Egg strings were rinsed in a solution of 1-part chlorine bleach to 250-parts water to prevent fungal growth and visually observed daily for larval development. Photographs were taken of 6-day egg strings in Stender dishes and a plastic ruler as a reference using an iPhone® and the length and width of seven egg strings were measured using ImageJ® software. To examine and measure individual undeveloped eggs, a two mm section of egg string was placed on a microscope slide in a drop of water and covered with a coverslip without crushing and the length and width was recorded using an ocular micrometer at 400× magnification.

Morphology of cysts. Field collected snails were returned to the laboratory where they were held in aged tap water for 24 hours and stored in a -80°C freezer prior to being examined. Allowing snails to sit in aged tap water for 24 hours allows waste contents to be excreted which could potentially affect analyses. Post frozen snails were processed for gordiid cysts following Harkins et al. (2016). Briefly, frozen snails were thawed, each snail's body was removed from its shell under a dissection microscope using forceps and pressed between two slides. A wet mount was prepared by removing the top slide and adding a drop of water and a coverslip. Slides were then examined with a Pleuger® light microscope by scanning the snail tissue. Eleven cysts were photographed at 400 × total magnification and the length and width of the cyst, cyst wall, and encysted larvae were obtained using ImageJ® software. Finally, the folding pattern of all encysted larvae was recorded. Procedures and terminology for cyst stages of gordiids follows Hanelt and Janovy (2002), Szmygiel et al. (2014) and Harkins et al. (2016).

RESULTS

Field collections

A total of 1,044 *Radix peregra*, were collected from 34 sites (Table I). Of those, snails from three sites contained gordiid cysts (Table II). Prevalence and mean intensity of cysts in *R. peregra* ranged from 27-43% and 4.2 ± 5 (1-20) to 14.9 ± 20 (1-77), respectively (Table II).

Four free-living adult worms along with juvenile gordiid infecting a ground beetle in the genus *Amara* (Coleoptera: Carabidae) were collected on July 19, 2018 from an unnamed stream on Uxahryggjavegur and adjacent to road 550 (64.4119, -20.99359). Additionally, two adult free-living worms were collected on July 29, 2018 from two streams located in the Hengill region (64.06789, -21.32822). Including samples of adult free-living worms obtained from Hólar University College and the Icelandic Institute of Natural History, adults and/or cysts of gordiids occurred at nine locations throughout Iceland (Fig. 1)

All adult free-living hairworms examined from Iceland conformed to the descriptions for the genus *Gordionus*. Below I provide the morphological descriptions for adult-free living male and female worms and egg strings and eggs. Additionally, I provide descriptions for the cysts and juvenile worm putatively identified as the non-adult stages of *Gordionus*.

Taxonomy

Gordionus sp.

Female Voucher. Female collected on July 19, 2018, from type locality. Deposited into the Museum of Southwestern Biology (MSB) Parasite Division, University of New Mexico (UNM), New Mexico, USA.

Male Voucher. Allotype: male specimen collected on July 29, 2018, from an unnamed stream in the valley, Hengladalir in the Hengill region of southwest Iceland (64.06792, -21.32823). Deposited into the Museum of Southwestern Biology (MSB) Parasite Division, University of New Mexico (UNM), New Mexico, USA.

Host. The putative final host is a ground beetle in the genus *Amara* (Coleoptera: Carabidae); and the putative paratenic host is the freshwater snail, *Radix peregra* (Pulmonate: Lymnaeidae).

Distribution. Current known distribution is based on collections from aquatic habitats from nine locations across Iceland. Adult and/or juvenile worms were collected from seven locations (64.41194, -20.99356; 64.06789, -21.32822; 65.78793, -19.82824; 64.49741, -21.41482; 64.10334, -21.88444; 63.48417, -19.05289; 65.44927, -21.90809). In addition, hairworm cysts putatively identified as this species were collected from snail hosts from three locations (64.41194, -20.9936; 65.66564, -19.0888; 65.66863, -19.0935; Fig. 1).

Material examined. Adults (n = 22), juveniles (n = 1), egg strings, eggs, and cysts. Eleven adult males and one adult female were examined and imaged using SEM. All other specimens were examined using an Olympus BX–51 upright research microscope (Olympus, Tokyo, Japan) configured for bright field and Nomarski differential interference contrast (DIC) microscopy with plain fluorite objectives at 400×

to 1000× total magnification for measurements and color pattern. A juvenile, eggs and cysts were examined using light microscopy and photographed.

Description of male. Adult free-living males (n = 15) were brown to dark brown in color on the dorsal side and creamy white in color on the ventral side (Fig. 3B). A dark dorsal and ventral medial line was present along the length of the cuticle being most distinct in the midbody region. Males were 78 ± 18 (45-107) mm in length and 0.40 ± .06 (.32 – .46) mm in width. The anterior end was slightly tapered and contained a white calotte with a dark collar.

The surface cuticle contained various areoles. However, areoles were variable in shape, depending on body region and individual males, but generally had similar characteristics. The ventral side of the anterior body region contained areoles that were polygonal in shape but not clearly defined. These areoles commonly connected and fused with neighboring areoles, reducing the interareolar furrows (Fig. 2A). Occasionally interareolar furrows contained rounded tubercles which were 3 µm in length (Fig. 2A). The midbody areoles showed the most variation among individuals, containing either (1) well defined polygonal areoles with distinct furrows, (2) flat well defined areoles that fused to form strips separated by interareolar features (Fig. 2B) or (3) flat undefined areoles with irregular, shallow furrows that fused to form strips. On the ventrolateral posterior region, well defined areoles were only present along the midline and positioned between the ventrolateral rows of adhesive warts (Fig. 2D). Adhesive warts were present on the ventrolateral area beginning 1.5 mm from the terminal ends of the tail lobes and continuing anteriorly for 3.6 mm (Fig. 2 D, E, F). Adhesive warts were positioned longitudinally on the body in 1-7 rows and increased in density anteriorly, and then

decreased in density. Adhesive warts were 24 ± 4 (19-32) µm in length, narrower at the center and wider at their ends located in a sulcus (Fig. 2F).

The posterior end was bilobed; and the tail lobes were 257 ± 27 (229-301) µm in length and 111 ± 20 (72-133) in width (Fig. 3A). The cloacal opening was centrally positioned but anterior to the separation of the tail lobes and appeared round in individuals that contained remnants of sperm within the cloaca (Fig 3A). The cloaca was surrounded by circumcloacal bristles that were approximately 7 µm long (Fig. 3D). Two linear bristle fields were positioned on the left and right side of the cloaca and arched over the cloaca but did not fuse above the cloacal opening (Fig. 3A,E,). The fields formed at an angle to the cloaca and stopped just anterior to where the tail lobes begin (Fig. 2A). Postcloacal spines occurred posteriorly to the cloacal opening and extended to the mid-region of inner ventral side of the tail lobes and were of 4.9 ± 0.7 (3.7-6.3) µm in diameter. The postcloacal spines were always accompanied by cuticular bristles (Fig. 2 A, B, C) that become increasingly denser at the terminal ends of the tail lobes (Fig. 2B).

Description of female. Adult females (n = 3) were creamy white in color and measured 68 ± 5 (62-72) mm long. Width was acquired for a single female which was 0.54 mm wide. The anterior end was slightly tapered and contained a white calotte with a dark collar. Females had a blunt posterior end with a terminal cloaca (Fig. 4G, 5B). The anterior areoles were flat, plate-like, polygonal in shape and with distinct borders. Posterior areoles were round and without distinct interareolar furrows (Fig. 2G). Posterior areoles measured 12 ± 1.5 (8.5-14) µm and 8 ± 1.3 (7-10) µm wide (Fig. 2I).

Description of juvenile. The single female juvenile recovered from the body cavity of a ground beetle in the genus *Amara* was 65 mm long and 1 mm thick (Fig. 4F).

The juvenile worm was white in color and contained a larval cuticle (Fig. 4G, H) which began to disintegrate when the worm was removed from the body cavity of its beetle host and placed in water.

Description of mating, oviposition, egg strings, and eggs. When males and females were placed together, male and female worms began mating. The male moved down the female's body with its coiled posterior end then released the female. After several attempts and within a few hours, the male deposited a sperm drop on the female's posterior end. Eggs strings were deposited by females within four days of receiving a sperm drop from a male. Newly deposited egg strings were white in color and deposited in a continuous string that broke as it emerged from the female's cloaca into short segments (Fig. 5D). Deposited egg strings were 1.6 ± 1 (0.8-3.7) mm in length and 0.4 ± 0.04 (0.4-.5) mm in width. Newly released eggs were tightly aggregated within egg strings and elliptical in shape (Fig. 5E). Eggs were 46 ± 3 (39-53) µm long by 32 ± 3 (27-37) µm wide (Fig. 5F). However, all egg strings developed a fungal infection and were destroyed.

Cyst morphology. Fully formed cysts contained a clear cyst wall of unknown composition surrounding the folded larva (Fig. 6). Cysts were 95 ± 33 (58-155) µm in length and 66 ± 19 (49-114) µm in width. Larvae within cysts were cylindrical in shape, superficially annulated, and folded on themselves only once, with small but distinct spines protruding from the preseptum (Fig. 6A, B). The preseptum was 26 ± 2 (22-31) µm in length and 14 ± 2 (9-16) µm, and the proboscis was laterally compressed (Fig. 6D). The postseptum was 27 ± 4 (23-35) µm in length and 11 ± 1 (9-23) µm in width and contained two pairs of terminal spines positioned ventrally (Fig. 6C).

Diagnosis and taxonomic comments. Of the 22-adult free-living *Gordionus* individuals examined during this study, all appeared to be morphologically similar, suggesting they belong to the same species. However, of the 40 Palearctic species that have been adequately described, the Iceland samples are most like *G. wolterstorffii* from Europe and *G. kimberleyae* from Canada (Schmidt-Rhaesa, 2001). As a result of these morphological similarities between the Iceland specimens and *G. wolterstorffii* and *G. kimberleyae*, it is not clear if these new collections represent a new species of *Gordionus*.

The genus *Gordionus* was originally characterized by a single type of areole. However, more recently Schmidt-Rhaesa (2001) evaluated the cuticular structures for multiple individuals of *G. wolterstorffii* and *G. violaceus* and identified intraspecific variation in areole pattern and shape. As a result, Schmidt-Rhaesa (2001) broadened the characters for the genus and defined three types of areoles, type I, type II and type III. Although three types of areoles were observed in the Iceland *Gordionus* sp. specimens, not all areole types were observed on every individual. As with the Iceland specimens, *G. wolterstorffii* contained all three types of areoles, but all types are not always present in each individual (Schmidt-Rhaesa 2001). This contrasts with the areole pattern for *G. kimberleyae* which only contain type I areoles (Ernst et al. 2016). In addition, areole size is distinct between *G. wolterstorffii* and *G. kimberleyae*. However, neither species descriptions indicate the variation in areole size on different body regions. This may be important because in the Iceland specimens, areole size differed depending on which region of the body they were located on.

In addition to the areole morphology issues, the interarolear setae, or bristles, are common in both the Iceland specimens and *G. wolterstorffii* but are lacking in *G.*

kimberleyae. These interarolear setae are important in defining another type of areole, the megaareole. Megaareoles are characterized by their large size and typically defined as the arrangement of two adjoining areoles enclosing a central tubercle and have been reported in some species of *Gordionus*. However, Schmidt-Rhaesa (2001) indicated that some megaareoles lack the central tubercle, making it difficult to distinguish megaareoles from type I areoles which are large in size. Of the 12 Iceland male specimens examined with SEM, one specimen contained the megaareolar pattern on the ventral side of the midbody. Although, descriptions of *G. wolterstorffii* and *G. kimberleyae* make no mention of megaareoles, it is likely that megaareoles may vary intraspecifically along different body regions or because they are not well defined.

Finally, adhesive warts, have been reported from many but not all *Gordionus* species. The Iceland *Gordionus* specimens contained adhesive warts along the ventrolateral sides of the posterior region beginning 1.5 mm from the terminal ends of the tail lobes and continuing approximately 3.5 mm anteriorly. *Gordionus wolterstorffii* is described as containing adhesive warts that begin at 0.6 mm from the terminal lobes while adhesive warts are lacking in *G. kimberleyae*. However, it is currently unclear if anyone has looked for adhesive warts 1.5 mm up from the terminal ends of the tail lobes in *G. kimerlayae*, a problem that is common among many descriptions of *Gordionus* (Bolek et al., 2015).

Non-adult stages. Unlike other genera of gordiids, currently no description of egg strings or eggs have been documented for the genus *Gordionus.* Females of *Neochordoes occidentalis* and all species of *Chordodes* for which oviposition behavior has been observed attach their egg strings in a zig-zag pattern to objects, such as rocks or

sticks. In contrast, species of *Gordius* and *Acutogordius* deposit short pieces of egg strings approximately 1–2 cm in length on the substrate or while in Gordian knots. Whereas, species of *Paragordius* deposit a single long egg string approximately 1–5 times the length of the worm's body in the water column and in algal mats (Bolek et al., 2015). The morphology of egg strings deposited by the Iceland *Gordionus* sp. were most similar to egg strings of gordiids in the genus *Gordius* and *Acutogordius* (Szmygiel et al. 2014; Chiu et al. 2017).

As with egg strings, three types of cyst folding patterns have been reported in the Gordiida (Hanelt and Janovy 2002; Szmygiel et al. 2014; Chiu et al. 2017). Work by Szmygiel et al. (2014) and Chiu et al. (2017) indicated that morphological differences in the three types of cyst folding patterns are correlated to three different morphologies in gordiid larvae. Larvae of *Gordius*, *Acutogordius* and *Paragordius* species have a post-septum, which is significantly longer than the pre-septum. As a result, larvae within cysts of these genera are always folded twice. However, the postseptum of *Gordius* and *Acutogordius* species is about three times as long as the preseptum and, consequently, the posterior end of the postseptum always reaches the posterior end of the preseptum. In contrast, the postseptum of *Paragordius* species is only 1.3 times as long as the pre-septum, and, as a result, the posterior end of the postseptum never reaches the posterior end of the pre-septum. Finally, larvae of *Chordodes* species and *N. occidentalis* have a post-septum to pre-septum ratio, which is almost equal in length; and, consequently, larvae of species in these genera only fold once. As a result, the folding pattern and morphology of cysts putatively identified as *Gordionus* sp. infecting freshwater snails and collected from the same locations as adult *Gordionus* species from Iceland are

significant for several reasons. First, based on the known folding pattern of gordiid larvae, Dorier‘s (1935) description of the larval stages of *Gordionus*, and as observed in this study, Szmygiel et al (2014) predicted the cyst morphology of *Gordionus* species should conform to the *Chordodes*/*Neochordodes* cyst type. Second, the occurrence of two pairs of terminal spines on the postseptum and a laterally compressed proboscis on the preseptum of larvae observed within cysts collected from Iceland, conform to the larvae description for the genus *Gordionus* by Dorier (1935).

Finally, the occurrence of an immature female worm, putatively identified as the same species of *Gordionus* sp., infecting a ground beetle in the genus *Amara* is also relevant. Although ground beetles are commonly reported as final hosts for gordiids, the only reports of hairworms infecting *Amara* species from North America, include two reports of *Gordionus* species and the closely related but controversial genus *Parachordodes* which contains megaareoles instead of adhesive warts, and may actually represent the genus *Gordionus* (Montén 1951; Poinar et al. 2004; Looney et al. 2012; Bolek et al. 2015; Ernst et al. 2016).

DISCUSSION

The major contribution of this work documents freshwater hairworms from Iceland for the first time. Twenty-two adult free-living specimens belonging to the genus *Gordionus* were documented across Iceland. In addition, I report the first description of egg strings and eggs for the genus *Gordionus*, and cysts and juvenile worms putatively identified as belonging to the genus *Gordionus*. Finally, the collection of a ground beetle

from the genus *Amara*, containing the juvenile stage of hairworms provides at least one final host for gordiids in Iceland.

Before the current study, evidence of hairworms from Iceland included a single photograph of a cross-section identified from Iceland but without providing any species or generic identification nor location information (Schmidt-Rhaesa 2013). Additionally, an early twentieth century publication by a naturalist, investigating the folklore of the world noted that in Iceland "hairworms are said to come down with the rain" (Annandale 1905). Therefore, this study is the first to provide credible documentation of gordiids from Iceland supplemented with voucher specimens that will be deposited in museum collections.

The genus *Gordionus* was first described by Müller in 1926 due to its unique characteristic of adhesive warts and described as lateral bumps that begin approximately 0.5-1.0 mm from the terminal end of the lobes, but whose function is unknown. The presence of adhesive warts was subsequently identified as a diagnostic character for the genus by Heinze (1937) and De Miralles (1976). However, adhesive warts have not been found in all species of *Gordionus* and in addition have been reported from species in the genus *Beatogordius* (Schmidt-Rhaesa and Bryant 2004; Begay et al. 2012). One reason adhesive warts may not be reported from the many *Gordionus* species descriptions is in the discrepancy of how specimens are prepared for SEM. In fact, both Schmidt-Rhaesa (2001) and Bolek et al. (2015) indicated that specimens cut for SEM should be long enough to include potential regions containing adhesive warts. Unfortunately, most species descriptions do not specify the exact length of the posterior end examined for adhesive warts using SEM. In addition, many species descriptions of *Gordionus* are

based on a single specimen or very few specimens (Heinze, 1952; Giudicelli and Nicoli 1962; de Villalobos and Voglino 2000; de Villalobos et al. 2001). As a result, our understanding of intraspecific and interspecific variation in adult free-living worms from several genera of gordiids is poorly understood (Schmidt-Rhaesa 2001). Finally, the lack of descriptions on non-adult stages for most gordiid species is also troublesome, because recent work clearly suggests that the morphology of these stages can provide new characters for understanding the relationships among gordiids (Hanelt and Janovy 2002; Szmygiel et al. 2014; Bolek et al. 2015; Chiu et al. 2017).

Using paratenic hosts such as snails as biodiversity indicators was a method developed to expand our understanding of the diversity and distribution of nematomorphs across large geographical regions (Hanelt et al. 2001; Hanelt et al. 2012; Bolek et al. 2013; Harkins et al. 2016;). In this study, snails were an effective tool for identifying cysts stages of gordiids in Iceland. However, compared to previous studies on snails as indicator hosts for gordiid cysts, the uncommon occurrence of cysts in snail paratenic hosts across Iceland was unexpected. Previous studies using physid and planorbid snails as indicator hosts for gordiid cysts in North America and Africa by Hanelt et al. (2001), Hanelt et al. (2012), Bolek et al. (2013) and Szmygiel et al. 2014, indicates that gordiids cysts were present at 60-70% of the streams sampled for snails. However, in my study, gordiid cysts were only present at 2 of the 34 locations sampled for snails. One reason for these discrepancies may be explained by differences in the feeding behavior and habits of the snail host examined in this study, *Radix peregra* (Lymnaeidea) and snails from other families (planorbids and physids) examined in previous studies (Hanelt et al. 2001; Hanelt et al. 2012; Bolek et al. 2013; Szmygiel et al. 2014). Previous work on North

American lymnaeid gastropods indicates that these snails are unselective benthic grazers that feed on detritus and phytoplankton (Brendelberger 1997). In contrast, physid snails that preferentially feed on carrion and detritus. Because lymnaeids are benthic, generalist feeders, one would expect them to commonly encounter sessile gordiid larvae in the benthic detritus layer of stream and ponds. However, during this study snails were commonly collected from rocks along the shoreline of streams. The structure of many streams in Iceland is unique with deep channels outlined with rocks on their periphery. As a result, snails that leave the rocks may be more likely to be carried away with the current. These observations suggest that the low occurrence of gordiid cyst in *R. peregra* may be a consequence of this snail species not encountering gordiid larvae on the stream bottom commonly.

Although this study represents the first credible report of hairworms and their non-adult stages recorded from Iceland. The inability to describe the adult free-living worms as a new species highlights our lack of understanding of intraspecific variation within the genus *Gordionus*. Because morphology appears to be difficult to decipher in this genus, my future work will include sequencing of the mitochondrial CO1 region and/or other barcoding mitochondrial and nuclear regions to compare intraspecific and interspecific variation in morphology of this neglected genus of gordiids. However, for these techniques to be successfully used in differentiating among species of *Gordionus*, additional sequencing must be obtained for other *Gordionus* species.

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LITERATURE CITED

Annandale N. (1904) Note on the hair-worms in oriental and European folklore. Journal of the Asiatic Society of Bengal 73: 75-77

Baylis HA (1944) Notes on the distribution of hairworms (Nematomorpha: Gordiidae) in the British Isles. Proceedings of the Zoological Society of London. 193-197.

Begay A, Schmidt-Rhaesa A, Bolek MG, Hanelt B (2012) Two new *Gordionus* species (Nematomorpha: Gordiidae) from the southern Rocky Mountains, USA. Zootaxa 3406: 30-38

Bolek MG, Coggins JR. (2002) Seasonal occurrence, morphology, and observations on the life history of *Gordius difficilis* (Nematomorpha: Gordioidea) from southeastern Wisconsin, United States. Journal of Parasitology 88: 287–294

Bolek MG, Schmidt-Rhaesa A, Hanelt B (2010) Redescription of the African *Chordodes albibarbatus* Montgomery 1898, and description of *Chordodes janovyi* n. sp. (Gordiida, Nematomorpha) and its non-adult stages from Cameroon. Zootaxa 2631: 36-50

Bolek MG, Szmygiel C, Kubat A, Schmidt-Rhaesa A, Hanelt B. (2013) Novel techniques for biodiversity studies of gordiids and description of a new species of *Chordodes* (Gordiida, Nematomorpha) from Kenya, Africa. Zootaxa 3717: 23–38

Bolek MG, Schmidt-Rhaesa A, De Villalobos LC, Hanelt B. (2015) Phylum Nematomorpha. *In:* Thorp, J., and Rogers, D.C. (Eds.), Volume I: Ecology and

General Biology: Thorp and Covich's Freshwater Invertebrates, 4th ed., Academic Press, 303–326

Brendelberger H (1997) Contrasting feeding strategies of two freshwater gastropods, *Radix peregra* (Lymnaeidae) and *Bithynia tentaculata* (Bithyniidae). Archives of Hydrobiologia 140: 1-21

Chiu MC, Huang CG, Wu WJ, Shiao SF (2016) Annual survey of horsehair worm cysts in northern Taiwan, with notes on a single seasonal infection peak in Chironomid larvae (Diptera: Chironomidae). Journal of Parasitology 102:319-326

Combes, C. (1991). Parasitism: the ecology and evolution of intimate interactions. University of Chicago Press, 728 pp

Dorier, A. (1930) Recherches biologiques et systématiques sur les Gordiacés. Trav. Lab. Hydrobiol. Piscicult. Univ. Grenoble 22: 1-183

Dorier, A. (1935) Sur le passage à la vie latente des larves de Gordiacés. C. R. Acad. Sci. Paris 200: 492-494

Ernst CM, Hanelt B, Buddle CM (2016) Parasitism of ground beetles (Coleoptera : Carabidae) by a new species of hairworm (Nematomorpha : Gordiida) in Arctic Canada. Journal of Parasitology 102: 327-335

Eversham B (2013) Identifying freshwater molluscs Version 2.2 https://www.naturespot.org.uk/sites/default/files/downloads/Pondsnails%20Key%20version%20%202.2%20iv2013%20illustrated%20PDF.pdf

Giudicelli J, Nicoli RM (1962) Un Gordionus nouveau *Gordionus cyrnensis* n. sp. (Gordioïdea) Contribution á l'étude du peuplement de la Corse. (10e note) 8: 616-622

Hanelt, B. and J. Janovy. (1999) The life cycle of a horsehair worm, *Gordius robustus* (Nematomorpha: Gordioidea) Journal of Parasitology 85: 139-141

Hanelt, B., L.E. Grother, and J. Janovy, Jr. (2001) Physid snails as sentinels of freshwater Nematomorphs. Journal of Parasitology 87: 1049-1053

Hanelt B and Janovy J (2002) Morphometric analysis of nonadult characters of common species of American gordiids (Nematomorpha: Gordioidea) 88: 557-562

Hanelt, B. and J. Janovy (2003) Spanning the gap: experimental determination of paratenic host specificity of horsehair worms (Nematomorpha: Gordiida). Invertebrate Biology 122: 12-18

Hanelt, B. and Janovy J. (2004a) Life cycle and paratenesis of American gordiids (Nematomorpha: Gordiida). Journal of Parasitology 90: 240-244

Hanelt, B. and J. Janovy (2004b) Untying a Gordian knot: the domestication and laboratory maintenance of a Gordian worm, *Paragordius varius* (Nematomorpha: Gordiida). Journal of Natural History 38: 939-950

Hanelt B, Thomas F, Schmidt-Rhaesa A (2005) Biology of the phylum Nematomorpha. Advances in Parasitology 59: 243–305

Hanelt B, Bolek MG, Schmidt-Rhaesa A (2012) Going solo: discovery of the first parthenogenetic gordiid (Nematomorpha: Gordiida). PlosONE https://doi.org/10.1371/journal.pone.0034472

Hanelt B, Schmidt-Rhaesa A., Bolek MG (2015) Cryptic species of hairworm parasites revealed by molecular data and crowdsourcing of specimen collections. Molecular Phylogenetics and Evolution 82: 211–218

Harkins C, Shannon R, Papeş M, Schmidt-Rhaesa A, Hanelt B, Bolek MG (2016) Using Gordiid cysts to discover the hidden diversity, potential distribution, and new species of Gordiids (Phylum Nematomorpha). Zootaxa 4088: 515-530

Heinze, K. (1937) Die Saitenwürmer (Gordioidea) Deutschlands. Eine systematisch-faunistische Studie über Insektenparasiten aus der Gruppe der Nematomorpha. Z. Parasitenkd. 9: 263-344

Heinze, K. (1952) Über Gordioidea, eine systematische Studie über Insektenparasiten aus der Gruppe der Nematomorpha. Z. Parasitenkd. 15: 183-202

Looney C, Hanelt B, Zack RS (2012) New Records of Nematomorph parasites (nematomorpha: Grodiida) of ground beetles (Coleoptera: Carabidae) and camel crickets (Orthoptera: Rhaphidophoridae) in Washington State. Journal of Parasitology 98: 554-559

May HG (1919) Contributions to the life histories of *Gordius robustus* Leidy and *Paragordius varius* (Leidy). Illinois Biol. Monogr. 5: 1-119

Meissner G. (1856) Beiträge zur Anatomie und Physiologie der Gordiaceen. Z. Wiss. Zool. 7: 1-144

De Miralles DB (1976) Nota sobre Gordiaceos Argentinos. Neotropica 22: 77-80.

Montén E (1951) Nematomorpha. Acta University Lund 46:162-170

Müller GW (1926) Über Gordiaceen. Z. Morph. Ökol. Tiere 7: 134-220

Ochiai N, Inoue I (1970) On the post-larval development of *Chordodes* (Gordiacea) - especially the formation of the intestine as well as its openings. Bull. Tokyo Gakugei Univ. 22: 78-87

Poinar, G. (2008) Global diversity of hairworms (Nematomorpha: Gordiaceae) in freshwater. Hydrobiologia 595: 79-83

Poinar GO, Doelman JJ (1974) A reexamination of *Neochordodes occidentalis* (Montg.) comb. n. (Chordodidae: Gordioidea): larval penetration and defense reaction in *Culex pipiens* L. Journal of Parasitology 60: 327-335

Poinar GO Jr. (1991) Hairworm (Nematomorpha: Gordioidea) parasites of New Zealand wetas (Orhtoptera: Stenopelmatidae). Canadian Journal of Zoology 69: 1592-1599

Poinar G, Rykken J, LaBonte, J (2004) *Parachordodes tegonotus* n. sp. (Gordioidea: Nematomorpha), a hairworm parasite of ground beetles (Carabidae: Coleoptera), with a summary of gordiid parasites of carabids. Systematic Parasitology 58: 139-148

Schmidt-Rhaesa A (2001) Variation of cuticular characters in the nematomorpha: studies on *Gordionus violaceus* (Baird, 1853) and *G. wolterstorffii* (Camerano, 1888) from Britain and Ireland. Systematic Parasitology 49: 41-57

Schmidt-Rhaesa A. (2009) Nematomorpha (Saitenwürmer). In: Endemiten - Kostbarkeiten in Österreichs Tier- und Pflanzenwelt (Rabitsch, W. & Essl, F., eds.). Naturwissenschaftlicher Verein für Kärnten und Umweltbundesamt GmbH, Klagenfurt: 381-383

Schmidt-Rhaesa A (2013) Nematomorpha. In: Schmidt-Rhaesa, A. (Ed.), Handbook of Zoology. Gastrotricha, Cycloneuralia and Gnathifera. Nematomorpha, Priapulida, Kinorhyncha and Loricifera. Vol 1. De Gruyter, Berlin, pp. 29–145

Schmidt-Rhaesa A, Bryant MS (2004) Filling the Gondwana gaps: new species and new reports of the genus *Beatogordius* (Nematomorpha) from Australia and Madagascar. Syst. Parasitol. 57: 173-181

Schmidt-Rhaesa A, Nishi H, Tanabe AS, Urabe M (2009) New records of hairworms (nematomorpha: Gordiida) from Japan. Species Diversity 14: 131-135

Szmygiel C, Schmidt-Rhaesa A, Hanelt B, Bolek MG. (2014) Comparative descriptions of non-adult stages of four genera of Gordiids (Phylum: Nematomorpha). Zootaxa 3768: 101-118

Thorne G (1940) The hairworm, *Gordius robustus* Leidy, as a parasite of the Mormon cricket, *Anabrus simplex* Haldeman. Journal of the Washington Academy of Sciences 30: 219-231

de Villalobos LC, Voglino D (2000) Description of *Gordionus porosus* n. sp. and Redescription of *Gordionus ondulatus* (Nematomorpha, Parachordodinae) with scanning electron microscopy. Journal of Parasitology 86: 358-365

de Villalobos LC, Ribera I, Bilton DT (2001) First data of Iberian Nematomorpha, with Redescription of *Gordius aquaticus* Linnaeus, G. plicatulus Heinze, *Gordionus wolterstorfii* (Camerano) and *Paragordius tricuspidatus* (Dufour) Contributions to Zoology 70:73-84

De Villalobos C, Ronderos, M (2003) *Dasyhelea necrophila* Spinelli et Rodriguez, 1999 (Diptera, Ceratopogonidae) a new potential paratenic host of *Paragordius varius* (Leidy, 1851) (Gordiida, Nematomorpha). Acta Parasitologica 48: 218-221.

Villot A (1872) Sur la forme embryonnaire des Dragonneaux (*Gordius*). C. R. Acad. Sci. Paris 75:363-365

White DA (1966) A new host record for *Paragordius varius* (Nematomorpha). Transactions of the American Microscopical Society 85: 579

White DA (1969) The infection of immature aquatic insects by larval *Paragordius* (Nematomorpha) Western North American Naturalist 29: 44

Table I. Location, and number of *Radix peregra* snails collected from streams and ponds across Iceland.

Site	Latitude	Longitude	No. of snails collected	No. of adult worms Collected
1	66.06361	-20.04694	30	0
2	65.73161	-19.61961	6	0
3	65.66564	-19.08876	22	0
4	65.49426	-19.38351	76	0
5	65.61686	-16.91673	30	0
6	65.47395	-19.27285	49	0
7	65.66476	-20.2294	7	0
8	65.06863	-19.09354	61	0
9	65.24345	19.44629	30	0
10	65.26116	-19.73919	32	0
11	65.18968	-18.82528	33	0
12	65.12992	-18.61673	38	0
13	65.10838	-18.52923	31	0
14	64.86639	-19.55361	48	0
15	66.038964	-19.343981	3	0
16	65.08636	-18.52817	39	0
17	65.10278	-18.525278	29	0
18	65.822652	-19.866209	30	0
19	63.55402	-20.06854	21	0
20	64.18177	-19.42561	40	0
21	63.93338	-20.98984	27	0
22	64.30449	-18.97916	20	0
23	64.52244	-18.65992	39	0
24	64.08499	-20.0087	30	0
25	64.21275	-20.72788	19	0
26	64.43644	-21.02169	31	0
27	64.14366	-21.94125	37	0
28	64.09318	-21.7945	14	0
29	64.66299	-21.29161	48	0
30	64.50028	-21.19444	34	0
31	64.93958	-21.06153	35	0
32	64.61847	-21.50138	7	0
33	64.4119	20.99359	34	4
34	64.06769	-21.33071	14	2
Total			1044	6

Table II. Prevalence and mean intensity (range) of gordiid cysts in *Radix peregra* and number of free-living adult *Gordionus* sp. collected at three different locations in Iceland.

Location	No. infected/no. examined	Prevalence % (no. infected/ no.examined	Mean Intensity	Adults found
3	15/35	43	4.2 ± 5 (1-20)	0
8	18/48	38	15 ± 20 (1-77)	0
33	6/22	27	10 ± 2 (1-4)	4

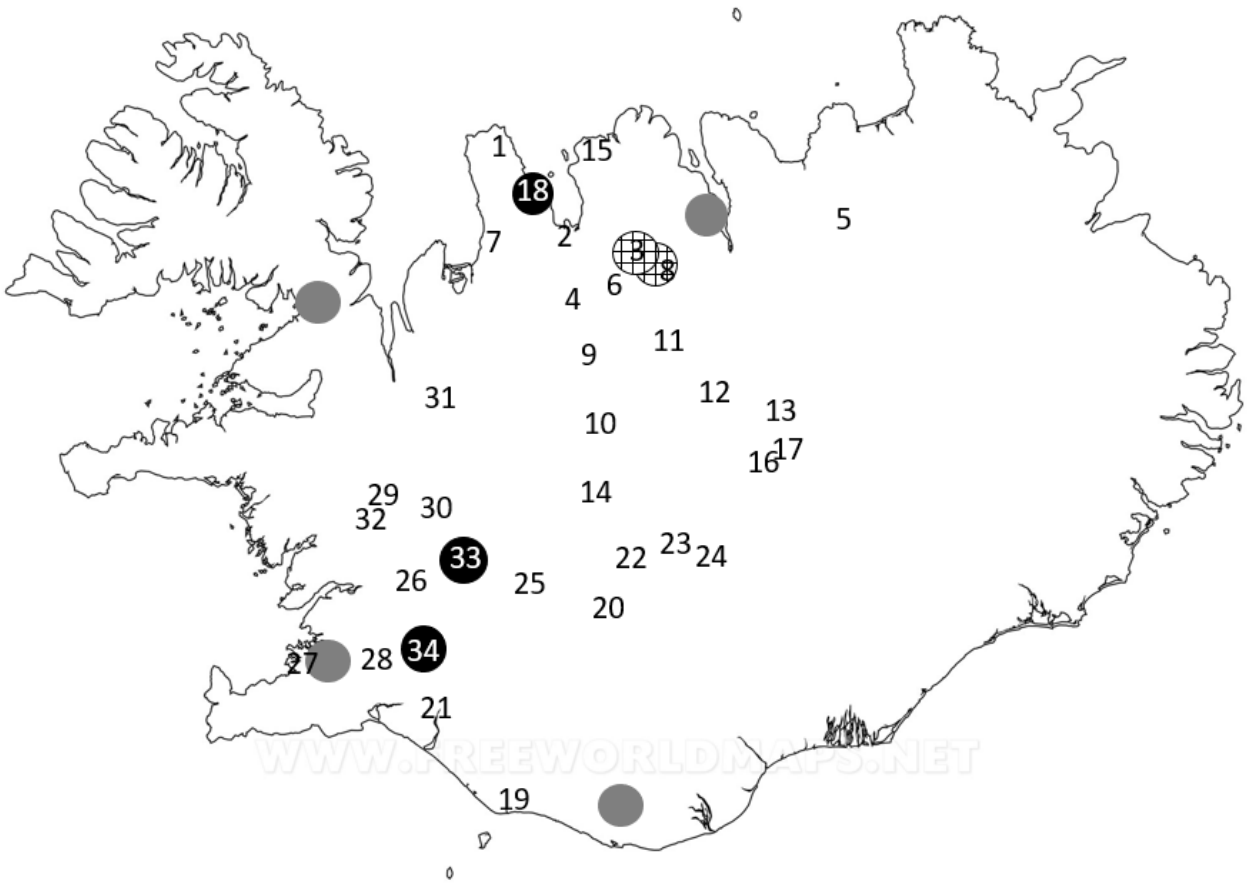

FIGURE 1**.** Locations sampled for the freshwater snail, *Radix peregra*. Black circles represent adult hairworms collected. Cross-hatch circles represent locations where snails were positive for hairworm cysts. Site 33 represents a location where adult free-living worms were found and cysts in *Radix peregra*. Dark grey circles represent locations where adults free-living worms were obtained from the Icelandic Institute of Natural History and were not assigned location numbers.

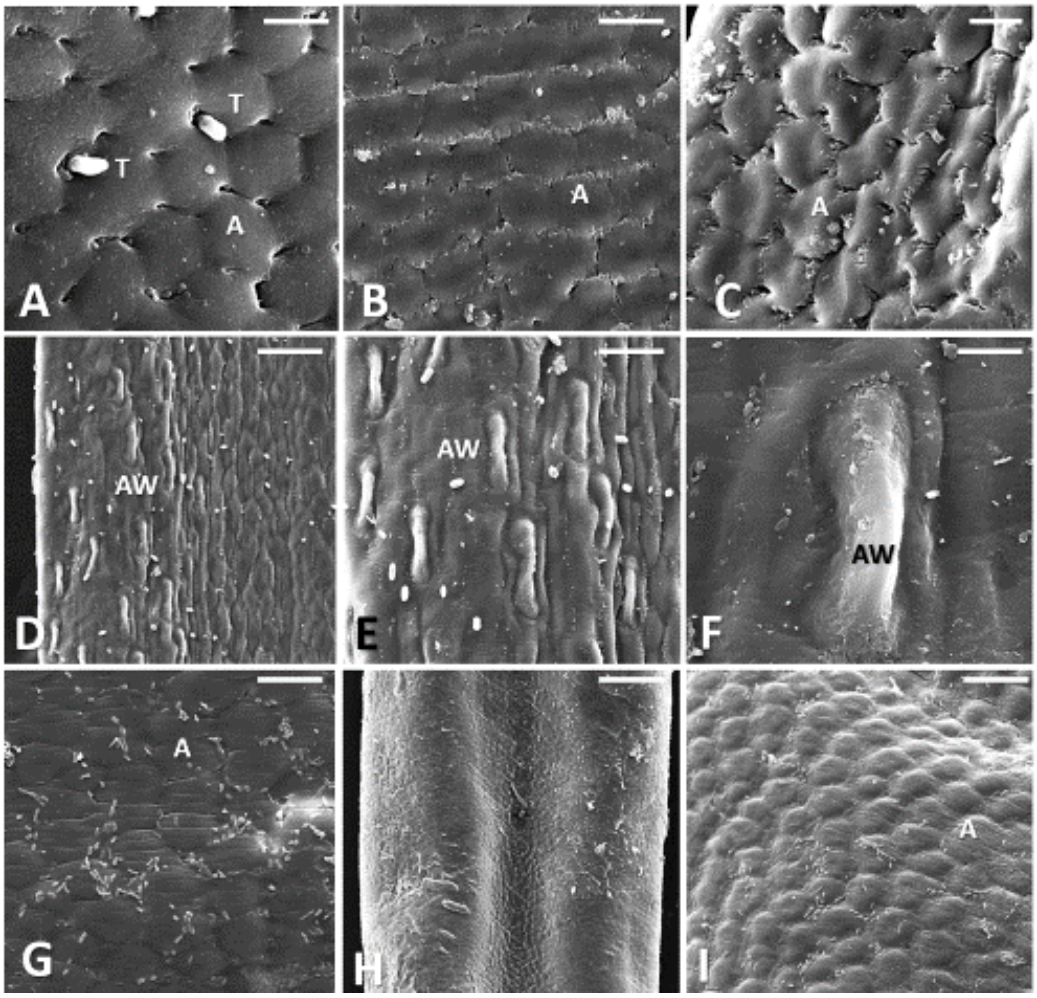

FIGURE 2. Cuticular structures of adult free-living male (**A-F**) and female (**G-I**) *Gordionus* sp. from Iceland. (**A**) Type I, polygonal areoles on the anterior body region. Note the blunt tubercles (T) within the interareolar furrows. Scale bar = 5 µm. (**B**) Type II, flat, plate-like areoles in the midbody region forming long strips separated by interareolar furrows resembling fused areoles. Scale bar = 10 µm. (**C**) Type I polygonal areoles located on the ventral side of the posterior region. Scale bar = 8 µm. (**D**) Lateroventral region showing rows of adhesive warts (AW). Note areoles are less prominent in this region. Scale bar = 42 µm. (**E**) Closeup of adhesive wart field showing arrangement of individual adhesive warts (AW). Scale bar = 22 µm. (**F**) Higher

magnification of an individual adhesive wart (AW) within a sulcus. Scale bar = 6 µm. (**G**) Type I polygonal, flat plate-like areole on the anterior body region. Scale bar = 16 µm. (**H**) Female midbody region with showing areoles. Scale bar = 105 µm. (**I**) Type I polygonal areoles without interareolar furrows located on the posterior body region. Scale bar = 16 µm.

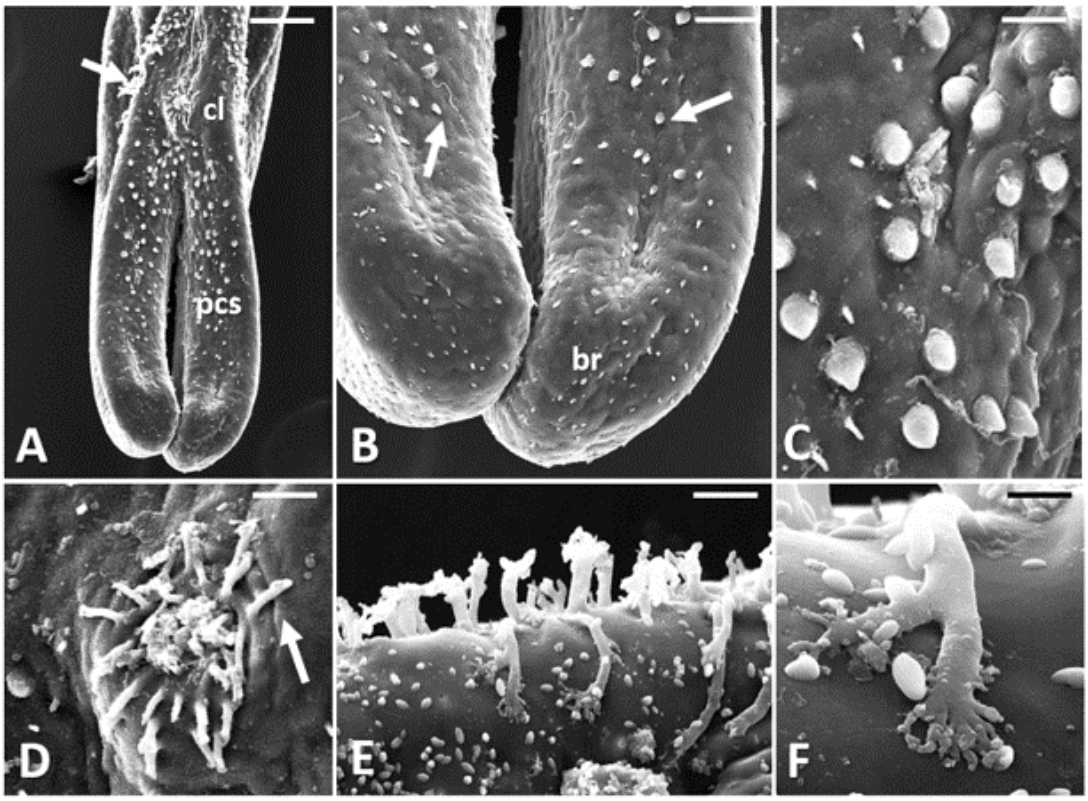

FIGURE 3. Ventral view of the posterior body region of a male *Gordionus* sp. from Iceland. (**A**) Ventral view of the posterior body regions showing cloaca (Cl), bristle fields (white arrow), and post-cloacal spines (pcs). Scale bar = 50 µm. (**B**) Posterior lobes showing postcloacal spines (white arrows) and bristles (br) on the terminal ends of the tail lobes. Scale bar = 20 µm. (**C**) Higher magnification of the postcloacal spines showing blunt ends. Scale bar = 9 µm. (**D**) Cloacal opening surrounded by circumcloacal spines (white arrow). Scale bar = 18 µm. (**E**) Lateral view of bristle fields showing branched apices on terminal ends of bristles. Scale bar = 10 µm. (**F**) Close-up of a single bristle showing the branching pattern. Scale bar 3 µm.

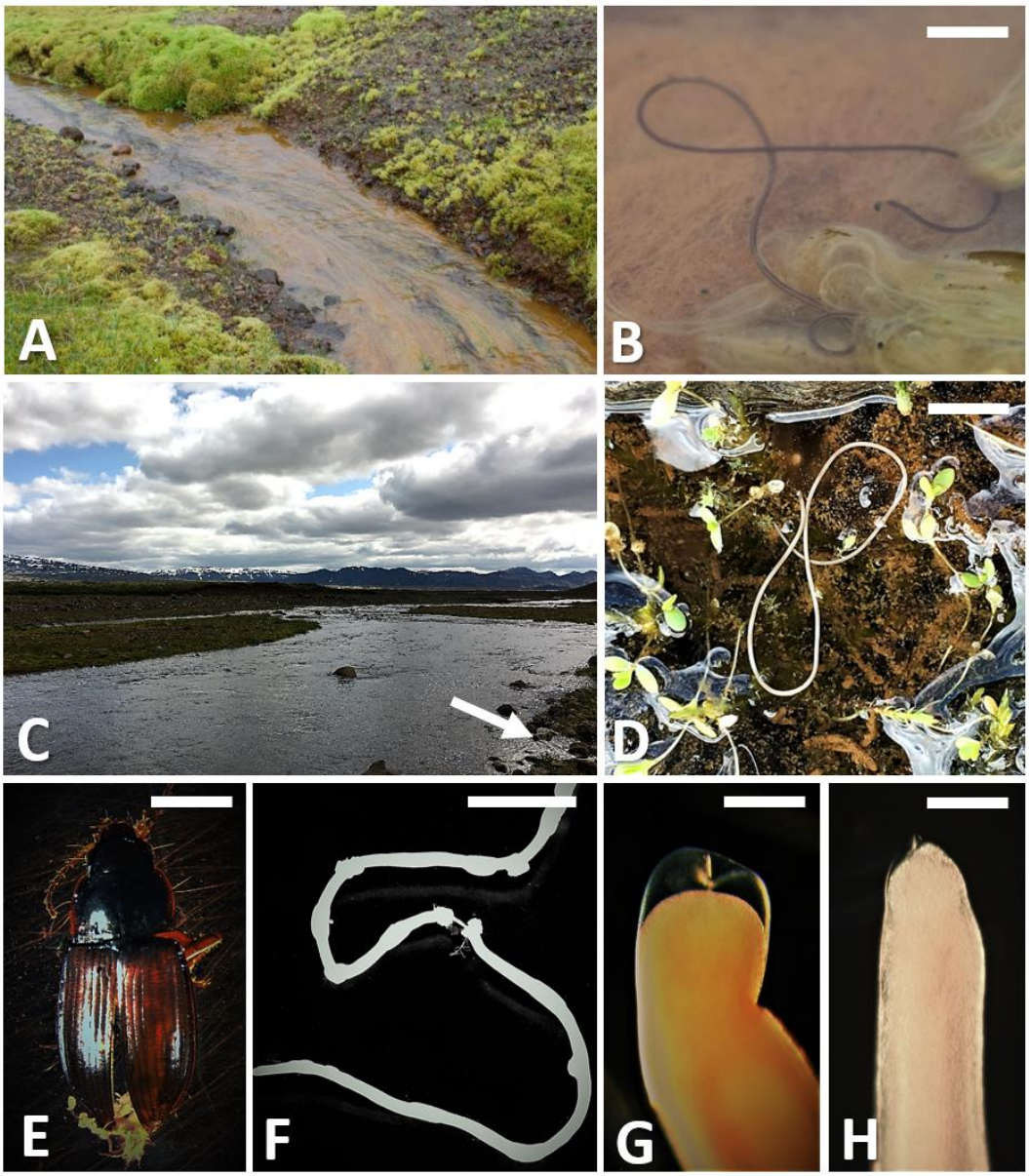

FIGURE 4. Typical habitats for gordiids and their arthropod final hosts in Iceland. (**A**-**B**) Unnamed stream in Hengladalir in the Hengill region of Southwestern Iceland. Note the algal mat with a hairworm in B. Scale bar = 5 mm. (**C**) Unnamed stream along Uxahryggjavegur road. Note small pool on periphery of stream where worms were found (white arrow). (**D**) Female worm in a small pool. Scale bar = 10 mm. (**E**) Ground beetle (*Amara* sp.) collected next to the small pool where a free-living adult female worm was

collected. Scale bar = 6 mm. (**F**) Immature hairworm recovered from the body cavity of *Amara* sp. Scale bar = 6 mm. (**G-H**) Posterior and anterior ends of an immature female worm recovered from *Amara* sp. Note the molting cuticle. Scale bar = 0.3 mm.

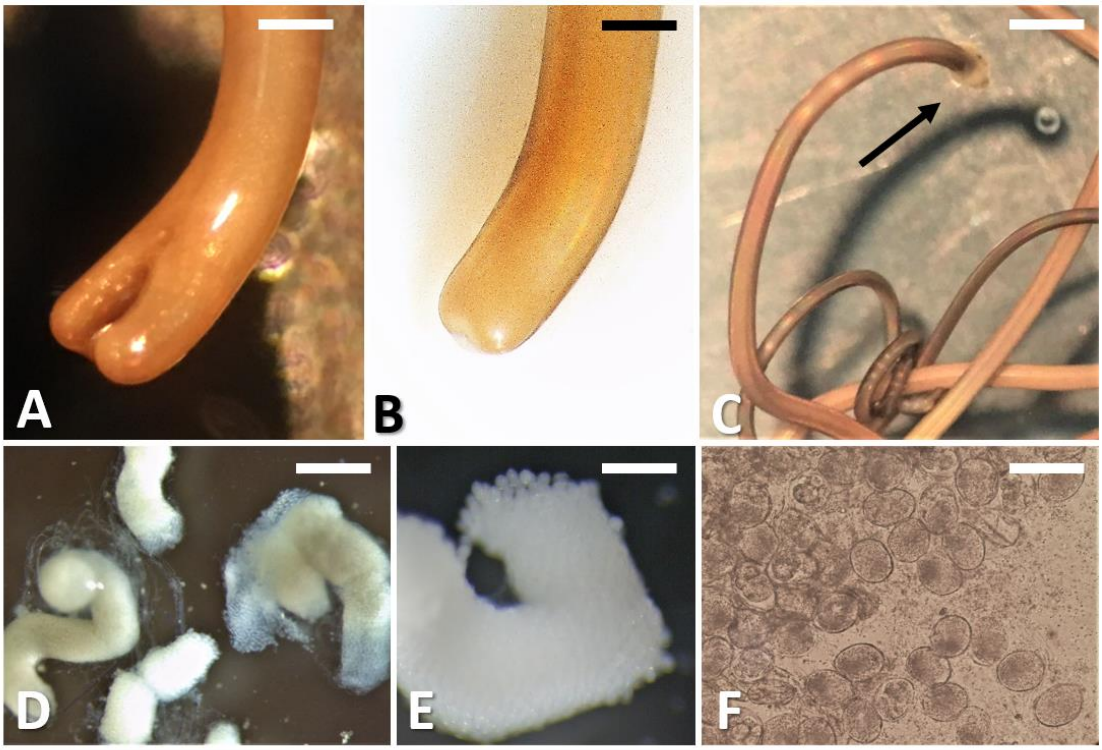

FIGURE 5. Light micrographs showing the posterior ends of a male and female, a Gordian knot, egg strings and eggs of *Gordionus* sp. from Iceland. (**A**) Male showing bifurcated posterior region. Scale bar = 0.2 mm. (**B**) Female showing the terminal end. Scale bar = 0.4 mm. (**C**) Mating behavior of male and female. Note the sperm drop on female terminal end (black arrow). Scale bar = 4 mm. (**D**) Egg strings. Scale bar 0.25 mm. (**E**) Higher magnification of egg strings showing tightly aggregated undeveloped eggs. Scale bar = 0.25 mm. (**F**) Typical shape of undeveloped eggs within egg strings. Scale bar = 50 µm.

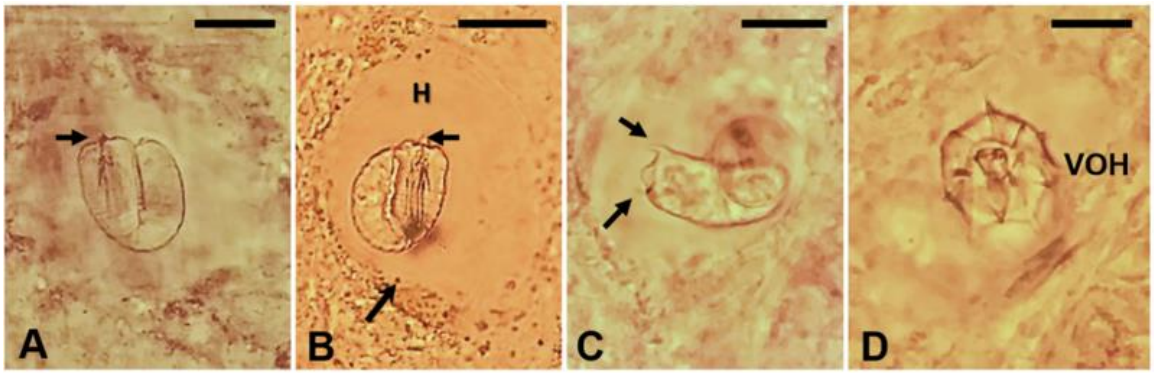

FIGURE 6. Photomicrographs of gordiids cyst infecting the freshwater snail *Radix peregra* from Iceland. (**A**) Fully formed cyst of showing a characteristic spine on the preseptum (black arrow). Note the postseptum is only folded once. Scale bar = 32 µm. (**B**) Fully folded larva within a cyst. Note the characteristic halo (H) surrounding the folded larva. Scale bar = 28 µm. (**C**) Posterior region of the postseptum showing two terminal spines (black arrows) Scale bar = 12 µm. (**D**) Preseptum showing laterally compressed proboscis in relation to the ventral outer hooks (VOH). Scale bar = 15 µm.

CHAPTER V

WHEN IS A PARASITOID A PARASITE? LONGEVITY AND OOGENESIS IN *ACHETA DOMESTICUS* (ORTHOPTERA: GRYLLIDAE) INFECTED WITH A SUSPECTED PARASITOID, *PARAGORDIUS VARIUS* (NEMATOMORPHA: GORDIIDAE)

ABSTRACT The significant costs parasites impose on hosts can lead to reductions in survival and fecundity, but few studies have evaluated the lasting impacts post-parasitism. Hairworms are parasites that infect terrestrial arthropods and then manipulate their host to seek water where the worm emerges as a free-living adult. As a parasitic juvenile, hairworms consume the fat body of hosts, shifting resources from host development to parasite growth. Until now, only one study had examined survivorship of hosts infected with hairworms. Using a different hairworm host system, I conducted experimental infections to examine survivorship and egg production in virgin female *Acheta domesticus* infected with the hairworm, *Paragordius varius*. In the first experiment, I found 41% (11/27) of female crickets died the first week after worms emerged. On average, infected crickets survived for 73 ± 32 days compared to control crickets that survived for 86 ± 45 days. Uninfected control crickets increased in length significantly more (6.5 ± 2 mm) than infected crickets (4.9 ± 2.6 mm; $t = 2.61$, $P = 0.012$). The mean intensity of hairworms was 2.3 ± 2 (range 1-11) and resulted in 47% cricket mortality

in the first week. In experiment two, I found 24% (7/33) of crickets died within the first week but 52% (17/33) of crickets produced eggs. When removing crickets that did not produce eggs in infected and control groups, infected crickets (N = 17) produced an average of 179 ± 134 (range: 20-483) eggs which was not significantly different from control crickets (N = 38) who had an average of 255 ± 195 (range: 19-754) eggs (t = 1.81, P = 0.078). Taken together, this suggests that female crickets infected with hairworms may experience less mortality than previous anecdotal evidence and one laboratory experiment suggests. Finally, I explore the history of parasitoid definitions and the conventions of parasitoids and suggest more field and laboratory research is required before suggesting hairworms are parasitoids.

INTRODUCTION

Parasites are known to incur significant reductions to host life history traits including fecundity and survival (Hurd 2001; 2009). These damaging effects can be the result of direct damage to host organs (Feldman 1998), or due to competition between host and parasite for host resources, among others (Combes 2001; Thomas et al. 2005). The damage experienced by the host can lead to differences in host fecundity and survival (Sorci 2013). For hosts, adaptive responses such as host tolerance, can lead to trade-offs that minimize damage by the parasite (Hurd 2001).

Members of the phylum Nematomorpha, known as hairworms, are macroparasites of arthropods such as beetles, crickets, and millipedes that have a manipulative component to their life cycle (Carvalho 1942; Dorier 1965; Townsend 1970; Blair, 1983; Poinar and Brockerhoff 2001). As the parasitic juvenile hairworm reaches adulthood

within the cricket abdomen, the hairworm begins to manipulate the behavior of its arthropod host to seek water, where worms emerge as free-living adults (Thomas et al. 2002). In dioecious species of gordiids, worms mate and females lay millions of eggs in the water. Eggs hatch releasing sessile larvae that may encyst in larval aquatic insects that act as paratenic (transport) hosts, bridging the gap between the aquatic and terrestrial systems (Hanelt and Janovy 2004). In the terrestrial system, an arthropod becomes infected when they consume the paratenic host harboring the hairworm cyst. In the arthropod's digestive system, the hairworm larva excysts and burrows into the hemocoel of the host where it begins feeding on the fat body (Von Linstow 1891; Studier et al. 1991).

Despite anecdotal evidence for more than a century that hairworms manipulate their host to seek water, where worms are released, empirical evidence for this was not presented until recently when Thomas et al. (2002), observed nine species of cricket displaying aberrant behavior of jumping into a swimming pool to release their worms. This led to the hypothesis that crickets commit 'suicide' by jumping into water to release worms (Thomas et al. 2002; Schmidt-Rhaesa et al. 2005). However, long-term studies on survivorship were not evaluate. In natural systems, hosts that jump into water risk being ingested by fish. For example, Sato et al. (2008) demonstrated the predation of hairworm-infected camel cricket (*Diestrammena* spp. and *Tachycines* spp.) by salmonids. However, several observational studies demonstrate that infected crickets do not discriminate in the type of water source they release worms in, and thus may not always fall prey to predators. In fact, puddles of water, shower stalls, and dog water bowls have been observed on multiple occasions to be opportunistic water sources for hairworms (Bolek

2000; Hanelt et al. 2005). Additionally, other water sources have been reported in the literature for gordiid worms including irrigation ditches, vernal pools, and freshwater shallow springs (Looney et al. 2012) all with variable depths, sizes, and organisms. Therefore, the possibility that arthropod hosts driven to water can survive after the worm exits its host is plausible, especially if the cricket can find its way out of the water. Unfortunately, only a single study has examined the survival of post-infected crickets. Biron et al. (2005) examined naturally infected crickets for survival post infection when he tested the manipulation hypothesis that collaborative crickets (bring the parasite to water) are more likely to survive than non-collaborative crickets.

The hairworm-arthropod system offers interesting insight into host-parasite interactions because during hairworm development, the microscopic (~40-60 micrometer) larvae grow up to 76 cm in length for some species of North American gordiids (Bolek et al. 2015) and up to 2 m for some species of gordiids from the tropics (Schmidt-Rhaesa, 2013; Bolek et al. 2015). This growth translates to an increase in hairworm body size of more than 5,000 times inside the arthropod host which may impose significant effects on the host as the parasite can make up 8-42% of the cricket's total body mass (Anaya pers. obs). Therefore, due to the large energy demands by the parasite, hosts should experience significant reductions in fecundity and/or survival due to the resources used by their parasites but with enough energy left over to deliver the parasites to the appropriate microhabitat where the worm emerges. However, Biron et al. (2005) found that 47% of female crickets survived and a portion of those were able to produce eggs post-infection.

As mentioned above, only a single study has examined host-parasite interactions in the hairworm-cricket system. Therefore, my aim was to add more information on nematomorph host-parasite interactions by investigating the effects of parasitism on host survival during and post hairworm infection using the laboratory hairworm-cricket model system. In experiment 1, I evaluated survivorship probabilities in crickets infected with the laboratory cultured hairworm, *Paragordius varius* and evaluated if parasite intensity or body size was a predictor of survival. I then evaluated if host body size and parasite intensity may contribute to survival probabilities of cricket hosts. In experiment 2, I tested if parasite intensity effected cricket survival but in addition, I evaluated the ability of crickets to produce eggs post-infection.

MATERIALS AND METHODS

Organism maintenance

Exposure of *Acheta domestic*us to *Paragordius varius* and rearing conditions

Physa gyrina snails were reared in 38 L aquaria filled with spring water and fed a diet of frozen iceberg lettuce and Tetra Min® fish food, ad libitum. *Paragordius varius* larvae used for snail infections came from a laboratory stock housed at Oklahoma State University and were stored at -80 °C. Approximately 200 thawed larvae were pipetted into a 1.5 ml wells of a 24-well culture plate filled with 1 mm filtered tap water. A single laboratory reared *Physa gyrina* snail was added to each well. Snails could feed on the larvae mixture for 48 hours in a dark cabinet. *Paragordius varius*-exposed snails were maintained in the laboratory as described above for two weeks allowing cysts to develop before exposing to crickets.

One hundred thirty-five, four-week old (seventh to eighth instar; 10 – 16 mm) female house crickets, *A. domesticus* (Armstrong Cricket Farms, West Monroe, LA), were isolated and starved for 48 hours. Starved crickets were exposed to *P. varius* cysts by offering each cricket a small piece of infected snail tissue. If crickets did not consume at least a portion of the snail tissue within 30 minutes, they were isolated and starved for an additional 12 hours and exposed to snail tissue. Additionally, 50 female control crickets were isolated and starved for 48 hours and fed uninfected snail tissues (sham-infected). All crickets were maintained individually in a 236 ml clear polystyrene container with 0.5 mm holes for air flow under a 12:12 light-dark photoperiod at 25° C. Crickets were fed a diet of ground Puppy Chow®, provided a 5mL plastic centrifuge tube of water with a cotton ball at the end, and a piece of egg carton for shelter. Food and water were provided *ad libitum*.

Experiment 1. Beginning at 30-days post exposure (PE), crickets were checked for hairworm emergence by first measuring the cricket body length to the nearest .1 mm. Crickets were then placed in water for a few minutes to check for worm emergence. If worms emerged, crickets were placed back into their individual housing, the number and sex of worms was recorded, and worms were measured to the nearest 0.1 mm. For crickets with previously emerged hairworms but that did not release worms on the first day, the abdomen was checked daily for darkening, indicating there were additional worms. Crickets were then placed in water to check for emergence. Because not all exposed crickets became infected, only successfully infected crickets were used in analyses. Survivorship was calculated as the number of days successfully infected crickets survived after being exposed to snail tissue. When crickets died during the

experiment, they were examined for developing worms. Infected crickets that died during the first two weeks post exposure but could not be identified as infected (as indicated by immature worms during necropsy), were not included in the analyses.

Experiment 2. All exposed, and control crickets were processed as described for experiment I. However, during necropsy crickets were also evaluated for egg production. If eggs were present in control and infected crickets, the eggs were removed from the abdomen of the cricket using a tapered micro spatula and placed in a petri dish filled with 2 mL of water. Eggs were then counted by eye under a stereomicroscope.

Descriptive Statistics and Analyses.

I used Kaplan-Meier survival analyses (Crowley and Breslow 1984) to compare infected and control cricket survivorship probabilities. Because I could not select a distribution of survival, I performed a nonparametric analysis which uses a step function with steps at the endpoints of each interval (Cantor and Shuster 1992). The survival function was then calculated using the Kaplan-Meier estimation method and the Wilcoxon log-rank test which is weighted by the number of samples that survive at each point in time and therefore, weights early death times more heavily than later ones (Philonenko and Postovalov 2015). All analyses were performed using Minitab 18™. To evaluate if parasite intensity effected host survival, I quantified intensity per host by the total length of hairworms because hairworm sizes within individual hosts can be variable (Hanelt 2009).

Experiment 1. A Student's *t*-test was used to compare the length gained between infected and control crickets. To test if cricket body length was a predictor of survival, I conducted a bivariate regression using cricket body length on the day of worm emergence

and the number of days crickets lived post exposure. For control crickets, I estimated the number of days it took worms to emerge from infected crickets and used the number of days to measure the body condition of control crickets at that time for comparative measurements.

Experiment 2. A Student's *t*-test was used to compare the average number of eggs produced by infected and control crickets. To evaluate if parasite intensity in individual crickets was a predictor of survival, I conducted a bivariate regression using the total length of hairworms and the number of days crickets were alive post exposure. I then tested if the number of days crickets remained alive post exposure effected the number of eggs that were produced.

RESULTS

Experiment 1. Of 135 crickets exposed, 29 crickets were successfully infected and survived through hairworm emergence. However, two crickets escaped after worms emerged and were not included in analyses. No infected crickets with juvenile worms died during the experiment. Of the sham-infected crickets, four escaped during the experiment. Hairworms emerged 46 ± 4 days (range 34-52) post exposure and the mean intensity worms was 2.3 ± 2 (1-11). Worms (N = 63) had an average length of 143 ± 60 mm (5-284). Of 27 crickets that released worms, 11/27 (41%) died within the first week of hairworms emerging. Of those, five crickets contained additional worms in the abdomen after death despite releasing worms previously.

Survivorship. Although control crickets survived longer than infected crickets (control: 86 ± 45 days, range 1—155, N = 46; infected: 73 ± 32, range 42—151, N = 27; Fig. 1),

these differences were not significantly different (Kaplan-Meier survival analysis: P = .089; Fig. 2).

Length gained. Infected crickets grew significantly less over the course of the experiment (infected N = 27, 4.9 ± 2.6 mm, range 0.5-10.7) then control crickets (N = 29, 6.5 ± 2 mm 2.5-10.5, $t_{(49)}$ = 2.61, P = 0.012; Fig. 3). However, both linear bivariate regression analyses showed that size of hairworms or cricket body size was not significantly related to the number of days crickets survived ($F_{(1,26)}$ = 0.22, P = 0.639; Fig. 4; $F_{(1,26)}$ = 0.95, P = 0.340; Fig. 5).

Experiment 2. Of 293 exposed crickets, 42 crickets were successfully infected. Of those, 33 survived through hairworm emergence, three died containing immature worms, two died with mature worms inside, two were misidentified as not infected, one escaped after worms emerged and were not included in analyses, and one date of death was not recorded. Crickets that did not become infected were excluded from analyses. Seventy-five sham-infected crickets were used as controls for comparisons. Hairworms emerged 38 ± 4 days (35-52; N = 34) post exposure and the mean intensity per cricket averaged 1.8 ± 1.3 worms (range 1-4). Of the 33 crickets that released worms, 8/34 (24%) died within the first week of hairworms emerging.

Eggs. Of all infected crickets, 52% produced eggs after releasing worms compared to 91% for uninfected control crickets. Infected crickets produced significantly fewer eggs (92 ± 132 eggs, 0-483, N = 33) compared to control crickets (231 ± 200 eggs, 0-754, N = 43; $t_{(72)}$ = 3.6, P < 0.001). When crickets that did not produce eggs were excluded from infected and control groups, no significant differences existed in egg production by infected crickets (N = 17; 179 ± 134, 20-483) and control crickets (N = 38; 255 ± 195,19-

754; $t_{(43)} = 1.81$, $P = 0.078$). Finally, cricket survival time had an effect on the number of eggs produced, with crickets that survived longer post worm emergence produced more eggs ($F_{(1,31)} = 14.80$, $P = 0.001$; Fig. 6).

DISCUSSION

My results indicated that crickets infected with hairworms are capable of surviving infection. Compared to uninfected controls, I found reduced survival in parasitized females. My results demonstrate that crickets infected with nematomorphs are not only capable of surviving the infection but can also produce eggs despite being infected. However, my prediction that survival is correlated to the number or length of worms within individual hosts and the number of days the host survived was not supported. Nor did I find a relationship between the size of crickets and the size of worms.

Previous studies indicate that parasites can alter host growth in a variety of organisms including insects (Hurd and Arme 1984; Kumar and Ballal 1992; Alleyne and Beckage 1997). Parasitism that leads to increases in host body size may increase body size of parasites leading to higher fecundity as body size is often a predictor of fecundity (Smith and Smilowitz 1976: Cotton 2006). Under controlled laboratory conditions, infected crickets grew less than uninfected crickets and I attribute this difference to hairworms sequestering energy from its host. This may be a result of the significant size increase of hairworms within the hemocoel of the hosts. The infective larval stage of *P. varius* starts at approximately 50 µm (Szmygiel et al 2014) and in this study, grew to an average of 143 mm. This is a 2,860 × increase in body size for the parasites suggesting

energy must be reallocated from the host to the developing worm leading to smaller bodies in infected crickets. Because of this, I analyzed the relationship between the total length of worms (parasite intensity) to the cricket body size but found no relationship (data not shown). Smaller host bodies are consistent with Harvey et al. (1999) who found two species of butterflies, *Pieris brassicae* and *P. rapae*, infected with the parasitoid wasp *Cotesia rubecula* grew smaller compared to uninfected controls but was dependent on the host larval stage at the onset of infection. Less weight gain has been attributed to illness-induced anorexia in which the host reduces dietary intake in an attempt to combat infection (Adamo et al 2007) and could result in smaller host bodies. I would predict that smaller bodies should result in reduced survival, as infected hosts would allocate resources to the developing parasites which leaves little for the growing host particularly for parasites that increase their body size 3000 × fold.

Despite some crickets surviving for several months after releasing worms, it is important to note that mortality was highest during the first week post emergence for both trials (41% and 24%). One explanation for this observation may be that infected crickets have been severely depleted of energy and could not recuperate from the infection. Relative to this, I predicted that crickets with a single worm infection should survive longer post emergence compared to crickets with multiple worm infections because a single worm may use less energy than multiple worms. However, I found no relationship between the total length of worms per cricket and the number of days crickets survived after being exposed to the parasite. Another reason for higher mortality during the first week is that infected crickets may have incurred internal damage because hairworms are folded upon themselves within the cricket abdomen and may become entangled in host

organs that are damaged during infection or emergence. My results are in agreeance with Biron et al. (2005) who found naturally infected wood crickets, (*Nemobius sylvestris*) infected with the hairworm, *Paragordius tricuspidatus* had a higher mortality rate during the first week post emergence compared to unparasitized control crickets. However, they found 47% of females died during the first week compared to the two trials in this study where mortality rate ranged from 24% to 41% during the first week. The differences between the Biron et al. (2005) study and my study may be attributed to the number of worms within cricket hosts; however, Biron et al. (2005) does not provide the intensity of hairworm infections to test a relationship of cricket mortality to the number of worms. However, when I examined the relationship of cricket survival to the intensity of infection, there was no relationship between the number of worms or the total length of hairworms within each cricket and cricket survival in both experiments.

In experiment 1, I then examined if host body size at the time of worm emergence is a predictor of survival. I predicted that hosts with larger bodies would be able to minimize damage to host resources there because there are enough resources to accommodate the developing worms and the hosts functional nutritional needs compared to smaller hosts. However, I found that although on average infected crickets grew less, during infection, there was no relationship between host body size and the probability of survival. Because a laboratory setting provides an environment free from predation and other constraints, it is possible my results are affected by this. In a natural environment, infected crickets jumping into water face additional risks of mortality including drowning or predation (Sato et al. 2011). However, more studies in natural systems are needed to determine the effects of hairworms on the survivorship of crickets in these settings. In

addition, future studies should evaluate the effect hairworm infection on male crickets in the *P. varius* - *A. domesticus* system. Because female crickets contain more fat body than male crickets, they may experience lower effects from hairworm infection due to their fat reserves (Lease and Wolf 2011). In fact, Biron et al. (2005) found this to be the case in the *N. sylvestris – P. tricuspidatus* system in which males survived on average, 11 days compared to female crickets which survived for an average of 37 days.

Finally, in trial 2, I was able to track the number of eggs that were produced by previously infected crickets but after they released their worms. I found that out of 37 infected crickets that survived emergence, 49% were able to produce eggs. This suggests that as soon as worms emerge, crickets begin storing energy to be used in egg production. In one particular case, a cricket died at six days post emergence and contained 20 eggs in necropsy. It is possible that crickets began producing eggs during infection but my laboratory observations over several years have never found eggs within crickets abdomens when necropsy was performed immediately after worm emergence (Anaya unpubl. data). These results are consistent with Biron et al. (2005) when they compared collaborative hosts (entering water to release worms) to non-collaborative hosts (not allowed to release worms). They found that non-collaborative hosts did not produce eggs and suggested that retaining worms within the host may damage host gonads prohibiting egg production. I propose that these results may also suggest that the hairworm, filling a large part of the cricket abdomen, affects stretch receptors inhibiting nutrient acquisition. Without adequate nutrition, crickets will not produce eggs. Alternatively, hairworms have been noted to consume host fat body which contain essential lipids used in egg production (Woodring, 1979). If lipids are sequestered by hairworms there may not be a

high enough level of lipids to produce eggs. Finally, inhibition of egg production during infection may simply be a mechanical issue. Surprisingly, one cricket from each trial contained eggs and one immature worm at necropsy. This may suggest that after the first worms emerged, other larvae that were present began growing but egg production was not inhibited because the immature worm either did not present a size burden or competition for nutrients was not severe enough to inhibit egg production. Further experimentation is needed to determine if females are capable of mating post infection and if those fertilized eggs are viable.

Hairworms as parasitoids?

Hairworms have been described as parasitoids despite a lack of empirical studies to support this claim (Brivio et al. 2000; Blaxter and Koutsovoulos 2015; Weinersmith et al. 2017). While there are examples presented online of showing the debilitating act of hairworms emerging, there are fewer examples of less dramatic emergence by hairworms from their hosts. This begs the question, is this the result of bias sampling, or true representation of the act of a "parasitoid"? As Biron et al. (2005) and my results indicate, some hosts can survive infection. Additionally, my personal field observations indicate that in the vicinity of adult hairworms (post-emergence) there is often no evidence of a dead arthropod hosts after the worms have emerged (Chapter II). The question then becomes: did the host, having recently released its worm, fall prey to a predator or did the host simply walk away? I believe the answer requires more field sampling and laboratory research to be conducted and may vary depending on the host parasite combination.

Definition of parasitoids historically

For the last century, the definition of parasitoid has been quite narrow as to only apply to insects or in some cases only describing the foraging habits of parasitoids (suggesting an insect parasitoid; Reuter 1913; Price 1984; Gauld and Bolton 1988). But even the historic definitions omitted some of the most common parasitoid groups (i.e. Strepsiptera) and depending on the definition used. Eggleton and Gaston (1990) derived that the most common traits of parasitoids are: 1) they are insects and their hosts are usually arthropods; 2) single larvae are associated with a single host; and 3) infection leads to host death. They argued that the traditional characteristic 1 is taxonomically constrained when in fact other taxonomic groups fit characteristic 2 and 3. Eggleton and Gaston (1990) proposed a functional definition to the term parasitoid that then presented entomophagous nematodes and fungi, some protists, some crustaceans, and a single turbellarian as parasitoids. The range of taxa are further expanded if Kuris' (1974) proposal of parasitic castrators are applied because castrators functionally remove the genes of host from the population.

Commonality of nonlethal parasitoids and are they really parasitoids?

Karban and English-Loeb (1997) found that 42% of caterpillars (*Platyprepia virginalis*) parasitized by the tachinid parasitoid fly (*Thelaira americana*) survived infection and pupated to adulthood when they changed their foraging habits from lupine to hemlock. But more importantly, they found these results to be consistent over four years of investigations on a particular population in laboratory and field studies. Interestingly, this phenomenon is not isolated (e.g. Worthley 1924; Richards and Waloff 1948; Clausen 1962; Harris and Todd 1982; DeVries 1984; Maure et al. 2015). In some host-parasite systems, host reproduction took place before parasites emerged. This would

imply that there are characteristics and/or behaviors that increase the chance of host survival or allow reproduction prior to the parasitoid imposing negative effects, in which case, should survivability be the defining factor in the definition of parasitoids or is there a more functional definition? Coevolutionary theory would predict that hosts should evolve phenotypes to combat or tolerate infection in which case, does not the parasitoid evolve to become less lethal in the process thus downgrading the parasitoid to the status of parasite?

LITERATURE CITED

Adamo, S.A., T. L. Fidler, C.A. Forestell. 2007. Illness-induced anorexia and its possible function in the caterpillar, *Manduca sexta*. Brain, Behavior and Immunity 21:292-300

Alleyne M and Beckage, NE (1997) Parasitism-induced effects on host growth and metabolic efficiency in tobacco hornworm larvae parasitized by *Cotesia congregate* Journal of Insect Physiology 43: 407-424

Biron, D.G., Ponton, F., Joly, C., Menigoz, A. Hanelt, B., and Thomas, F. 2005. Water seeking behavior in insects harboring hairworms: should the host collaborate? Behavioral Ecology 16:656-660

Biron, D.G., F. Ponton, L. Marché, N. Galeotti, L.Renault, E. Demey-Thomas, J. Poncet, S.P. Brown, P. Jouin, and F. Thomas. 2006. 'Suicide' of crickets harbouring hairworms: a proteomics investigation. Insect Molecular Biology 15:731-742

Blair, D. 1983. Larval horsehair worms (Nematomorpha) from the tissues of native freshwater fish in New Zealand. New Zealand Journal of Zoology. 10: 341-344.

Blaxter M., Koutsovoulos G. (2015) The evolution of parasitism in Nematoda. Parasitology 142:S26-S39

Bolek MG (2000) Records of horsehair worms Paragrodius varius, Chordodes morgani and Gordius robustus (Nematomorpha) from Indiana. Journal of Freshwater Ecology 15:421-423. DOI: 10.1080/02705060.2000.9663760

Brivio MF, de Eguileor M., Grimaldi A., Vigetti D, Valvassori R., Lanzavecchia G. 2000. Structural and biochemical analysis of the parasite *Gordius villoti* (Nematomorpha, Gordiacea) cuticle. Tissue & Cell 32:366-376

Cantor AB and Shuster JJ (1992) Parametric versus non-parametric methods for estimating cure rates based on censored survival data. Statistics in Medicine 11:931-937. https://doi.org/10.1002/sim.4780110710

Carvalho, J.C.M. 1942. Studies on some Gordiacea of North and South America. Journal of Parasitology 28: 213-222

Clausen CP (1962) Entomophagous insects. Hafner, New Yourk, New York USA

Coombes, C. 2001. Parasitism: the ecology and evolution of intimate interactions. University of Chicago Press, 728 pp

Cotton S, Small J, Pomiankowski A. (2006). Sexual selection and condition-dependent mate preferences. Current Biology 16:R755-R765.

Crowley J and Breslow N (1984) Statistical analysis of survival data. Annual Review of Public Health 5:385-411

DeVries PJ (1984) Butterflies and Tachinidae: does the parasite always kill its host? Journal of Natural History 18:323-326

Dorier. A. 1965. Clsse des Gordiacés. Pp. 1201-1222 in: Grassé P.P. ed Traité de Zoologie Vol. 4(3). Paris Masson et Cie.

Eggleston P and Gaston KJ (1990) “Parasitoid” species and assemblages: convenient definitions or misleading compromises? Oikos 59:417-421

Feldman RM (1998) Parasitic castration of the crab, *Tumidocarcinus giganteus* Glaessner, from the Miocene of New Zealand: coevolution within the Crustacea. Journal of Paelontology 72:493-498. doi.org/10.1017/S0022336000024264

Gauld ID and Bolton B (1988) The Hymenoptera – BM(NH) & Oxford University Press

Hanelt B and Janovy JJ. 2004. Untying a Gordian knot: the domestication and laboratory maintenance of a Gordian worm, *Paragordius varius* (Nematomorpha: Gordiida). Journal of Natural History 38:939-950 doi.org/10.1080/0022293021000058718

Hanelt B, Thomas F, Schmidt-Rhaesa A (2005) Biology of the Phylum Nematomorpha. Advances in Parasitology 59:243-309

Harris VE and Todd JW (1982) Longevity and reproduction in the southern green stinkbug, *Nezara viridula* as affected by parasitization by *Trichopoda pennipes*. Entomologica experimentalis et applicate 31:409-412

Harvey JA, Jervis MA, Gols R, Jiang N, Vet LEM (1999) development of the parasitoid, *Cotesia rubecula* (Hymenoptera: Braconidae) in *Pieris rapae* and *Pieris brassicae* (Leipidoptera: Pieridae): evidence for host regulation. Journal of Insect Physiology 45:173-182.

Hurd, H. 2001. Host fecundity reduction: a strategy for damage limitation? Trends in Parasitology 17:363-368

Hurd, H. 2009. Evolutionary drivers of parasite-induced changes in insect life-history traits: from theory to underlying mechanisms. Advances in Parasitology 68:85-110.

Hurd, H. and C. Arme. 1984. *Tenebrio molitor* (Coleoptera): effects of metacestodes of *Hymenolepis diminuta* (Cestoda) on haemolymph amino acids. Parasitology 89:245-251

Karban R and English-Loeb G (1997) Tachinid parasitoids affect host plant choice by ceterpillars to increase caterpillar survival. Ecology 78:603-611

Kumar P, and Ballal CR (1992) The effect of parasitism by *Hyposoter didymator* (Hym.: Ichneumonidae) on food consumption and utilization by *Spodoptera litura* (Lep.: Noctuidae) Entomophaga, 37 (1992), pp. 197-203

Kuris A (1974) Trophic interactions: similarity of parasitic castrators to parasitoids. The Quarterly Review of Biology 49:129-148. doi.org/10.1086/408018

Lease, H.M. and Wolf, B.O. 2011. Lipid content of terrestrial arthropods in relation to body size, phylogeny, ontogeny and sex. Physiological Entomology 36: 29-38

Looney C, Hanelt B, Zack RS (2012) New records of Nematomorph parasites (Nematomorpha: Gordiida) of ground beetles (Coleopter: Carabidae) and camel crickets (Orthoptera: Rhaphidophoridae) in Washington state. Journal of Parasitology 98: 554-559. doi.org/10.1645/GE-2929.1

Maure F, Thomas F, Doyon J, Brodeur J (2015) Host nutritional status mediates degree of parasitoid virulence. Oikos 125:1314-1323 doi.org/10.1111/oik.02944

Minchella DJ (1985) Host life-history variation in response to parasitism. Parasitology 90:205-216. doi.org/10.1017/S0031182000049143

Philonenko P and Postovalov S (2015) A new two-sample test for choosing between log-rank and Wilcoxon tests with right-censored data. Journal of Statistical

Computation and Simulation 85:2761-2770. doi.org/10.1080/00949655.2014.941533

Poinar, G., and A.M. Brockerhoff. 2001. *Nectonema zealandica* n. sp. (Nematomorpha: Nectonematoidea) parasitizing the purple rock crab *Hemigrapsus edwardsi* (Brachyura: Decapoda) in New Zealand, with notes on the prevalence of infection and host defense reactions. Systematic Parasitology 50:149-157

Richards OW and Waloff N (1948) Toe hosts of four British Tachinidae (Diptera). Entomologist's Monthly Magazine 84:127

Sato T, Arizono M, Sone R, Harada Y (2008) Parasite-mediated allochthonous input: do hairworms enhance subsidized predation of stream salmonids on crickets? Canadian Journal of Zoology 86:231-235. doi.org/10.1139/Z07-135

Sato T, Watanabe K, Kanaiwa M, Niizuma Y, Harada Y, Lafferty KD (2011) Nematomorph parasites drive energy flow through a riparian ecosystem. Ecology 92:201-207

Schmidt-Rhaesa, A., (2012) Nematomorpha. In: Schmidt-Rhaesa, A. (Ed.), Handbook of Zoology. Gastrotricha, Cycloneuralia and Gnathifera. Nematomorpha, Priapulida, Kinorhyncha and Loricifera, vol. 1. De Gruyter, Berlin, pp. 29–145

Smith CL, Smilowitz Z (1976) Growth and development of *Pieris rapae* larvae parasitized by *Apanteles glomeratus***.** Entomol. Exp. Appl., 19 (1976), pp. 189-195

Sorci, G (2013) Immunity, resistance and tolerance in bird-parasite interactions. Parasite Immunology 35:350-361. doi.org/10.1111/pim.12047

Studier, E. H., K. H. Lavoie, and C. M. Chandler. 1991. Biology of cave crickets, *Hadenoecus subterraneus*, and camel crickets, *Ceuthophilus stygius* (Insecta: Orthoptera): parasitism by hairworms (Nematomorpha). Journal of Helminthological Society of Washington. 58:248-250

Szmygiel, C., A. Schmidt-Rhaesa, B. Hanelt, and M.G. Bolek. 2014. Comparative descriptions of non-adult stages of four genera of Gordiids (Phylum: Nematomorpha). Zootaxa 3768:101-118

Thomas F, Schmidt-Rhaesa A, Martin G, Manu C, Durand P, Renaud F (2002) do hairworms (Nematomorpha) manipulate the water seeking behaviour of their terrestrial hosts? Journal of Evolutionary Biology 15:356-361 doi.org/10.1046/j.1420-9101.2002.00410.x

Thomas F, Adamo S, Moore J (2005) Parasitic manipulation: where are we and where should we go? Behavioural Processes 68:185-199

Townsend, J.I. 1970. Records of Gordian worms (Nematomoprha) from New Zealand Carabidae. New Zealand Entomologist 4:98-99

Von Linstow, O. (1891b): Ueber die Entwicklungsgeschichte von *Gordius tolosanus* Duj. Centralbl. Bacteriol. Parasitenkd. 9: 760-762

Weinersmith KL, Liu SM, Forbes AA, Egan SP (2017) Tales from the crypt: a parasitoid manipulations the behaviour of its parasite host. Proceedings of the Royal Society B 284:1-8. doi.org/10.1098/rspb.2016.2365

Wilson RA and Denison J (1980) The parasitic castration and gigantism of *Lymnaea truncatula* infected with the larval stages of *Fasciola hepatica*. Parasitology Research 61:109-119

Woodring, J.P., C.W. Clifford, and B.R. Beckman. 1979. Food utilization and metabolic efficiency in larval and adult house crickets. Journal of Insect Physiology 25:903-912

Worthley NH (1924) The biology of *Trichopoda pennipes* Fab. (Diptera, Tachinidae), a parasite of the common squash bug. Psyche 31:7-16

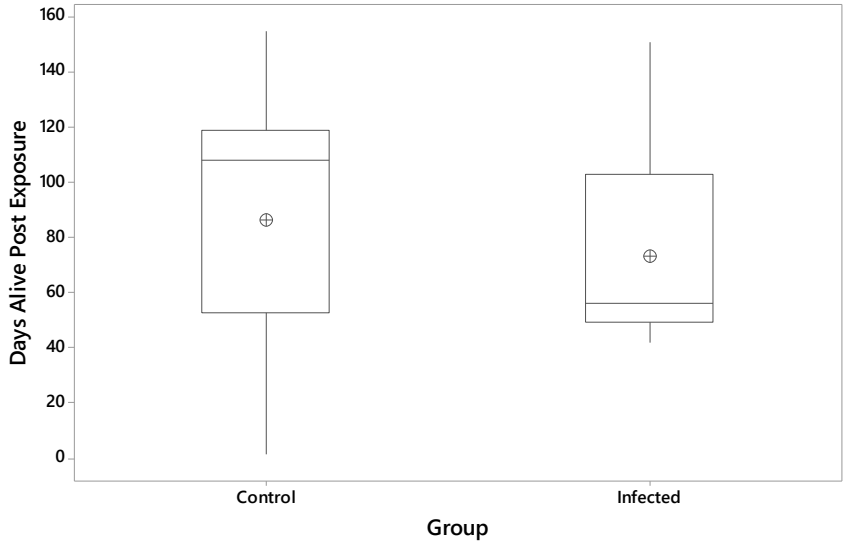

FIGURE 1**.** Total days alive post-exposure.

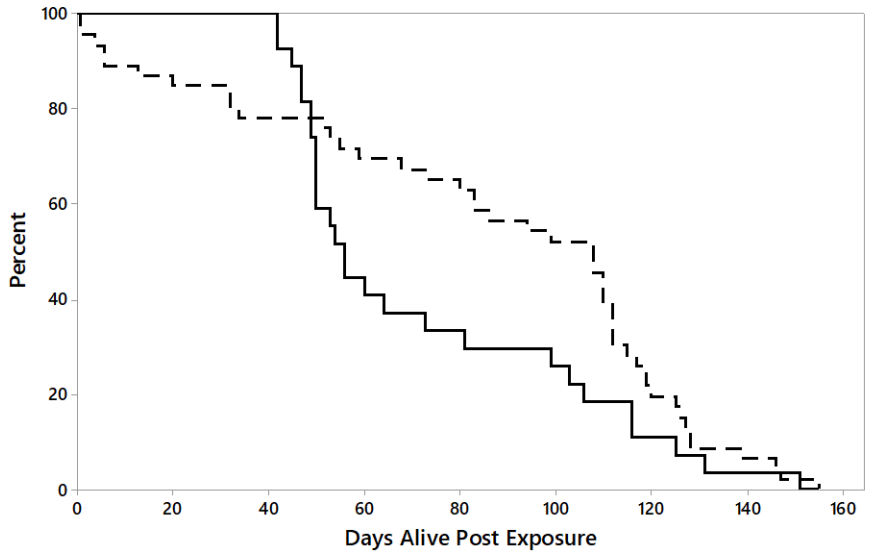

FIGURE 2**.** Infected female cricket survivorship (solid line) was lower than uninfected control crickets (dashed line). Lines represent Kaplan-Meier analysis cumulative survivorship curves beginning at exposure to the parasite.

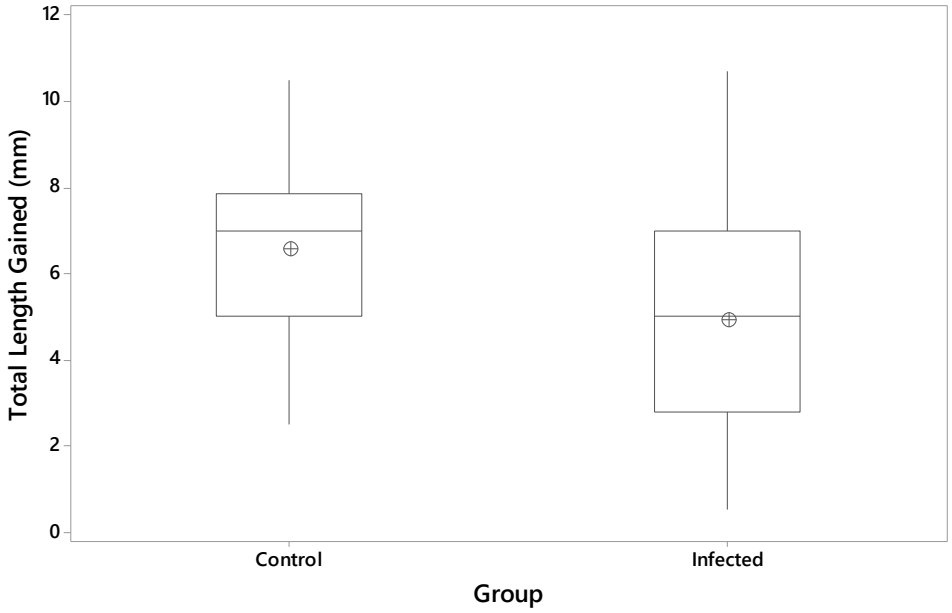

FIGURE 3**.** Box and whisker plot of total length gained by control (N = 29) and infected crickets (N = 27). The central box spans from the lower to the upper quartile, the middle line represent the median, the whiskers extend from the 10^{th} to the 90^{th} percentile lengths gained.

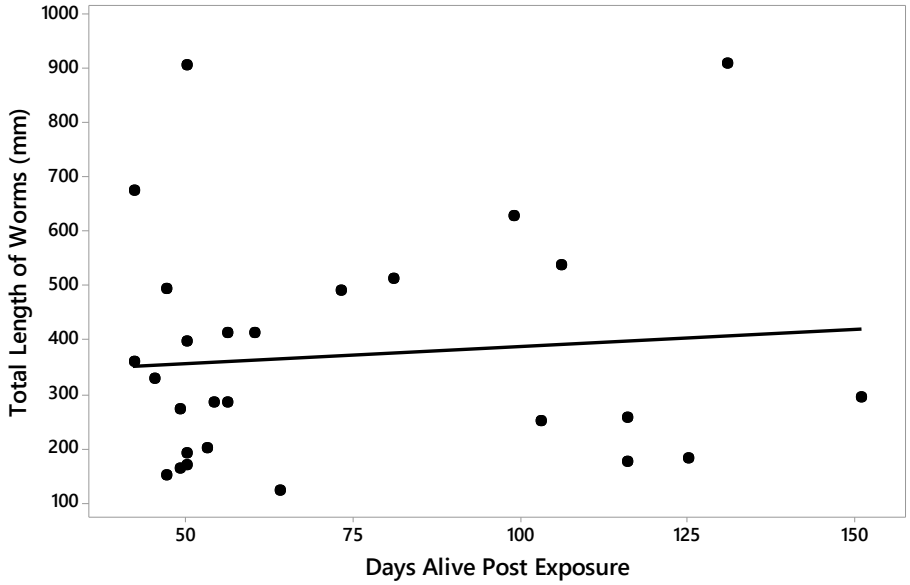

FIGURE 4. Parasite intensity represented as total length of worms within individual crickets as a function of the number of days crickets remained alive post exposure.

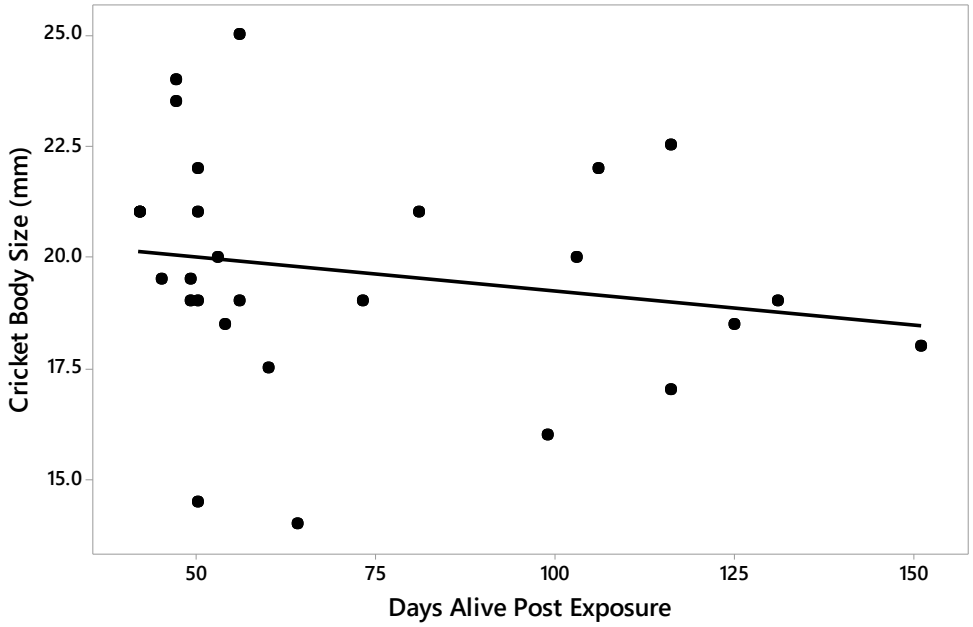

FIGURE 5. Female cricket body size in mm as a function of the number of days crickets stayed alive post exposure.

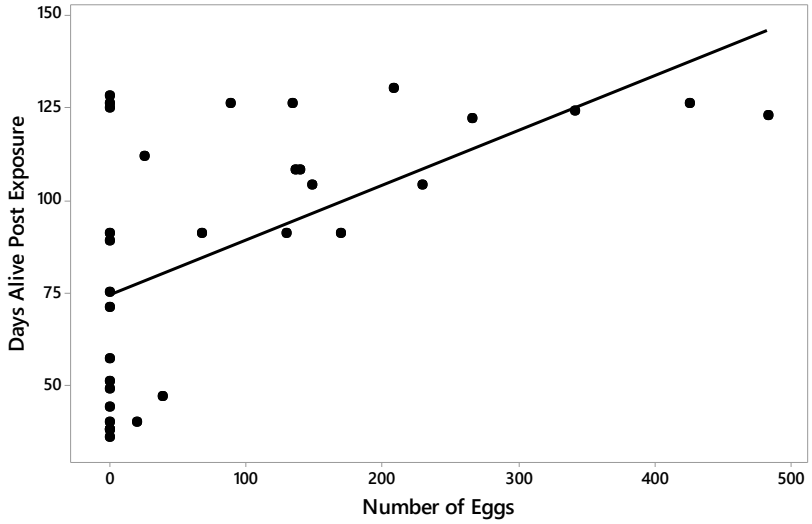

FIGURE 6. Days alive post exposure as a function of the number of eggs produced by female crickets.

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