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NEMATOMORPHA

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Phylum Nematomorpha

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Chapter 57

Nematomorpha (Phylum): Horsehair Worms

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Introduction

Nematomorphs are commonly known as horsehair worms because of their resemblance to and the common myth of worms arising from horse tail or mane hairs that fall into water. Additionally, because of their habit of becoming entangled in masses of many individuals while mating, horsehair worms are also known as Gordian worms after the Gordian knot from Greek mythology (Figure 1) (Bolek et al., 2015).

The phylum Nematomorpha consists of species that can be allocated into 2 major classes, the freshwater and terrestrial Gordiida and the marine Nectonematida. These animals are unique in several ways and they are 1 of 3 entirely parasitic animal phyla that include the Cestoda—the tapeworms—and the Acanthocephala—the thorny-headed worms (Hanelt et al., 2005). At the current time, the nematomorphs include approximately 360 species that have been described globally and are included in 19 extant and 2 extinct genera (Poinar, 1999; Poinar and Buckley, 2006; Yadav et al., 2018). The 5 known marine species belong to the genus *Nectonema*, and all infect decapod crustaceans (phylum Crustacea: class Decapoda) (see Schmidt-Rhaesa, 2013). Among the Gordiida, both dioecious and parthenogenetic species are known and at least 1 species occurs in terrestrial habitats (Hanelt et al., 2012; Anaya et al., 2019). The freshwater and terrestrial gordiids have complex life cycles, which means that the life cycle can be completed by using multiple hosts with final free-living larvae and adults. Species of gordiids that infect an insect such as a cricket, appear to influence the cricket to go near or into water where the adult worm then emerges from the insect to continue its life in a free-living phase (Thomas et al., 2002; 2003). After emerging from their host, dioecious species form large mating assemblages, also called gordian knots, where they mate and females deposit egg strings on substrate in the water. Those species that are parthenogenetic immediately deposit egg strings after emerging from their host (Hanelt et al., 2012; Bolek et al., 2013a). Larvae develop in the water and infect various species of aquatic invertebrate animals (Bolek and Coggins, 2002; Hanelt and Janovy, 2003). Some of these infected animals (such as aquatic insect larvae) act as paratenic or transport hosts, and when the insects metamorphose, they can carry the cysts to a terrestrial environment where they may be consumed by omnivorous or predatory arthropods, including millipedes, orthopterans (crickets, grasshoppers, etc.), beetles, cockroaches, and mantids (Figure 2).

Figure 1. Free-living adult gordiids. A) Adult free-living male *Gordius* sp.; B) A typical Gordian knot containing numerous individuals of *G. terrestris*. Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

Horsehair worms are commonly found in domestic water sources such as swimming pools, toilet bowls, cow troughs, pet water bowls, and more, thus making human interactions with them quite common (Bolek, 2000; Hanelt et al., 2005). However, besides the trauma people experience when they discover nematomorphs in their toilet or pet's water bowl, they have no medical or economic importance, although their potential as biological control agents has been suggested (Schmidt-Rhaesa, 2013). There are a few reports of adult horsehair worms from humans, but all of these observations are most likely the result of people swallowing infected arthropods or arthropod hosts releasing free-living worms into drinking water (Bolek et al., 2015). Additionally, there is one odd report of larval horsehair worms in human facial tissue resulting in orbital tumors (Singh and Rao, 1966). However, this report is questionable because juvenile worms contain few if any morphological characteristics of gordiids (see Schmidt-Rhaesa, 2013).

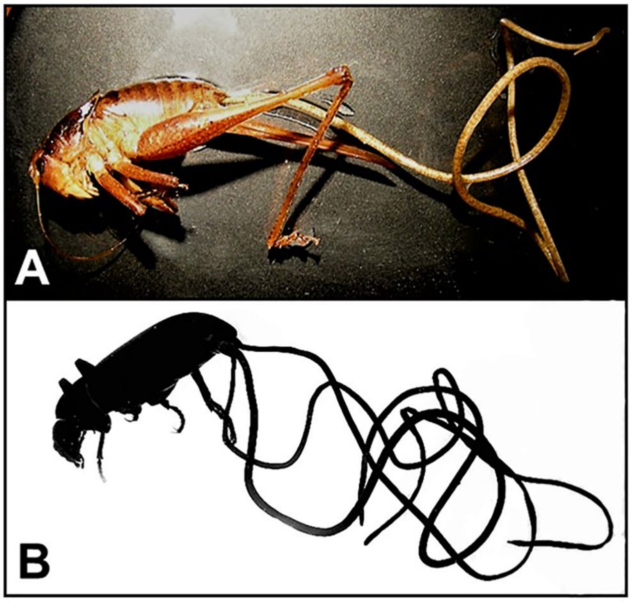

Figure 2. Examples of typical arthropod definitive hosts for gordiids. A) An undescribed species of shieldback katydid *Atlanticus* sp. with an emerging female *Chordodes morgani*; B) an unidentified tenebrionid beetle with 3 emerging individuals of a new species of *Parachordodes*. Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

Chief Morphological Characters

The Nematomorpha belong to the superphylum Ecdysozoa. As with all ecdysozoans (which means molting animals), horsehair worms molt their **cuticle** at least once during their life history. As free-living adults, nematomorphs are very long, cylindrical, and thin, and range from a few cm to over 2 m in length and less than 1 to 3 mm in diameter (Bolek et al., 2015). However, most free-living worms are 20–40 cm in length (Bolek and Coggins, 2002; Schmidt-Rhaesa, 2013).

Among the freshwater and terrestrial gordiids, adult females are usually longer and thicker than adult males (Bolek and Coggins, 2002). Adult free-living worms vary in color from white to shades of dark brown. In some species of *Chordodes*, some individuals have dark patches on a lighter background, producing a leopard-skin pattern, whereas in species of the genus *Gordius* some individuals may have white spots on a darker background (Figure 4C,D). However, color is not a good characteristic for species identification, and most species for which information is available contain various color morphs within and among populations (Schmidt-Rhaesa, 2013).

The anterior end in free-living adults is spheroid or distinctly tapering (Figure 4E,F). The **mouth** may be visible or closed (Figure 4H), and no other structures are present on the anterior end. In some species, there is a distinctly lighter colored area on the anterior end known as a **calotte**, followed by a darkly pigmented ring (Figure 4G). The posterior end is trilobed or unbranched in females and bi-lobed or unbranched in males (Figure 5A–D). In males, the **cloacal opening** is always situated on the ventral side and may contain cuticular structures, such as **circumcloacal spines**. The **cloaca** of males is usually surrounded by either post-cloacal **crescents** or **spines** and/or pre-cloacal **bristles**, and these structures are genus- and/or species-specific (Figure 5E). The cloaca in females is terminal or slightly subterminal and circumcloacal spines have not been reported for females of most species (Bolek and Coggins, 2002; Schmidt-Rhaesa et al., 2003; Bolek et al., 2010; Begay et al., 2012; Schmidt-Rhaesa, 2013).

The marine *Nectonema* species are morphologically similar to the gordiids, wormlike, long, 10–270 mm for males and 30–960 mm for females and approximately 1 mm in diameter (Schmidt-Rhaesa, 2013). The anterior and posterior ends are rounded in females, whereas the posterior end is curved ventrally and distinctly tapered in males. Unlike most gordiids, the cuticle is smooth and does not contain areoles or other surface structures, but instead the dorsal and ventral longitudinal midlines contain natatory bristles (Figure 3). In addition to the ventral longitudinal nerve cord, a dorsal nerve cord is present. The intestine is incomplete and forms a blind gut. Unlike the freshwater gordiids, the anterior end of nectonematids contains a body cavity with conspicuous large cells of unknown function known as **giant cells**. Additional work by Schmidt-Rhaesa (1996a; 1996b) and Restelli and colleagues (2002) indicates that gordiids and nectonematids differ in their muscle cell structure. Freshwater gordiids have thick and thin contractile filaments which are concentrated in bundles as thick sheets and myofibrils enclose the cell body; whereas nectonematid muscle cells are coelomtyarian, as in some nematodes.

General larval morphology. Larvae are 60–100 µm by 14–30 µm in length and width, respectively, cylindrical in shape, and superficially annulated. A septum divides the larval body into 2 regions, the pre-septum and the post-septum (Figure 10). The pre-septum contains 3 rings of cuticular **hooks** and an eversible **proboscis**, supported by 3 internal **stylets** and various sets of **muscles** (Müller et al., 2004). The outer **cuticular ring** contains 6 hooks, 1 of which is positioned ventrally and bifurcated; whereas the middle and inner rings contain 6 hooks, none of which is bifurcated (Figure 11) (Szmygiel et al., 2014). The post-septum contains 1–4 terminal spines among some gordiid genera (Szmygiel et al., 2014). Internally, the post-septum contains the **pseudointestine**, which is subdivided into unequal portions and opens to the outside of the body via a small duct (Hanelt and Janovy, 2002; Szmygiel et al., 2014). The pseudo-intestine is assumed to have a glandular function and empties during cyst formation.

Cuticular Features

In most species of Nematomorpha, the surface of the cuticle is smooth or structured into elevated thickenings called **areoles**. When present these are separated by interareolar furrows; in addition, a variety of short spines and/or bristles may be present on the surface of the cuticle. In most gordiid species, 1 or 2 types of areoles are present. These are known as simple areoles that form a regular pattern on the cuticle (Figure 5F). In species of some genera, such as those in the genus *Gordius*, areoles are lacking or are weakly developed, while in species of other genera, such as those in the genus *Chordodes*, up to 6 different types of areoles can be present and include the characteristic crowned areoles which define the species in this genus (Figure 5I–J) (Schmidt-Rhaesa et al., 2003; Bolek et al., 2013b). Finally, in some species, 2 or more areoles may be fused and form structures referred to as mega-areoles and super-areoles, and these are considered synapomorphies that group species into defined genera (Figure 5G,H).

Sexual dimorphism is common in the areole pattern among gordiids. For proper descriptions and to make firm identifications to the level of species, it is necessary to have examples of and descsribe the characters of both sexes for complete species descriptions (Bolek and Coggins, 2002; Bolek et al., 2010; 2013b).

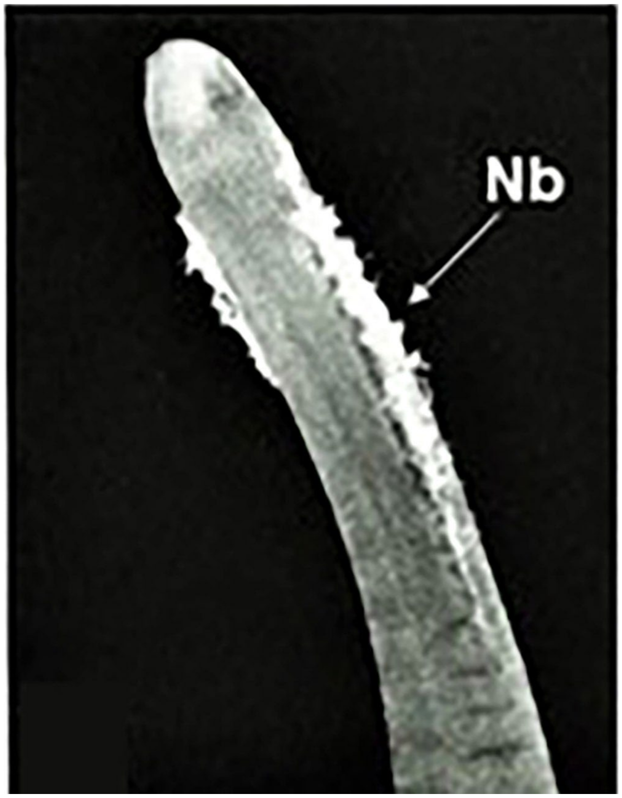

Figure 3. Light micrograph showing natatory bristles (Nb) on the anterior part of a mature fee-living gordiid.

Body Wall

The body wall of adult gordiids is composed of a thick cuticle containing an outer homogeneous region and an inner fibrous region. The fibrous region consists of 25–45 layers of thick **fibrils** that are arranged in a crisscross pattern alternating at an angle of 60–65° (May, 1919; Schmidt-Rhaesa, 1997; 2013). Studies on the chemical composition of the cuticle by Brivio and colleagues (2000) and Protasioni and colleagues (2003) indicate that the makeup of the fibrils is not collagen but some other proteinaceous components. Below the cuticle is a very thin epidermis, which secretes the cuticle layers during development within the definitive host (Schmidt-Rhaesa, 2013). The musculature in all nematomorphs consists of **longitudinal muscles** and, as in species of the phylum Nemata, circular muscles are absent (Schmidt-Rhaesa, 1996a; 1996b; Restelli et al., 2002). The body cavity in free-living adults is mostly filled with **gonads** and vacuolated **parenchyma cells** filled with lipids and glycogen, and the **digestive track** is greatly reduced (Reutter, 1972).

Nervous System

The nervous system consists of a **brain** (basically a circumesophageal **nerve commisure**), a ventral longitudinal **nerve cord**, which emerges from the ventral part of the brain, and a number of peripheral basi-epidermal **nerves**. The brain forms a ring-like structure similar to those possessed by species in the phylum Nemata and surrounds the anterior region of the **alimentary canal** (Schmidt-Rhaesa, 2013). The ventral nerve cord is connected to the epidermis by a thin lamella (Schmidt-Rhaesa, 1997). Simple **sensory organs** in the cuticle are not fully understood, but some studies indicate that integumentary receptors are present. However, it is unclear if these function as mechanoreceptors or if they have other functions (Schmidt-Rhaesa, 2013).

Digestive System

In free-living adults, a **mouth** may or may not be present and, depending on the gordiid species, the **pharynx** can be a cuticularized tube (Figure 4H), cellular in structure, or absent altogether. The **intestine** is located dorsally to the ventral nerve cord and consists of layers of cuboidal cells that manifest **microvilli** on the lumen side of the tissue. Work on *Paragordius varius* indicates that the organization of the intestine changes during development in the definitive host, decreasing in free-living adults compared to parasitic juveniles. In both sexes, the intestine and reproductive system fuse and form the **cloaca**, which is lined with **cuticle** (Schmidt-Rhaesa, 1997; 2005; 2013).

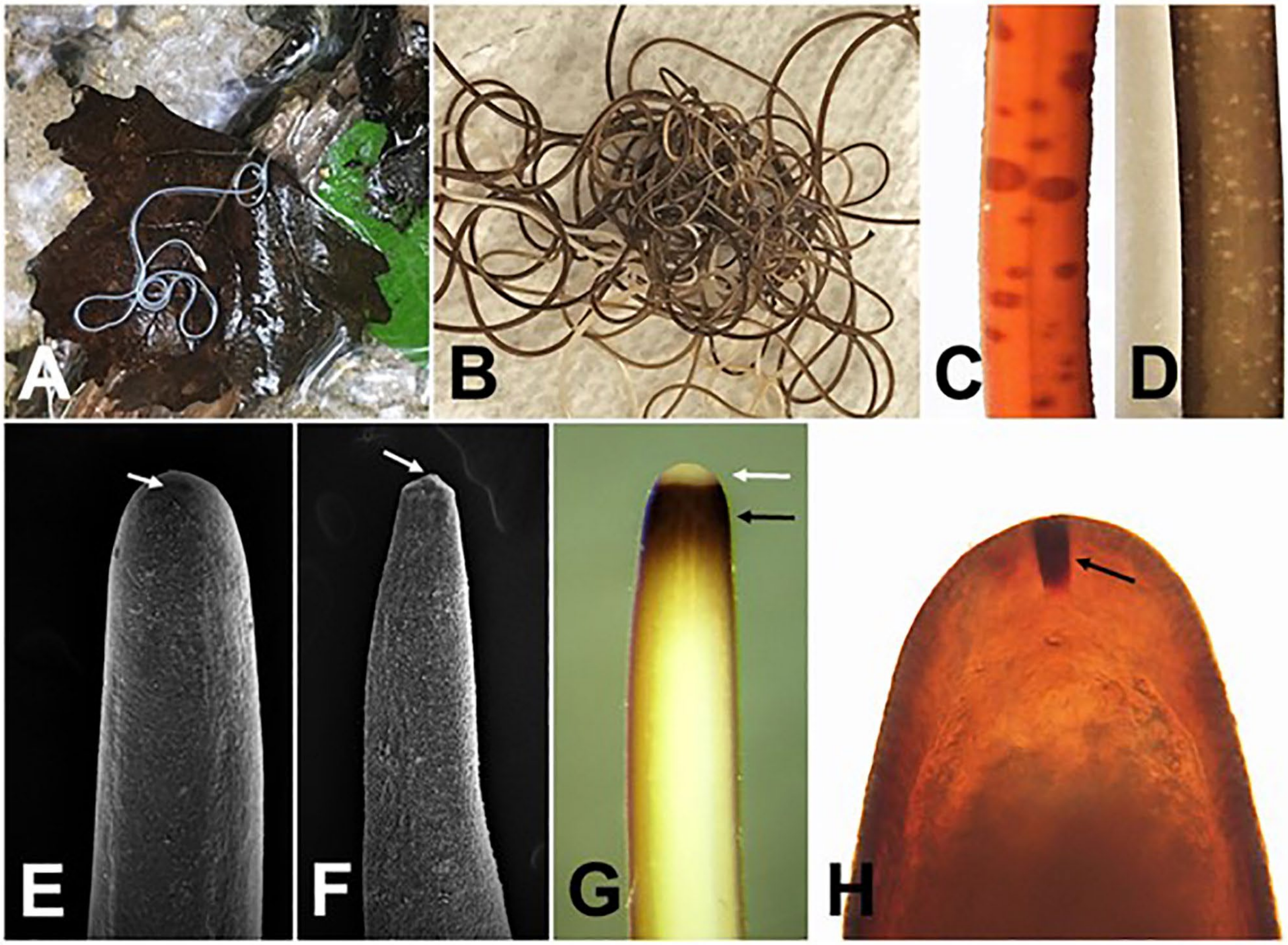

Figure 4. Color and anterior morphology of adult free-living gordiids. A) Typical white color of a female *Gordius difficilis*; B) a Gordian knot of *G. terrestris* showing variation in color of worms ranging from white to dark brown; C, D) mid-body region of a free-living adult female *Chordodes morgani* showing the leopard pattern and the mid-body region of a free-living adult male *G. terrestris* showing white spots on a darker background; E, F) scanning electron micrograph of the E) anterior region being spherical in *G. difficilis* and F) distinctly tapering in *C. morgani*. Note the degenerate mouth (white arrows); G) anterior end of a female *G. terrestris*. Note the calotte (white arrow) followed by a by a dark pigmented ring (black arrow); H) anterior end of a male *C. morgani*. Note the cuticularized pharynx (black arrow). Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

Reproductive System

The **gonads** are arranged as 2 long dorsolateral tubes, surrounded by **parenchymal cells**, and extend almost the entire length of the body. In mature males, the 2 **testes** are full of spermatozoa but may be empty after males complete mating with several females. In developing females, the 2 dorsolateral tubes contain **ovarial ducts** with numerous extensions called **ovaries** (Schmidt-Rhaesa, 1997).

Little information is available about the structure of gonads in the marine Nectonema species, including no information on mature spermatozoa (Schmidt-Rhaesa, 2013).

Reproduction

Once worms enter water, in dioecious species, male and female worms must find each other to mate. Observations on *Paragordius varius* in the laboratory indicate that male worms begin mating with females even before they completely exit their arthropod definitive host (Hanelt and Janovy, 2004b). However, field studies indicate that most arthropods that are shown to be infected by horsehair worm larvae are infected with a single worm and these individuals must somehow find the opposite sex for copulation after they emerge from their hosts (Bolek and Coggins, 2002; Looney et al.,

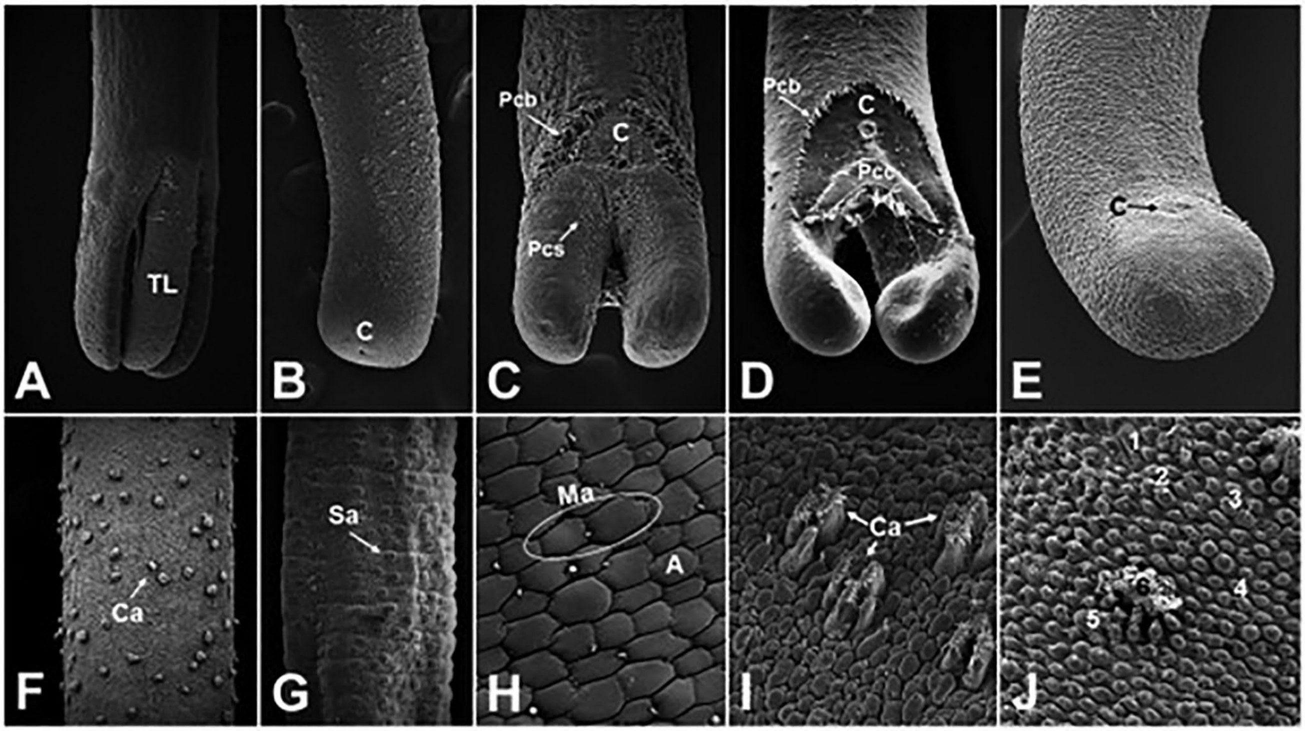

Figure 5. Scanning electron micrographs of the posterior ends and cuticle structures of adult free-living gordiids. A) Posterior end of a female *Paragordius* sp. Note the 3 tail lobes (TL); B) posterior end of a female *Chordodes* sp. showing a round posterior end and a terminal cloaca; (C); C) posterior end of a male *Parachordodes* sp. showing 2 tail lobes, cloacal spines surrounding the ventrally located cloaca (C) and rows of precloacal bristles (Pcb), and postcloacal spines (Pcs); D) posterior end of a male *G. difficilis*. Note the ventrally located cloaca (C), precloacal bristles (Pcb), and postcloacal crescent (Pcc); E) a male *Neochordodes* sp. Note the absence of distinct tail lobes and the cloacal opening (C); F) mid-body region of an adult free-living female *Chordodes* sp. Note the distinct crown areoles (Ca) among simple areoles; G) mid-body region of an adult free-living male *Parachordodes* sp. showing characteristic super-areoles (Sa); H) higher magnification of the mid-body cuticle of a male *Neochordodes* sp. Note the simple (A) and mega-areoles (Ma); I, J) higher magnification of the mid-body cuticle of 2 species of *Chordodes* showing interspecies variation in crown areoles (Ca and 6). Note the 6 types of areoles (1–6) on the cuticle of the *Chordodes* species in (J). Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

2012). Currently, it is unknown whether both sexes find each other by the use of attractants, such as pheromones, or simply by chance. However, field studies using daily collections of individual female *Gordius difficilis* indicate that most females mate within a day of emerging from their host (Bolek and Coggins, 2002).

Both field and laboratory observations indicate that male and female worms initiate typical Gordian knots within hours to days of being placed together (Bolek and Coggins, 2002; Bolek et al., 2013b). During mating, males move up and down the female's body with their coiled posterior end. In genera with bi-lobed posterior ends, such as *Gordius*, a male will spread its tail lobes and glide along the female's body (Figure 8). Once a male's cloaca is in proximity of the female cloaca, the male deposits a mass of sperm referred to as a **sperm drop** or **spermatophore** (Figure 8). Field studies on *Gordius difficilis* indicate that sperm drops can remain on the posterior region of females for at least a week (Figure 8) (Bolek and Coggins, 2002).

The spermatozoa of gordiids are unique and change shape during sperm transfer between a male and a female. They contain a **nucleus** and compartments, which have been named the **acrosomal tube**, **acrosomal sheath**, and **multivesicular complex** (Figure 8). It is unclear if and how these spermatozoa move because they lack a flagellum or pseudopods (Schmidt-Rhaesa, 2013).

After dioecious species mate, females produce up to 8 million eggs during their 2-week to 2-month adult life span (Bolek and Coggins, 2002; Hanelt, 2009). Females in the genus *Gordius* and *Acutogordius* deposit short pieces of **egg strings** approximately 1–2 cm in length on the substrate or while within Gordian knots. In contrast, females of species of *Euchordodes*, *Chordodes*, and *Neochordodes* deposit their egg strings in a zigzag pattern to objects such as sticks or rocks in water. Finally, females of *Paragordius* species deposit a single long egg string approximately 1–5 times the length of the worm's body in the water column and/or in algal mats (Figure 9) (Poinar, 2010; Szmygiel et al., 2004; Bolek et al., 2015; Chiu et al., 2017).

Eggs are elliptical to round in shape, with a distinct shell and a thin inner membrane surrounding the developing larva. This inner membrane is relatively thin in aquatic species but is much thicker in gordiids that reproduce in terrestrial habitats (Figure 10) (Anaya et al., 2019). After hatching, the free-living gordiid larvae are semi-sessile and not capable of moving great distances.

In order for aquatic gordiid larvae to reach their terrestrial arthropod hosts, 3 transmission strategies have been proposed. These include: 1) Direct consumption of larvae by the definitive hosts while drinking water; 2) larvae encysting on vegetation/detritus and being ingested accidently while the definitive host ingests vegetation/detritus; and 3) larvae entering and encysting within a paratenic host, which is preyed on or scavenged by the definitive hosts (Hanelt et al., 2005; Bolek et al., 2015). A number of studies show that when definitive arthropod hosts ingest suspended larvae when they drink water, the definitive host becomes infected (May, 1919; Inoue, 1962; Hanelt and Janovy, 2004b). However, comparative work by Inoue (1962) and Hanelt and Janovy (2004a) strongly suggests that prevalence and intensities of these infections in definitive arthropod hosts are much lower compared to those that occur when definitive hosts are exposed to gordiid cysts in paratenic hosts. Finally, observations on gordiid larvae of European and North American *Gordius* species

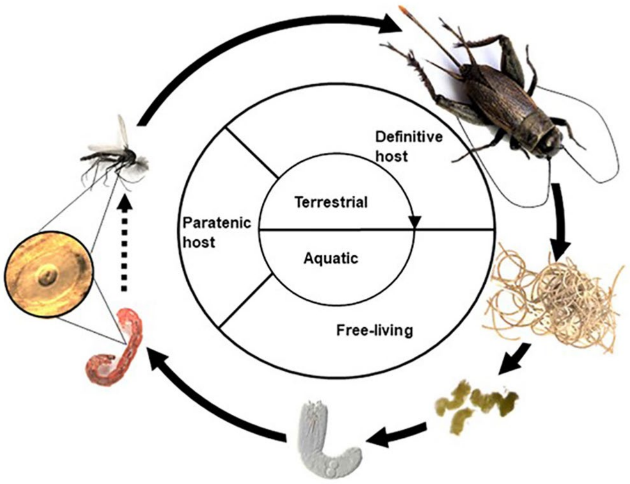

Figure 6. Diagram of a typical gordiid life cycle. The life cycle takes 4–8 weeks to complete in the laboratory depending on the species of nematomorph involved. Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

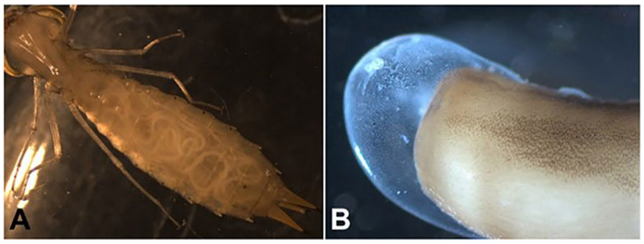

Figure 7. Development of gordiids in their arthropod definitive host. A) An infected larval comet darner *Anax longipes*, showing developing *Neochordodes* species within the hemocoel; B) a developing worm removed from the hemocoel of an arthropod host, showing the thin larval cuticle. Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

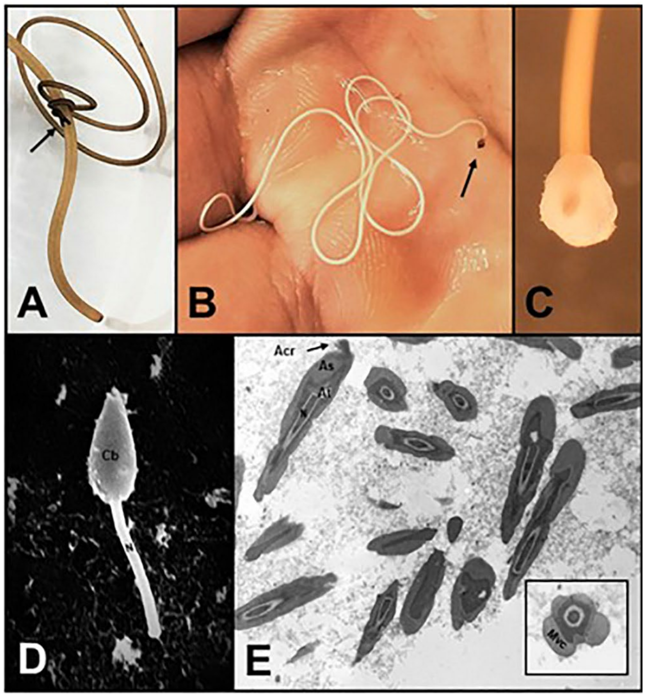

Figure 8. Mating behavior and the morphology of the sperm drop and sperm of gordiids. A) A male *Gordius terrestris* in the process of initiating mating with a female. Note the thinner size and bi-lobed posterior end (arrow) of the male; B) a field-collected female *G. difficilis* with a sperm drop (arrow) on the posterior end; C) a higher magnification of the posterior end of a female *Paragordius varius* with a deposited sperm drop; D) a single sperm on the posterior region of the cloaca of a female *G. difficilis*. Note the round end (Cb) and rod-shaped end (N) where part of the nucleus is located; E) cross- and longitudinal sections of a spermatozoon from the reproductive system of a *Gordius* sp. Note the numerous compartments and organelles, including Acr = acrosome, As = acrosomal sheath, At = acrosomal tube, Mvc = multivesicular complex, and N = nucleus. Sources: A–C) M. G. Bolek; D) Bolek and Coggins, 2002; E and insert) A. Schmidt-Rhaesa. License for all: CC BY-NC-SA 4.0.

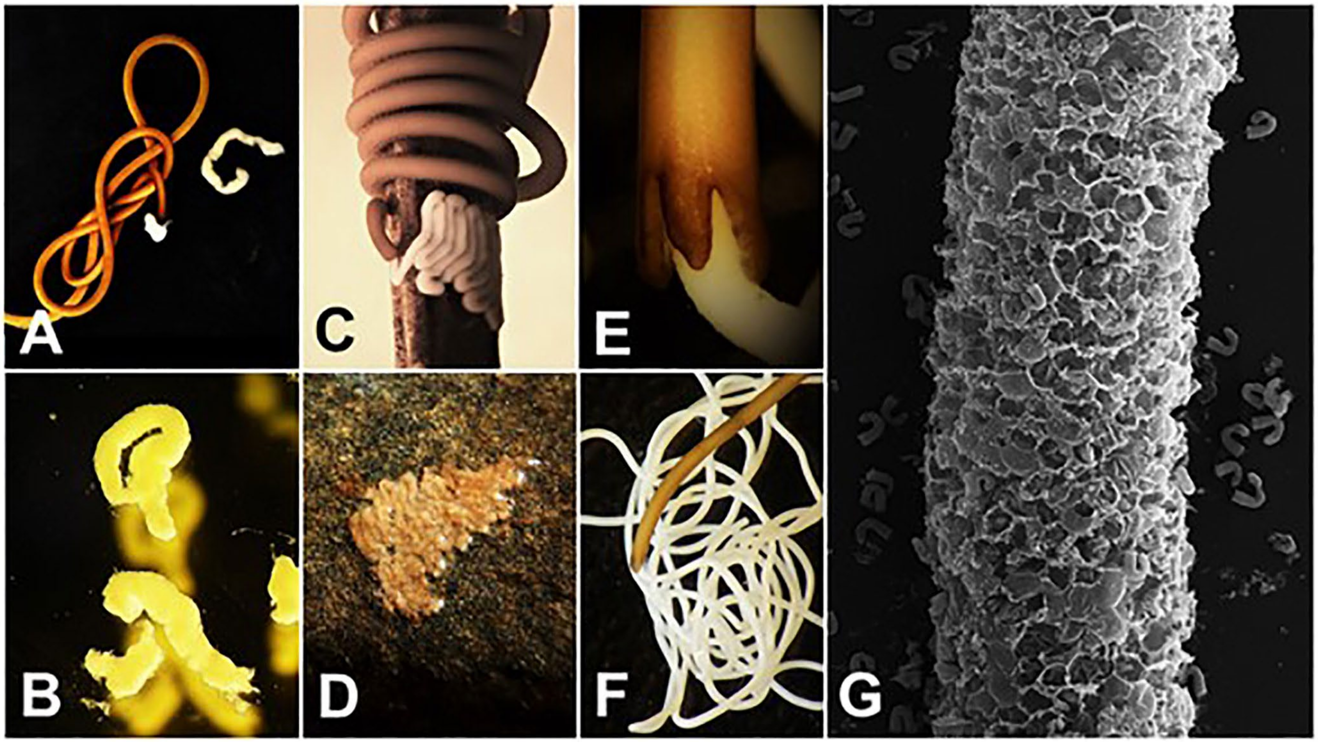

Figure 9. Examples of gordiid egg strings and eggs. A) A female *Gordius terrestris* (brown) in the process of depositing short pieces of egg strings (white); B) higher magnification of pieces of *G. terrestris* egg strings; C) a female *Chordodes kenyaensis* in the process of laying an egg string on a stick. Note the zigzag pattern of the egg string; D) egg string of *Chordodes* sp. deposited on a rock in a zigzag pattern; E) posterior end of a female *Paragordius varius* showing an egg string (white) excreted from between the 3 tail lobes; F) posterior end of a female *P. varius* (tan) in the process of depositing a single and very long egg string (white); G) a scanning electron micrograph of a partial egg string (ES) of *P. varius*, with hatched larvae scattered around the periphery of the egg string. Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

and Asian *Acutogordius* species indicate that these larvae can encyst freely on the surface of aquatic vegetation, or within the egg strings deposited in the environment, but the role of these cysts in transmission is unclear (Dorier, 1930; Bolek et al., 2015; Chiu et al., 2017).

Studies on larvae of other gordiid species indicate that larvae of these species never encyst on vegetation or detritus (May, 1919; Inoue, 1960; Hanelt and Janovy, 2002; Bolek et al., 2010; Hanelt et al., 2012; Bolek et al., 2013a; Szmygiel et al., 2014). In fact, most reports of gordiid cysts have been reported from aquatic metazoan animals including molluscs, annelids, arthropods, fish, and amphibians (Harkins et al., 2016; Chiu et al., 2016; Yamashita et al., 2017). More importantly, experimental studies by Hanelt and Janovy (2004a) demonstrate that 3 phylogenetically distinct species of gordiids indiscriminately infect and form cysts in a variety of aquatic invertebrates and fish. These authors also demonstrated that within non-biting midge paratenic hosts, gordiid cysts survived metamorphosis of these aquatic insects, and when these insects were fed to crickets, the crickets became infected and released adult worms. Taken together, the numerous reports of gordiid cysts infecting a variety of aquatic animals, their ability to survive insect metamorphosis and their ability to infect terrestrial arthropod hosts, supports the paratenic host strategy in the life cycles of gordiids.

Once paratenic hosts ingest larvae, the larvae penetrate the gut and begin forming cysts within the tissue of their paratenic host. During cyst formation, larvae empty the contents of their pseudo-intestine. Laboratory studies by Dorier (1930), Poinar and Doelman (1974), Hanelt and Janovy (2003), Hanelt et al. (2012), and Bolek et al. (2010; 2013a; 2013b) report that during cyst formation, larvae secrete a jelly-like material from the pseudo-intestine and a clear halo-like structure appears around the folded larva (Figure 11). Transmission electron microscopy studies of gordiid cysts in tadpole paratenic hosts indicate that the clear halo-like cyst wall is multilayered (Poinar, 2010). Cyst development can take a few days up to a few months (Hanelt and Janovy, 2002; De Villalobos and Ronderos, 2003). More importantly, if an animal other than a definitive host ingests a paratenic host, the cysts are digested out and the larvae re-penetrate into the

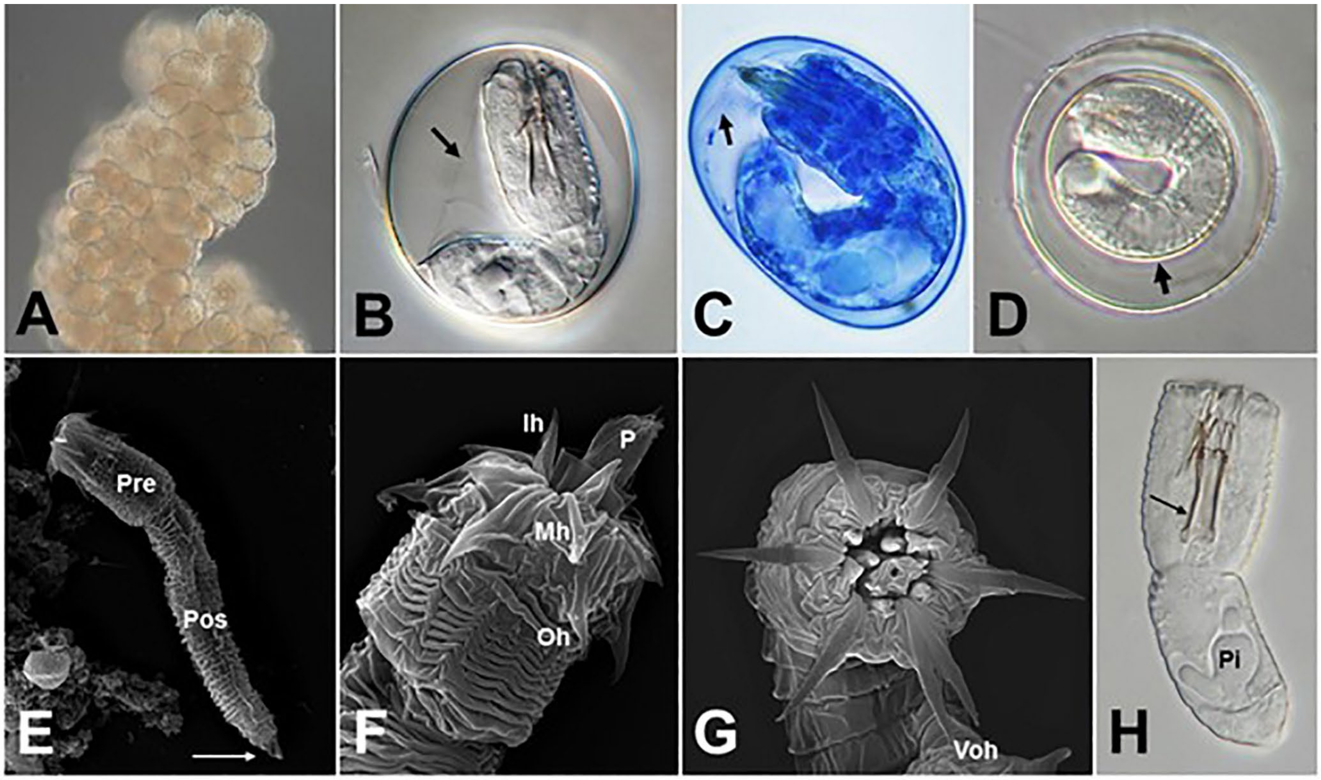

Figure 10. Eggs and larvae of gordiids. A) Higher magnification of pieces of *Gordius difficilis* egg strings showing the concentration of eggs; B, C) typical eggs of freshwater gordiids. Note the developed larva surrounded by a thin inner membrane (arrow) in each egg; D) an unusual egg of *G. terrestris*, a gordiid that lays eggs in the soil. Note the outer shell separated by distinct space from a thick inner membrane (arrow) surrounding the larva; E) scanning electron micrograph of a typical *Gordius* larva. Note the pre-septum (Pre), post-septum (Pos), and terminal spine (arrow) on the post-septum; F) scanning electron micrograph of the anterior end of a *Gordius* larva. Note the proboscis (P) and 3 rings of cuticular hooks including the inner, middle and outer hooks (Ih, Mh, Oh); G) scanning electron micrograph of the anterior end of a *Paragordius varius* larva. Note the dorsoventrally compressed proboscis in relationship to the ventral outer hooks (Voh); H) larva of *Chordodes kenyaensis.* Note the 3 internal stylets (arrow), and V-shaped pseudo-intestine (Pi). Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

second paratenic hosts and re-encyst (De Villalobos and Ronderos, 2003; Hanelt and Janovy, 2003).

It is unknown if nematomorph larvae have any impact on mortality of paratenic hosts in nature. However, several laboratory studies and field observations indicate that insect paratenic hosts mount some type of immune reaction to horsehair worm larvae and cysts (Poinar and Doelman, 1974; De Villalobos and Ronderos, 2003; Hanelt and Janovy, 2003). Host reactions usually involve humoral mediated melanization of larvae (Figure 11) and/or cysts. Melanization of gordiid larvae and cysts have been reported in a variety of aquatic larval insects including mosquitoes, chironomids, caddisflies, mayflies, stoneflies, as well as larval beetles (Poinar and Doelman, 1974; Poinar, 1991; Hanelt and Janovy, 2003; Bolek et al., 2015).

Most definitive hosts for gordiids are predaceous or omnivorous arthropods, which capture infected paratenic arthropod hosts after they metamorphose from an aquatic habitat or scavenge on dead infected paratenic hosts (Figures 2, 6, and 7). Laboratory studies indicate that the maturation of gordiids within the definitive host takes several months. For example, the development within the definitive arthropod hosts can take as long as 8 months for *Gordius tolosanus* (Svábeník, 1925), 2 to 3 months for *Chordodes japonensis*, *C. kenyaensis*, and *Paragordius obamai* (Inoue, 1962; Hanelt et al., 2012; Bolek et al., 2013a), to as short as 1 month for *P. varius* (Hanelt and Janovy, 2004b).

Field studies indicate that after worms emerge from their hosts, only the gut remains within the host's body cavity (Linstow, 1891; Thorne, 1940), whereas, other studies indicate that the production of eggs by female definitive hosts is inhibited or absent altogether (Tanner, 1939; Baker, 1985; Studier et al., 1991; Chiu et al., 2015). Only 1 report found that naturally infected female hosts might be capable of reproducing (Poulin, 1995). A more recent experimental study by Biron et al. (2005b) using naturally infected crickets showed

Figure 11. Cysts of gordiids. A) A larval *Gordius terrestris* in the process of folding into a cyst in its earthworm paratenic host; B, C) typical *Gordius* type cyst, not the folding pattern of the larva; D) fully developed *Gordius* type cyst. Note the clear halo-like structure surrounding the folded larva; E) 2 types of gordiid cysts (arrows) in the hemocoel of a non-biting midge larva; F) a *Paragordius varius* cysts in the tissue of an aquatic snail. Note the prominent spines on the folded larva within the cyst, which are characteristic for the genus *Paragordius*; G) a *Chordodes* like larva on the outside gut wall of an aquatic beetle larva in the process of being melanized (orange-brown pigment). Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

that female crickets were capable of producing eggs only after they released worms and were provided with food ad libitum. However, all female crickets that released worms and produced eggs had difficulties mating with male crickets and/or ovipositing. In contrast, all infected male crickets were castrated by horsehair worms and did not regain the ability to produce sperm after they released worms.

Life Cycle

One fascinating aspect of gordiid biology is their complex life cycle which includes both free-living and parasitic phases (May, 1919; Inoue, 1962; Hanelt and Janovy, 1999; 2004b; Hanelt et al., 2012; Bolek et al., 2013b; Swanteson-Franz et al., 2018) (Figure 6). As juveniles, gordiids are parasites of terrestrial arthropod hosts from which free-living adults emerge into aquatic or semi-aquatic environments, such as waterlogged fields, streams, rivers, and lakes (Hanelt et al., 2005; Anaya et al., 2019). Three species of gordiids (*Paragordius varius*, *P. obamai*, and *Chordodes kenyaensis*) have been domesticated in the laboratory including dioecious and parthenogenetic species (Hanelt and Janovy, 2004b; Hanelt et al., 2012; Bolek et al., 2013a; 2013b). Studies on these domesticated nematomorphs indicate that life cycles of gordiids involve 5 distinct life stages (Figure 6) 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; 2004b; Hanelt et al., 2012). Juvenile gordiids are obligate parasites of predominantly terrestrial arthropods, whereas a number of species of aquatic animals serve as paratenic hosts for the cyst stage (Hanelt et al., 2001; Bolek and Coggins, 2002; Hanelt and Janovy, 2003; 2004a).

As noted above in general, gordiids commonly infect 4 major groups of terrestrial arthropods, including beetles, orthopterans, praying mantids, and cockroaches. Additional confirmed records exist from earwigs (Dermaptera) and aquatic larval trichopterans and larval dragonflies (Schmidt-Rhaesa, 2013). All gordiids develop in the hemocoel of their arthropod host where they grow from a small length of 60–100 µm to a length of over 2 m for some species (Schmidt-Rhaesa, 2013) (Figure 7). During development in the arthropod definitive host, 2 cuticles are present, a thin white larval cuticle which is replaced by a robust dark adult cuticle (Figure 8) (Schmidt-Rhaesa, 2005). Before emergence from their host, adult gordiids form an open wound on the posterior end of the host's abdomen and once the infected arthropod enters water, the worms emerge head-first (Hanelt and Janovy, 2004b; Hanelt et al., 2012; Bolek et al., 2013a).

As noted, field observations indicate that infected terrestrial arthropods, such as crickets and beetles, deliberately enter water, suggesting that worms may be manipulating the behavior of their arthropod hosts (McCook, 1885; Müller, 1926; Jolivet, 1945; 1948). More recently, Thomas and colleagues (2003) discovered differences in the brains and concentrations of neurotransmitters among infected and uninfected field-collected crickets. Additional studies by Biron and colleagues (2005a; 2005b; 2006) show that several brain proteins are altered in crickets infected with gordiids. It is not known whether the gordiids' mere presence or something emitted by the gordiids affects the hosts' behavior.

Distribution and Diversity

Within the freshwater/terrestrial Gordiida, approximately 360 species of horsehair worms have been described worldwide from 18 extant and 2 extinct genera (Poinar, 1999; Poinar and Buckley, 2006; Yadav et al., 2018). In addition, 5 species of marine horsehair worms have been described from a single genus (Schmidt-Rhaesa, 2013). However, current estimates suggest that only 18% of the horsehair worm diversity has been documented across the world, with another 2,000 species awaiting discovery (Poinar, 2008). The earliest reported and credible fossil Nematomorph, was described from 100 million year-old Lower Cretaceous Burmese amber and belongs to the extinct species *Cretachordodes burmitis* (Poinar and Buckley, 2006). Additionally, 2 individuals of the fossilized species *Paleochordodes protus* (Poinar, 1999) emerging from a cockroach have been described from Dominican amber dated between 15 and 45 million years-old (Figure 12). However, obtaining knowledge on the diversity of horsehair worms has been difficult due to their unusual life cycles, where free-living adult worms exit their hosts, and the lack of reliable ways of collecting the free-living adults over large geographic areas from aquatic and terrestrial habitats (Bolek and Coggins, 2002; Bolek et al., 2013a; Bolek et al., 2015).

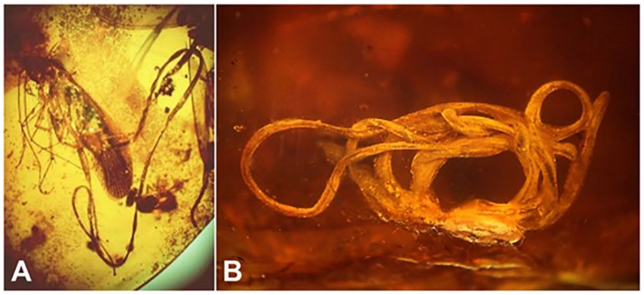

Figure 12. Fossil nematomorphs. A) Two specimens of *Paleochordodes protus* in the process of emerging from a cockroach host in Dominican amber, 15–45 Ma (= million years old); B) the oldest known hairworm fossil *Cretachordodes burmitis* recovered from Early Cretaceous amber, 100 Ma, from Myanmar. Source: G. Poinar. License: CC BY-NC-SA 4.0.

The freshwater and terrestrial horsehair worms have been reported from all continents except Antarctica; whereas the marine genus *Nectonema* is known from several locations worldwide including both coasts of the northern Atlantic Ocean, as well as the Indian Ocean, and the Pacific Ocean from the southern coast of New Zealand (Poinar and Brockerhoff, 2001; Hanelt et al., 2005; Bolek et al., 2015). The fauna of North American, European, and Argentinean nematomorphs has been relatively well studied. However, nematomorph diversity in Africa, Asia, and most of South America has received comparatively little attention (Hanelt et al., 2005; Schmidt-Rhaesa, 2013; Bolek et al., 2015; Schmidt-Rhaesa et al., 2016; Swanteson-Franz et al., 2018; Anaya et al., 2019; Zanca et al., 2020). Among the Nearctic freshwater and terrestrial horsehair worms, 24 species from 7 genera have been described (Schmidt-Rhaesa et al., 2003; Poinar and Chandler, 2004; Begay et al., 2012; Swanteson-Franz et al., 2018; Anaya et al., 2019). However, evidence from molecular barcoding techniques indicates there are numerous hidden, cryptic species. For example, the common Nearctic species *Gordius robustus* represents a large species complex composed of at least 8 distinct genetic lineages (Hanelt et al., 2015). These recent molecular studies indicate the importance of genetic data and limitations of morphological characters in determining some gordiid species within the phylum.

Taxonomy and Phylogeny

The phylum Nematomorpha consists of 2 subphyla, the marine Nectonematida and the freshwater and terrestrial Gordiida comprising 1, and 18 extant and 2 extinct genera, respectively (Schmidt-Rhaesa, 2013). Nematomorphs have few morphological characters that can be used by taxonomists for species determination. Macroscopic characters include the shape of the posterior end being bilobed or round in males, and trilobed or round in females. In addition, the presence of cuticular structures such as crescents near the cloacal opening and/or areas of dense bristles and/or spines on the posterior region of worms are useful for some genera delimitations

(Figure 4) (Schmidt-Rhaesa, 2013). All other characters, including areoles and intra-areoles spaces are found on the cuticle, many of which are so small that scanning electron microscopy (SEM) is necessary to visualize these characters. As such, SEM has become the standard protocol for nematomorph identification (Hanelt et al., 2005; Bolek et al., 2015).

Horsehair worms are placed in the superphylum Ecdysozoa and are considered the sister phylum to the phylum Nemata (Hanelt et al., 2005). However, and unlike nematodes, cephalic papillae, lateral epidermal cords, secretory-excretory systems, amphids, and spicules are lacking in horsehair worms. Other differences between horsehair worms and nematodes include genital openings located on the posterior end of female horsehair worms instead of near the middle of the body as in nematodes. Additionally, and unlike nematodes, these animals have a true larval stage that undergoes drastic morphological tissue reorganization during development in their host (Schmidt-Rhaesa, 1997; 2013).

Of 3 phylogenic hypotheses based on molecular (DNA) sequencing discussed here, the ancestor-descendant relationships of multiple genera and species within the Nematomorpha were analyzed (Bleidorn et al., 2002; Chiu et al., 2017; Tobias et al., 2017). Bleidorn and colleagues (2002) uses a combination of morphological and molecular (18S rRNA gene) data indicating a sister-group relationship between the marine genus *Nectonema* and the freshwater Gordiida. However, within the Gordiida, all species within the basal genus *Gordius* and all species within the sister genus *Paragordius* are monophyletic. The remaining derived genera are not well supported, and some appear as polyphyletic. For example, the more derived *Neochordodes occidentalis* is nested within species of *Chordodes*. More recent molecular phylogenetic analyses using mitochondrial markers (*CO1*) and/or nuclear markers (8S rRNA) indicate that the freshwater genus *Paragordius* is basal to the remaining freshwater and terrestrial gordiids. More importantly, these molecular phylogenetic hypotheses are in agreement with the traditional morphological relationships of freshwater and terrestrial gordiids including the genera *Gordius* and *Acutogordius* within the family Gordiidae and the remaining genera within the family Chordodidae (Chiu et al., 2017; Tobias et al., 2017).

Ecology and Behavior

The ecology of nematomorphs is closely tied to the biology of their arthropod definitive hosts and the aquatic or terrestrial habitats of the adult free-living worms. However, few studies have sampled for free-living adult worms throughout the year and even fewer studies have examined multiple arthropod species for nematomorph infections (Bolek and Coggins, 2002; Poinar and Weissman, 2004; Looney et al., 2012). As a general rule and depending on the gordiid species, nematomorphs vary in their definitive host specificity, and free-living adults are seasonal and have a male-biased sex ratio (Hanelt et al., 2005; Bolek et al., 2015).

Host specificity for most nematomorph species is poorly understood, and most host records are based on field observations (Schmidt-Rhaesa, 1997; 2013; Schmidt-Rhaesa et al., 2003). Field studies indicate that some, but not all, nematomorph species appear to be host-specific at the definitive arthropod host level (Poinar, 1991; Bolek and Coggins, 2002; Schmidt-Rhaesa et al., 2003; Chiu et al., 2011; Looney et al., 2012). For example, the North American *Chordodes morgani* has been reported from 4 phylogenetically distinct orthopteran and cockroach species, suggesting that some horsehair worms are generalists at the definitive host level (Schmidt-Rhaesa et al., 2003). However, other species, particularly in the *Gordius* cf. *robustus* complex, appear to be more specific at the definitive host level and are restricted to a single or a few closely related species of arthropod hosts (Hanelt et al., 2015). Molecular evidence from mitochondrial (*CO1* and *cytB*) and nuclear (partial 28S, ITS1, 5.8S, and ITS2) DNA suggests that at least 8 species occur across North America. However, this group is paraphyletic, since the European *G. aquaticus* and *G. balticus* group among the *G. robustus* lineages form 2 distinct clades, A and B (Figure 13B). When all known arthropod definitive hosts are mapped onto this phylogeny it appears that species within clade A infect various species of orthopterans; whereas species in clade B infect millipedes and ground beetles (Figure 13).

Once emerged from their arthropod hosts, free-living adult worms are seasonal (Bolek and Coggins, 2002; Schmidt-Rhaesa et al., 2005; Salas et al., 2011; Anaya, 2019). For example, Bolek and Coggins (2002) reported the occurrence of free-living adults of *Gordius difficilis* in Wisconsin, United States from June to October; whereas Anaya (2019) reported *G. terrestris* (incidentally, the only known species of gordiid consistently collected from terrestrial habitats) from Oklahoma, United States during October through March. Additionally, Salas and colleagues (2011) examined the seasonal occurrence of free-living individuals of 4 species of sympatric gordiids over a 1-year period from a stream in Argentina. In their study, free-living worms of all 4 species occurred in the stream during the fall, winter, and spring. However, *Noteochorododes cymatium*, *N. talensis*, and *Pseudochordodes dugesi* were most abundant during the winter and spring; whereas *Chordodes brasiliensis* was most abundant during the fall.

The explanation for these seasonal patterns includes the short life span of free-living adult worms (2–8 weeks) and the abundance of their arthropod definitive hosts. For example, in a 3-year study, Schmidt-Rhaesa and colleagues (2005)

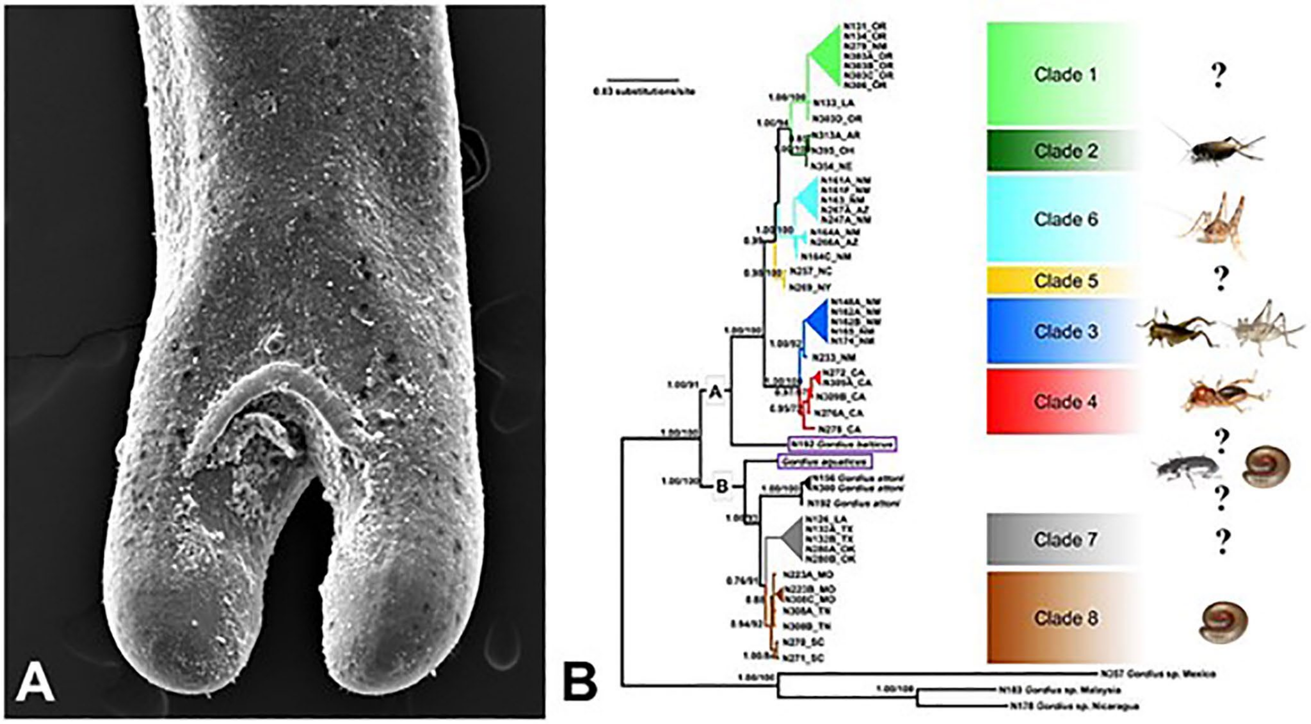

Figure 13. Hosts and phylogenetic relationships of the *Gordius* cf. *robustus* complex. A) Posterior end of a male *Gordius* cf. *robustus*, with the characteristic bilobed end, post cloacal crescent, and poorly developed areoles on the cuticle; B) phylogenetic hypothesis (partial CO1 and cytB sequences) of the *Gordius* cf. *robustus* group and diverse group of arthropod definitive hosts. Note that the *Gordius* cf. *robustus* linages are paraphyletic with European species (purple brackets) group among the *G. robustus* lineages and form 2 distinct clades A and B. Species in clade A appear to infect orthopteran arthropod hosts, whereas, species in clade B infect ground beetle and millipede arthropod hosts. Sources: A) M. G. Bolek; B) adapted from Hanelt et al., 2015. License: CC BY-NC-SA 4.0.

collected data on recently emerged adults of 2 species of nematomorphs and their definitive arthropod hosts around a swimming pool in southern France. Most adults of *Pseudochordodes tricuspidatus* emerged from their hosts during June through August, whereas most adults of *Spinochordodes tellinii* emerged from their hosts during August through September. At their study site, both gordiid species infected different species of definitive hosts and their occurrence was correlated with the abundance of these hosts.

Free-living adults do not feed and are found in various aquatic habitats including water sources in caves, puddles, ponds, lakes, and small and large streams and rivers (Reeves, 2000; Hanelt et al., 2005; Schmidt-Rhaesa, 2013; Bolek et al., 2015). Within these habitats, free-living worms can be located in the sediment, among moist fallen leaves, under rocks, in algal mats, and/or on aquatic vegetation where they form Gordian knots and mate (Hanelt et al., 2005; Bolek et al., 2015). Additionally, free-living adults of *Gordius terrestris*, a terrestrial species, appear during rain events on wet lawns and pools of water on streets and sidewalks, where the worms copulate. After the rains stop, adult free-living worms can be found entangled in the roots of grasses and in the soil where females deposit egg strings (Anaya, 2019; Anaya et al., 2019; Figure 14).

The sex ratio of free-living adult gordiids is usually but not always male biased, with a few field studies indicating equal sex ratios (De Villalobos and Camino, 1999; Valvassori et al., 1988; Salas et al., 2011). For example, Cochran and colleagues (1999) reported that of 1,391 individuals of *Gordius difficilis* collected during a 32-year period in 6 Midwestern states of the United States, 1,205 were males. In contrast, Watermolen and Haen (1994) reported 67 individuals of *G. robustus* from Wisconsin of which 66 were females. These field-skewed sex ratios are in contrast to laboratory life cycle studies on dioecious nematomorph species. Hanelt and Janovy (2004b) and Bolek and colleagues (2013b) each found no statistically significant differences in the sex ratios of *Paragordius varius* or *Chordodes kenyaensis* emerging from laboratory-reared and -infected cricket definitive hosts.

A few hypotheses have been proposed for this strongly skewed sex ratio in the field, including differences in the development times of male and female worms in their final hosts,

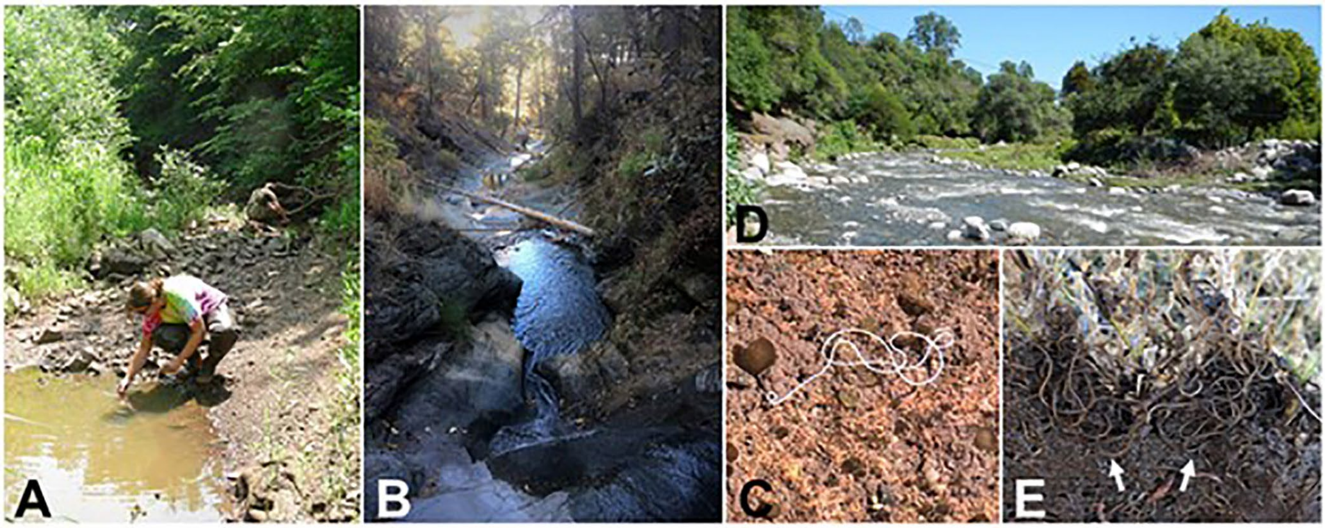

Figure 14. Typical habitat for free-living adults, larvae, and cysts of freshwater and terrestrial gordiids. A) Second-order stream in Payne County, Oklahoma, United States; B) a typical first-order stream in the Chiricahua Mountains, Arizona, United States; C) a white free-living adult male *Gordius* sp. on the bottom of a first-order stream in the Chiricahua Mountains, Arizona, United States; D) a third-order stream in the Córdoba, Argentina. Many of the nematomorphs glue their egg strings on rocks in this habitat; E) adult free-living *G. terrestris* entangled in grass roots under the soil, in a suburban environment, Stillwater, Oklahoma, United States. Source: M. G. Bolek. License: CC BY-NC-SA 4.0.

and/or behavior differences among free-living males and females (Poulin, 1996; Bolek and Coggins, 2002). More recently, Anaya (2019) documented behavioral differences among male and female *Gordius terrestris*, a species with an extremely male biased sex ratio (5.4:1.0) observed in the field. In the laboratory, when male and female worms are placed on the surface of the soil, significantly more females burrow into the soil than males. Once females burrow, they begin ovipositing. This observation is important and provides a plausible explanation for the extremely male-biased sex ratio observed for *G. terrestris* in the field. Taken together, these observations suggest that unlike males, after mating female horsehair worms may be moving to specific locations in the environment to oviposit and be more difficult to locate than males.

Little information is available on the physiological constraints of free-living stages of horsehair worms to their external environment. However, Bolek and colleagues (2013b) indicate that in laboratory cultures adult free-living *Chordodes kenyaensis*, worms die within 24 hours if they emerge from their hosts in cages without a water source, suggesting that adult free-living worms must remain moist to survive. In addition, Achiorno and colleagues (2008) examined the survival of eggs, larvae, and free-living adults of *C. nobilii* to extreme temperatures. They demonstrated that all eggs, most larvae, and all adult gordiids die at a high temperature of 40.5 °C, and all eggs and most adult worms (89%) die at a low temperature of −3 °C. In contrast, larvae frozen at −3 °C for 48 hours survived freezing and are capable of infecting mosquito larva paratenic hosts. Finally, Bolek et al. (2013a) evaluated the survival of larvae and cysts of North American and African gordiids in the genus *Paragordius* when super cooled and/or frozen at −20 °C or −80 °C for up to 7 months. Their work demonstrates that post-frozen larvae and cysts of these species have the ability to infect and develop in the next host in the life cycle. It is currently unclear why larvae and cysts of gordiid species from Africa have the ability to survive super cooling and/or freezing for such periods.

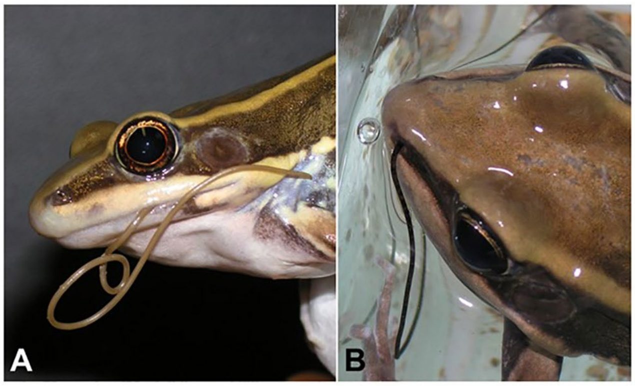

Figure 15. Predators and epibionts of gordiids. A, B) A free-living *Paragordius tricuspidatus* escaping from the mouth (A) and nose (B) of the common green frog (*Rana erythraea*) after it ingested an infected cricket. Source: F. Thomas. License: CC BY-NC-SA 4.0.

Finally, birds, fish, and frogs occasionally eat free-living adult nematomorphs and their infected hosts (Cochran et al., 1999; Bolek and Coggins, 2002; De Villalobos et al., 2008; Fair et al., 2010). However, work by Sato and colleagues (2008; 2011) in Japan indicates that gordiids are only found in the stomachs of trout (*Salvelinus leucomaenis japonicus*) when those fish also consume their camel cricket hosts. As a result, Sato and colleagues (2011) hypothesized that horsehair worm infections and their manipulations of terrestrial arthropod hosts can increase energy inputs into aquatic ecosystems. Using estimates of seasonal prey abundance, they argue that, during worm peak emergence times, infected orthopterans account for 60% of the annual energy intake of Japanese trout (Sato et al., 2011). Other studies by Ponton and colleagues (2006a; 2006b) indicate that when trout and frogs consume orthopterans infected with these nematomorphs, 18–35% of the ingested nematomorphs can escape the predators of their hosts, through the mouth or nose of frogs and the mouth or gills of fish (Figure 15).

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