Phylum Nematomorpha

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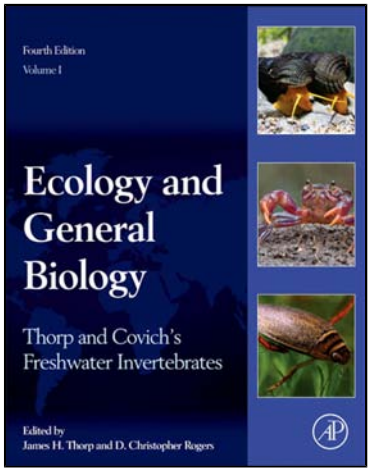

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Chapter 15

Phylum Nematomorpha

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Chapter Outline

INTRODUCTION TO NEMATOMORPHA

General Introduction

Members of the phylum Nematomorpha are commonly known as horsehair worms or hairworms because of their resemblance to and the common myth arising from horse tail hairs that fell into water. Additionally, because of their habits of becoming entangled in masses of two to many individuals, hairworms are also known as Gordian worms after the Gordian knot episode in Greek mythology (Figure 15.1; Roberts et al., 2013).

The phylum consists of freshwater and marine species, and represents one of three entirely parasitic animal phyla (Hanelt et al., 2005). Among the freshwater species, dioecious and parthenogenetic species exist (Hanelt et al., 2012). The freshwater gordiids have complex life cycles, which include multiple hosts and a free-living aquatic phase. At the end of their parasitic phase, gordiids manipulate the behavior of their terrestrial arthropod hosts, causing them to enter aquatic environments where adult worms emerge at the expense of the host committing suicide (Thomas et al., 2002, 2003). After emerging from their host, dioecious

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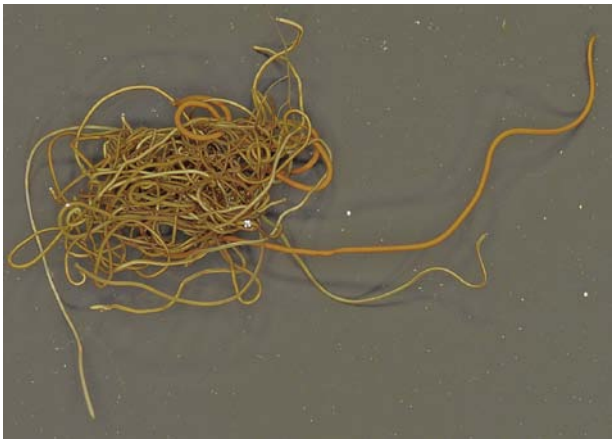

FIGURE 15.1 A typical Gordian knot containing numerous individuals of *Gordius* cf. *robustus*.

species form Gordian knots, mate, and females deposit egg strings as free-living forms. In contrast, females of parthenogenetic species deposit egg strings after emerging from their host (Hanelt et al., 2012; Bolek et al., 2013a). Within weeks, larvae develop, infect, and encyst indiscriminately within a variety of aquatic vertebrate and invertebrate animals (Bolek and Coggins, 2002; 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 definitive hosts, including millipedes, orthopterans (crickets, grasshoppers, etc.), beetles, cockroaches, and mantids.

Freshwater hairworms can be over 2 m long, and they seem to appear suddenly in domestic sources of water (swimming pools, toilets, pet bowls, etc.), thus making human interactions with them quite common (Bolek, 2000; Hanelt et al., 2005). There are a few odd reports of adult hairworms from humans, but these strange cases are most likely the result of people swallowing infected arthropods or arthropod hosts releasing free-living worms into drinking water (Roberts et al., 2013). Additionally, there is one strange report of larval hairworms in human facial tissue resulting in orbital tumors (Singh and Rao, 1966). However, this report is questionable because juvenile worms contained few if any morphological characteristics of gordiids (see Schmidt-Rhaesa, 2012).

General Systematics

The phylum Nematomorpha is divided into two taxa, the marine Nectonematida consisting of five species and the freshwater Gordiida comprised of 19 extant and two extinct genera (Schmidt-Rhaesa, 2012). Nematomorphs are relatively poor in characters important for genus and species determinations. General macroscopic characters important for systematics of adult free-living worms include the posterior ends of males and females and the presence of cuticular structures on both sexes. Most generic and species characters are based largely on the surface pattern and sculpturing of the cuticle. Many of these characters are relatively small and difficult to detect. Therefore, scanning electron microscopy (SEM) and differential interference contrast (DIC) microscopy have become standard tools in recent species descriptions and redescriptions (Bolek et al., 2010; Hanelt et al., 2012; Bolek et al., 2013a).

Crucial for the justification of genera is that they represent monophyletic taxa recognized by autapomorphies (evolutionary novelties). However, a morphological study of 15 gordiid genera represented by multiple species by Schmidt-Rhaesa (2002a) found such autapomorphies for only five of the 15 genera examined. Clearly, detailed morphological work along with molecular markers will be critical for deciphering the systematics and relationships of hairworms in the future.

Phylogenetic Relationships

Nematomorph phylogenetic relationships are far from being resolved. Nematomorphs are usually considered the sister group to nematodes and are placed in the superphylum Ecdysozoa (Hanelt et al., 2005). However, hairworms lack cephalic papillae, lateral epidermal cords, secretory-excretory systems, amphids, and spicules, which are all present in nematodes. Other differences between hairworms and nematodes include genital openings located on the posterior end of female hairworms instead of near the middle of the body as in nematodes. Finally, in contrast to the juvenile stages of nematodes, hairworms have a true larval stage that undergoes considerable morphological change during development in the host (Schmidt-Rhaesa, 1997, 2012). Other phylogenetic and molecular studies place nematodes, tardigrades, onychophorans, and loriciferans as potential close relatives to the nematomorphs (Malakhov and Adrianov, 1995; Sørensen et al., 2008; Roberts et al., 2013).

The only available molecular phylogenic hypothesis on the relationships of multiple genera and species within the Nematomorpha was presented by Bleidorn et al. (2002). A combination of morphological and molecular (18S rRNA gene) data supports 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* were monophyletic. The remaining derived genera were not well supported, and some were polyphyletic. For example, the more derived *Neochordodes occidentalis* was nested within species of *Chordodes*.

Distribution and Diversity

Within the Gordiida, approximately 350 species have been described worldwide from 19 extant and two extinct

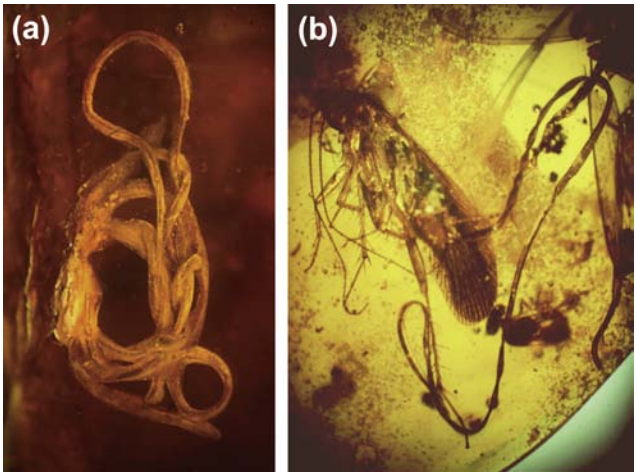

FIGURE 15.2 Examples of fossil hairworms. (a) The oldest known hairworm fossil, *Cretachordodes burmitis* recovered from Early Cretaceous amber (100 million years old) from Burma. (b) Two specimens of *Paleochordodes protus* in the process of emerging from a cockroach hosts in Dominican amber (15–45 million years old). *Both photographs are courtesy of George Poinar.*

genera (Poinar, 1999; Poinar and Buckley, 2006; Schmidt-Rhaesa, 2012). However, estimates suggest that only 18% of the hairworm diversity has been documented globally (Poinar, 2008). The earliest reported and credible fossil Nematomorph is *Cretachordodes burmitis*, described from 100-million-year-old Lower Cretaceous Burmese amber (Poinar and Buckley, 2006). Additionally, two individuals of *Paleochordodes protus* (Poinar, 1999) emerging from a cockroach have been discovered in Dominican amber dated at 15–45 million years old (Figure 15.2). Of the 350 freshwater species, more than 50 were described after 1990 (Schmidt-Rhaesa, 2012). Acquisition of knowledge on the diversity of hairworm species has been hindered by the lack of reliable ways of collecting adult hairworms over large geographic areas and the relatively short life span of the free-living adults, making them difficult to collect (Bolek and Coggins, 2002; Bolek et al., 2013a).

Among the Nearctic Gordiida, 22 species from seven genera are formally recognized (Schmidt-Rhaesa et al., 2003; Poinar et al., 2004; Begay et al., 2012). However, evidence from molecular barcoding indicates that there are numerous hidden, cryptic species within this phylum. For example, the common Nearctic species *Gordius robustus* represents a large species complex composed of at least eight distinct genetic lineages (Ben Hanelt, personal observations). These recent molecular studies indicate the importance of genetic data and limitations of morphological characters in determining some gordiids species and highlight the use of molecular data for taxonomic markers for species identification within the phylum.

GENERAL BIOLOGY

External Anatomy

The body of adult free-living hairworms is circular in cross-section and very long. Adult free-living worms are usually <1 mm in diameter and their body lengths vary from a few centimeters to more than 2 m. However, most free-living worms are 20–40 cm in length (Bolek and Coggins, 2002; Schmidt-Rhaesa, 2012). Usually, adult females are longer and thicker than adult males (Bolek and Coggins, 2002). Adult free-living worm color varies from shades of dark brown to white with some individuals containing dark patches on a lighter background, producing what is known as the 'leopard' pattern (Figure 15.3). Color is not a good characteristic for species identification, however, and most species for which information is available contain various color morphs within and among populations (Schmidt-Rhaesa, 2012).

The anterior end in free-living adults is spherical or distinctly tapering (Figure 15.3). The mouth opening may be visible or closed (Figure 15.3), and no other structures are present on the anterior end. The anterior end is always lighter in color than the rest of the body. In some species, there is a distinctly lighter colored area on the anterior end known as a calotte; this is followed by a dark pigmented ring (Figure 15.3). The posterior ends are trilobed or unbranched in females and bilobed or unbranched in males (Figure 15.4). 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 15.4). In females, the cloaca 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, 2012; Bolek et al., 2013a).

The surface of the cuticle of gordiids may be smooth or structured into thickenings called areoles. Areoles are separated by interareolar furrows and a variety of short spines and/or bristles can be present on the surface of the cuticle (Figure 15.4). These structures and their arrangement are important for species identification. Areoles are elevated thickenings of the cuticle; and in most gordiid species, one or two types of areoles are present. These are known as simple areoles that form a regular pattern on the cuticle (Figure 15.4). In *Gordius*, areoles are lacking or are poorly developed; whereas in *Chordodes*, up to six different types of areoles are present and include the characteristic crowned areoles for the genus (Figure 15.4; Schmidt-Rhaesa et al., 2003; Bolek et al., 2013a). In some genera, two or more areoles may be fused and form structures referred to as

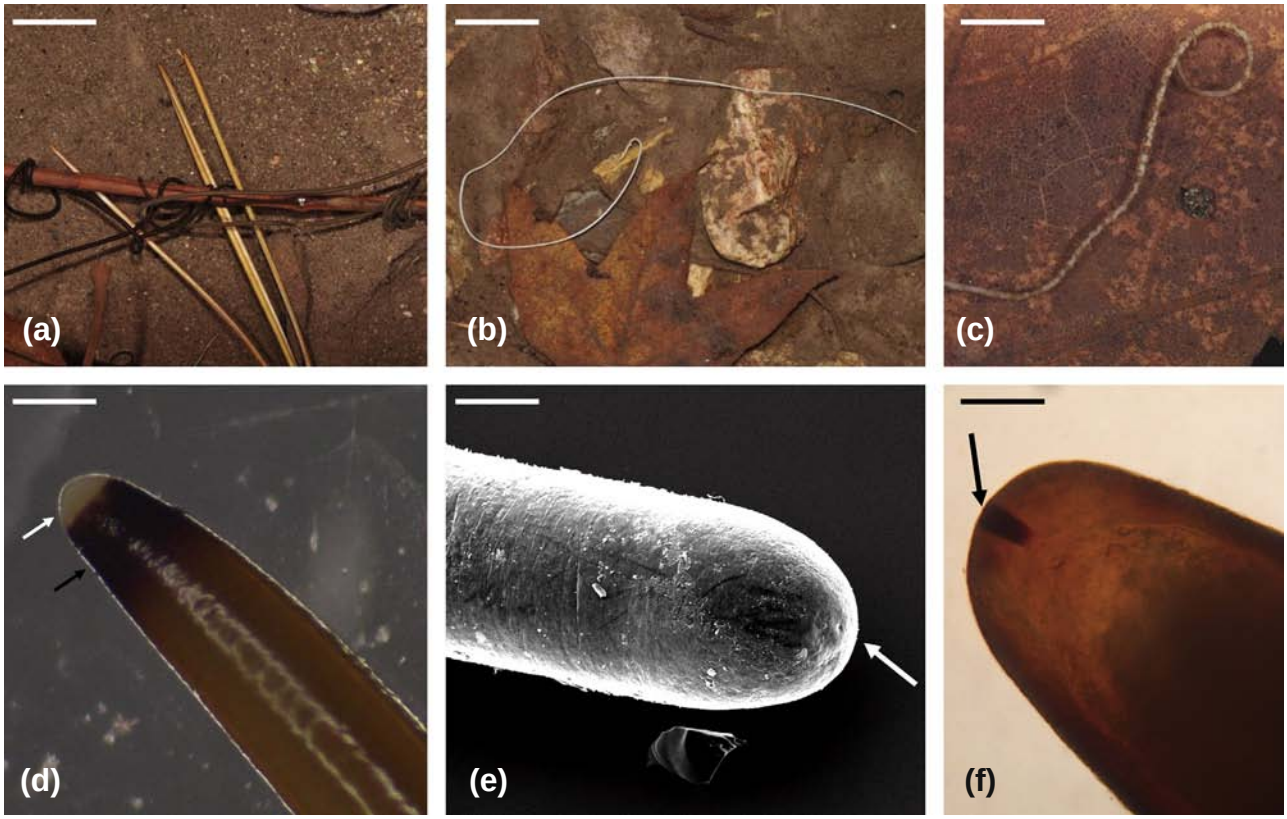

FIGURE 15.3 Color and anterior morphology of adult free-living gordiids. (a) Variation in brown coloration of free-living adults of *Paragordius varius*. Scale bar = 1.0 cm. (b) Typical white color of a female *Gordius difficilis*. Scale bar = 1.0 cm. (c) Typical 'leopard' pattern of a free-living male *Chordodes morgani*. Scale bar = 1.0 cm. (d) Anterior end of a female *G.* cf. *robustus*. Note the calotte (white arrow) followed by a by a dark pigmented ring (black arrow). Scale bar = 150.0 µm. (e) Scanning electron micrograph of the anterior region of a male *G. difficilis*. Note the degenerate mouth (white arrow). Scale bar = 50.0 µm. (f) Anterior end of a male *C. morgani*. Note the cuticularized pharynx (black arrow). Scale bar = 50.0 µm.

megareoles and superareoles (Figure 15.4). Sexual dimorphism is common in the areole pattern among gordiids, and both sexes are necessary for complete species descriptions and proper identifications (Bolek and Coggins, 2002; Bolek et al., 2010, 2013a).

Internal Anatomy

Body Wall and Musculature

The body wall of free-living adults consists of a thick cuticle containing an outer homogeneous 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° (Figure 15.5; May, 1919; Schmidt-Rhaesa, 2012). The number of fiber layers can vary within species and within different body regions among individuals (Schmidt-Rhaesa, 1997). Investigations on the chemical nature of the cuticle by Brivio et al. (2000) and Protasioni et al. (2003) suggested that the composition of the fibrils is not collagen but some other proteinaceous components. Below the cuticle is a very thin epidermis, cellular in structure and functions in secreting the cuticle layers during development within the definitive host (Schmidt-Rhaesa, 2012).

The musculature in all nematomorphs consists of longitudinal muscles; circular muscles are absent. Ultrastructural work on the musculature of the European *Gordius aquaticus*, the South American *Pseudochordodes bedriagae*, and the marine *Nectonema munidae* were provided by Schmidt-Rhaesa (1996a,b) and Restelli et al. (2002). These studies indicated that gordiids and nectonematids differ in muscle cell structure. Studies on freshwater gordiids indicate that thick and thin contractile filaments are concentrated in bundles as thick sheets and myofibrils enclose the cell body. The pseudocoelom in free-living adults is mostly filled with gonads and parenchyma cells, and the digestive track is greatly reduced (Figure 15.5). Parenchymal cells contain vacuoles filled with lipids and glycogen (Reutter, 1972).

Neural System

The nervous system of all gordiids consists of: (1) a brain in the anterior region; (2) a ventral longitudinal nerve cord (Figure 15.5), which emerges from the ventral part of the brain; and (3) a number of peripheral basiepidermal nerves. The

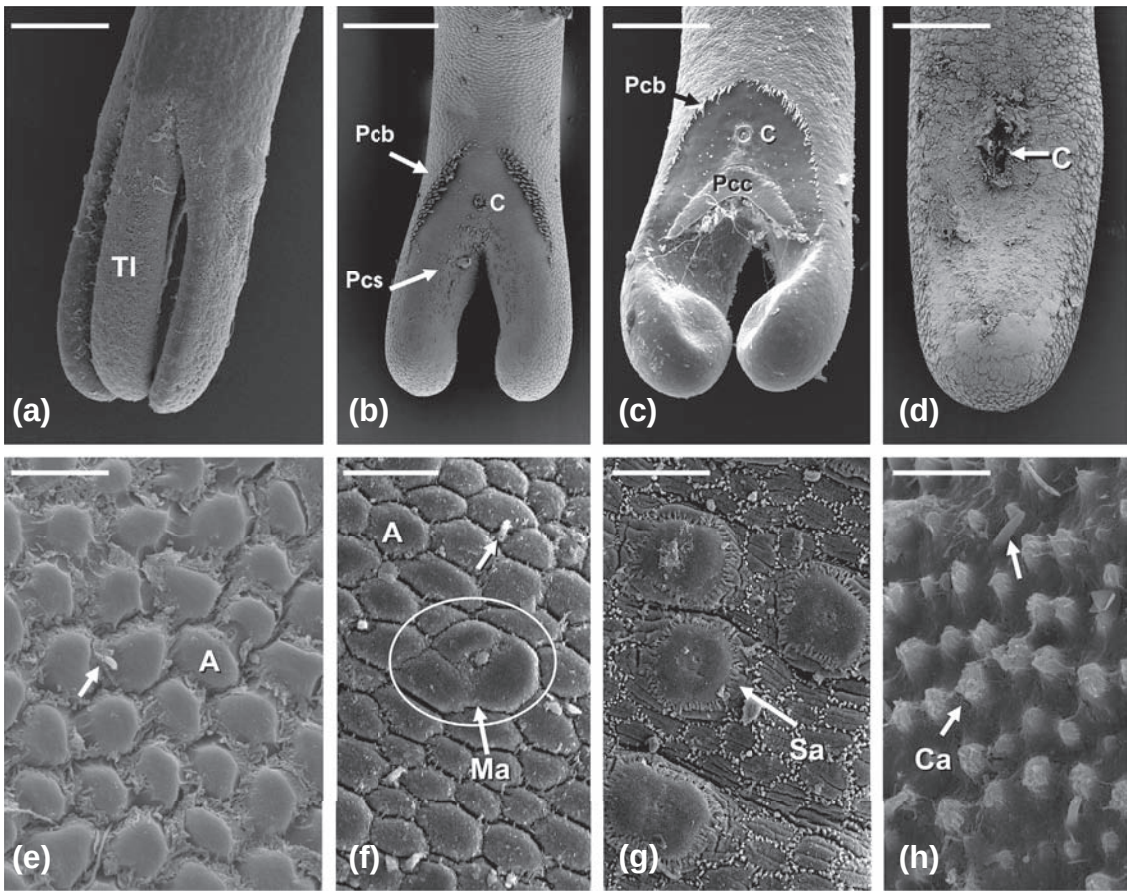

FIGURE 15.4 Scanning electron micrographs of the posterior ends and cuticle structures of adult free-living gordiids. (a) Posterior end of a female *Paragordius* sp. Note the three tail lobes (Tl). Scale bar = 200.0 μm. (b) Posterior end of a male *Gordionus lokaaus* showing two tail lobes, cloacal spines surrounding the cloaca (C) and rows of precloacal bristles (Pcb) and postcloacal spines (Pcs). Scale bar = 300.0 μm. (c) Posterior end of a male *G. difficilis*. Note the ventrally located cloaca (C) and precloacal bristles (Pcb) and postcloacal crescent (Pcc). Scale bar = 150.0 μm. (d) Posterior end of a male *Neochordodes* sp. Note the absence of distinct tail lobes and the cloacal opening (C). Scale bar = 150.0 μm. (e) Simple areoles (A) and spines (white arrow) on the cuticle of a female *Neochordodes occidentalis*. Scale bar = 15.0 μm. (f) Spines (white arrow), simple (A) and megareoles (Ma) on the cuticle of a female *Gordionus* sp. Scale bar = 20.0 μm. (g) Characteristic suprareoles (Sa) on the cuticle of a male *Parachordodes* sp. Scale bar = 40.0 μm. (h) Spines (white arrow) and characteristic crowned areoles (Ca) on the cuticle of a female *C. morgani*. Scale bar = 15.0 μm.

brain has a ring like structure surrounding the anterior region of the alimentary canal (Schmidt-Rhaesa, 2012). The ventral nerve cord is connected to the epidermis by thin lamella (Schmidt-Rhaesa, 1997). In females, the ventral nerve cord terminates in a caudal ganglion; whereas in males with bifurcated posterior ends it divides and branches into each tail lobe (Schmidt-Rhaesa, 2012). Ultrastructural studies on marine hairworms indicate that a peripheral nervous system is present, and some studies indicate that it also occurs in species of gordiids (Montgomery, 1903; May, 1919). Simple sensory organs in the cuticle are not fully understood, but studies indicate that integumental receptors are present. However, it is unclear if these function as mechanoreceptors or if they have other functions (Schmidt-Rhaesa, 2012). All gordiids have a lighter colored area (calotte) on the anterior region of the body, which blends in with the normal body coloration or is followed by a dark pigmented ring. A number of studies report that this lighter region might function as a photoreceptor (Von Linstow, 1891a; Montgomery, 1903; Rauther, 1905). Although the calotte is extensively innervated, there is no evidence based on ultrastructural studies that it functions as a photoreceptor (Schmidt-Rhaesa, 2005, 2012).

The anterior region of the alimentary track is reduced in free-living adults in some gordiid species. A mouth opening may or may not be present; and, depending on the gordiid species, the pharynx can be a cuticularized tube (Figure 15.3), cellular in structure, or absent all together (Schmidt-Rhaesa, 2012). The intestine is positioned dorsal of the ventral nerve cord (Figure 15.5). It consists of layers of cuboidal cells and studies indicate that cells within the lumen have microvilli (Schmidt-Rhaesa, 1997). Ultrastructural work on *Paragordius varius* indicates that the organization of the intestine

changes during development in the definitive host. The diameter of the intestine decreases in free-living adults compared to parasitic juveniles, and cross-sections of free-living worms indicate that the intestine is collapsed (Schmidt-Rhaesa, 2005). In both sexes, the intestine and reproductive system fuse and form the cloaca, which is lined with cuticle.

Reproductive Organs

The gonads are arranged in two long dorsolateral tubes and are surrounded by parenchymal cells (Figure 15.5). Gonads extend almost the entire length of the body. In mature male individuals, the two testes are full of spermatozoa but may be empty after males complete mating with several females (Figure 15.5). In developing females, the two dorsolateral tubes contain ovarial ducts with numerous extensions called ovaries. In free-living adult females, the oocytes fill the entire body cavity, and there is little parenchyma (Schmidt-Rhaesa, 1997).

Physiology

Very little is known about the physiology of nematomorphs (Schmidt-Rhaesa, 2012). Internal transport is most likely by diffusion through the pseudocoelom and cuticle, and it is likely aided by body movement. The long threadlike body of an adult free-living hairworm, results in short diffusion distances between the moist external environment and the body organs and tissues. Excretory and osmoregulatory functions most likely operate on a cellular level. Histological studies on nematomorph gut epithelium and malphighian tubules of insects have suggested that the gut may play an excretory function in free-living adult hairworms (Hyman, 1951), however, these observations have never been confirmed (Schmidt-Rhaesa, 2012).

Reproduction and Life Cycle

Life Cycle

One of the most fascinating aspects of gordiid biology is their complex life cycles. The life cycle includes free-living and parasitic phases (Figure 15.6). Gordiids are parasites as juveniles in terrestrial arthropod hosts from which free-living adults emerge into aquatic environments, such as streams, rivers, and lakes (Hanelt et al., 2005). The life cycles of three species of gordiids (*P. varius*, *Paragordius obamai*, and *Chordodes kenyaensis*) have been domesticated in the laboratory (Hanelt and Janovy, 2004a; Hanelt et al., 2012; Bolek et al., 2013a). 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 these domesticated hairworms indicate that life cycles of gordiids involve five distinct life stages (Figure 15.6) 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,b; Hanelt et al., 2012; Bolek et al., 2013a). Juvenile gordiids are obligatory parasites of mostly 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).

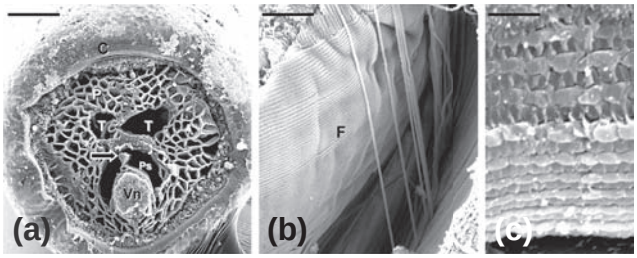

FIGURE 15.5 Scanning electron micrographs of internal organs and cuticle of adult free-living gordiids. (a) Cross-section of the midbody region of a male *G. difficilis*. Note the cuticle (C), ventral nerve cord (Vn), intestine (arrow), testes (T) and reduced pseudocelome (Ps) filled with parenchyma (P). Scale bar = 100.0 µm. (b) Lateral view of the fibers (F) in the fibrous layer of the cuticle in a male *G. difficilis*. Scale bar = 8.0 µm. (c) Alternating fiber layers in the midbody region of the cuticle of a male *G. difficilis*. Scale bar = 2.0 µm.

Hosts and Emergence

Although definitive arthropod hosts are not known for most species of gordiids, a recent review of the literature indicates that gordiids commonly infect four major terrestrial groups of arthropods, including beetles, orthopterans, praying mantids, and cockroaches (Figure 15.7). Additional confirmed records exist from earwigs (Dermaptera) and aquatic larval trichopterans (Schmidt-Rhaesa, 2012). In the Nearctic region, confirmed definitive hosts for gordiids include four families of orthopterans (Gryllidae, Rhaphidophoridae, Stenopelmatidae, and Tettigonidae), two families of cockroaches (Blattidae and Ectobidae), one family of beetles (Carabidae), and two families of diplopods (Cambalidae and Spirobolidae) (Hanelt and Janovy, 2000; Bolek and Coggins, 2002; Schmid-Rhaesa et al., 2003; Poinar and Weissman, 2004; Poinar et al., 2004; Looney et al., 2012; Schmidt-Rhaesa et al., 2009).

All gordiids for which definitive host information is available develop in the hemocoel of their arthropod host. Incredibly, within the definitive host gordiids grow from a small length of 60–100 µm to a length of over 2 m for some species (Schmidt-Rhaesa, 1997, 2012). During development, two cuticles are present. Studies on morphogenesis of *P. varius* within cricket hosts indicate that a thin white larval cuticle is replaced by a robust dark adult cuticle about a

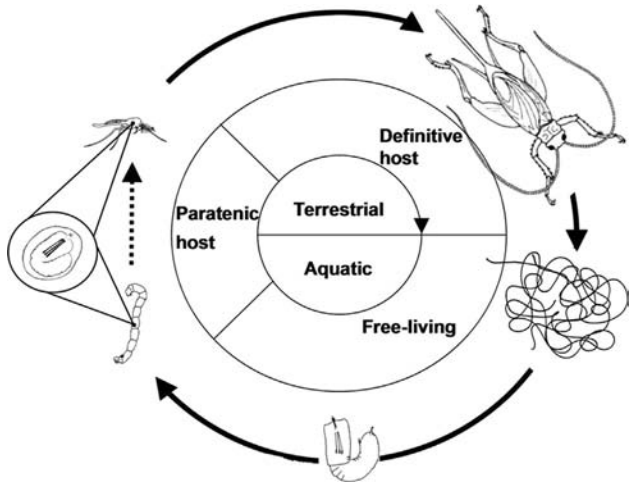

FIGURE 15.6 Diagram of a typical gordiid life cycle. The entire life cycle takes 4–8 weeks to complete in the laboratory, depending on the species of gordiid involved.

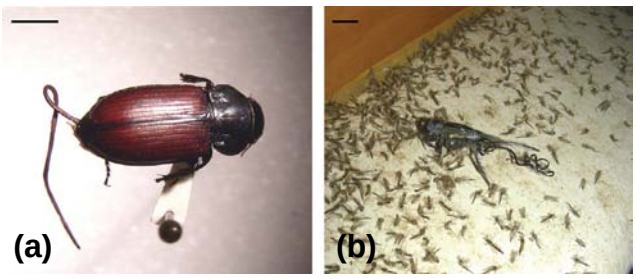

FIGURE 15.7 Examples of typical arthropod definitive hosts for gordiids in the Nearctic. (a) *Amara alpina* (family Carabidae) collected in Canada. Note the gordiid (*Gordionus* sp.) emerging from the posterior region of the beetle's abdomen. (b) A female ground cricket, *Gryllus* sp. which apparently died while feeding on dead midges (Chironomidae) and from which a *P. varius* attempted to emerge but also died. Scale bars = 0.5 cm.

week before worms emerge from the host (Schmidt-Rhaesa, 2005; Figure 15.8). Before emerging from the hemocoel of their host, developed adult gordiids form an open wound on the posterior end of the host's abdomen; and once the infected arthropod enters water, worms emerge head first (Hanelt and Janovy, 2004a; Hanelt et al., 2012; Bolek et al., 2013a; Figure 15.8).

In order for gordiids to emerge from their hosts, the infected terrestrial arthropod must enter water to release the free-living adult worms. Once a host enters water, developed worms emerge within 30–60 s (Hanelt and Janovy, 2004a; Hanelt et al., 2012; Bolek et al., 2013a). Field observations suggest that infected terrestrial arthropods deliberately enter water. For example, McCook (1884) indicated that infected crickets place their abdomens into water and allow gordiids to emerge. Moreover, Müller (1920) and Jolivet (1945, 1948) observed carabid beetles searching for and entering water where worms emerged. More recent studies by Thomas et al. (2002) demonstrated that infected crickets actively jumped into a French swimming pool more frequently than uninfected crickets suggesting that hairworms may manipulate the behavior of their infected hosts. However, until recently, the mechanisms of this assumed parasite-induced altered host behavior have been unclear. Studies by Thomas et al. (2003) have elucidated some of these mechanisms. Thomas et al. (2003) compared the brains of infected and uninfected field collected cricket. They discovered differences in the mushroom body (responsible for olfactory learning and memory in arthropods) and concentrations of neurotransmitters and neuromodulators among infected and uninfected crickets. Additional studies by Brion et al. (2005a, 2005b, 2006) indicated that several cricket brain proteins are differentially expressed in infected versus uninfected crickets suggesting that either gordiids or their presence within infected crickets alter the behavior of infected hosts. More importantly, Sanchez et al. (2008) showed that field collected crickets infected with juvenile gordiids have erratic behaviors, but these crickets only enter water and release worms when worms are fully mature.

Fertilization

In dioecious species, male and female worms must find each other for fertilization soon after emerging from the host. Laboratory observations on *P. varius* indicated that male worms may begin mating with females even before they completely exit the host (Figure 15.4; Hanelt and Janovy, 2004a). Observations by Looney et al. (2012) on 38 naturally infected cricket and beetle hosts indicated that 32 hosts each contained a single worm, four hosts each contained two worms, and two hosts each contained three worms. Five of the six hosts infected with multiple worms had at least one male and one female worm present, suggesting that this mating strategy may sometimes occur in nature. Other studies indicate that up to 8–15% of hosts can be infected with multiple worms (Poulin, 1995; De Villalobos et al., 1999; Thomas et al., 2002). In the remaining cases, hosts release single worms and these individuals must somehow find the opposite sex for copulation after they emerge from their hosts (Bolek and Coggins, 2002; Looney et al., 2012). Laboratory studies and some field observations indicate that both males and females swim relatively well after exiting their host (Bolek and Coggins, 2002; Hanelt and Janovy, 2004a; Bolek et al., 2010, 2013a). 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* revealed that most female worms (91%) contain sperm, indicating that most female worms mate within a day or so of emerging from their host (Bolek and Coggins, 2002).

Although copulatory structures have been described for a few species by Kirjanova (1958), these have not been

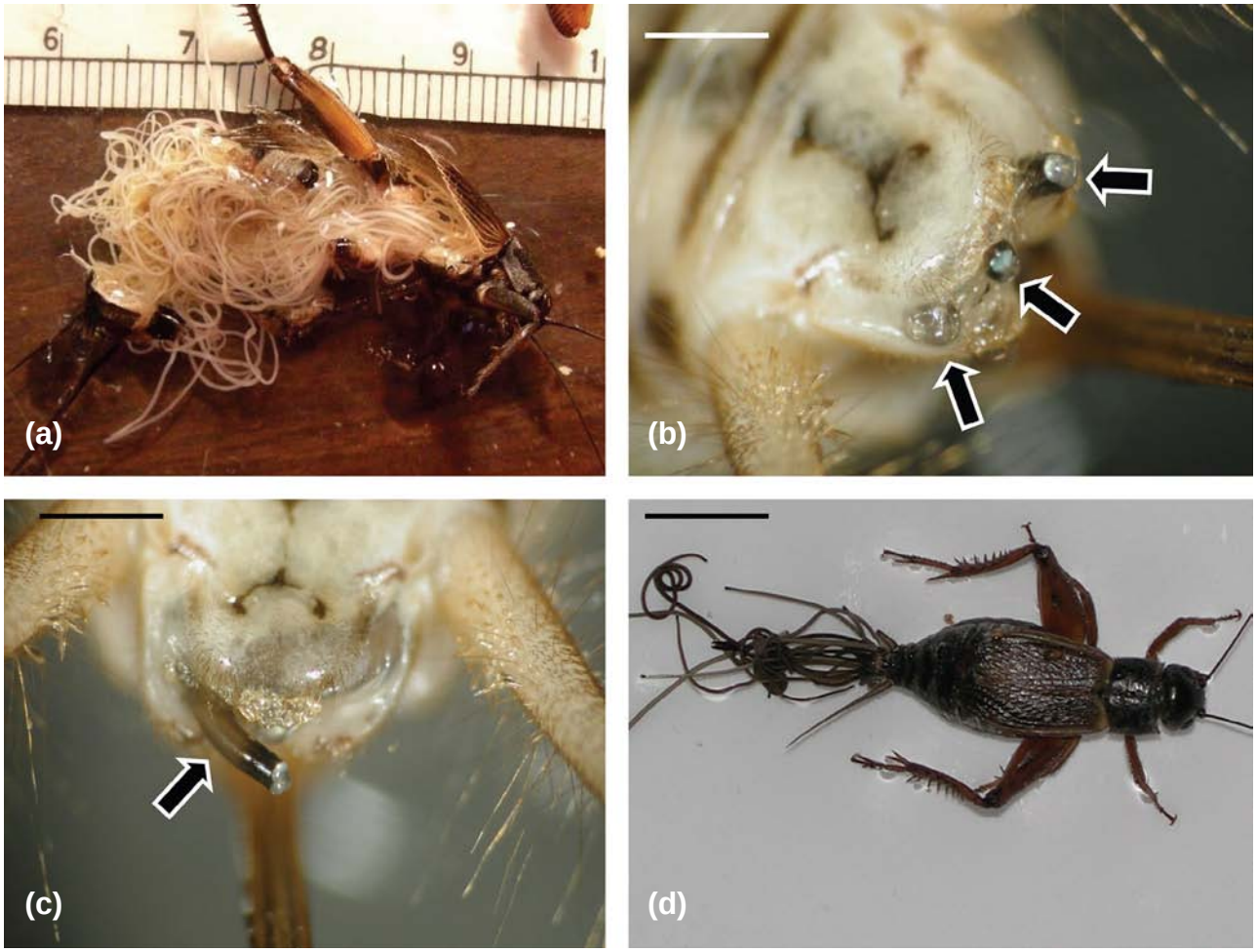

FIGURE 15.8 Development and emergence of *P. varius* in laboratory infected cricket definitive hosts. (a) A female *Gryllus firmus* which died 20 days postexposure to cysts of *P. varius*. Note the white color of the immature worms, which fill the entire hemocoel of the cricket. (b and c) Ventral view of the posterior ends of two female *Acheta domesticus* infected with 30 day old *P. varius* worms ready to emerge. Note the open wounds created by the worms on the abdomen, from which worms poke their heads out and sample the environment. Scale bars = 0.25 cm. (d) A female *Gryllus firmus* submerged in water for 30 s, from which a large number of worms are emerging. Note that worms begin forming Gordian knots even before they completely emerge from the cricket. Scale bar = 1.0 cm.

confirmed in other investigations (see Schmidt-Rhaesa, 2012). Once emerged from their host, free-living males curl their posterior ends ventrally and grasp onto any object, including aquatic vegetation, branches, and other worms where they form characteristic Gordian knots (Figure 15.9). Observations on field collected and laboratory cultured free-living worms clearly show that individual male and female worms initiate typical Gordian knots within hours to days of being placed together (Bolek and Cogins, 2002; Bolek et al., 2013a). During mating, males move up and down the female's body with their coiled posterior end. In genera with bilobed posterior ends, such as *Gordius*, a male will spread its tail lobes and glide along the female's body; whereas species where males lack distinct posterior lobes, such as *Chordodes* and *Neochordodes*, a male will coil its posterior end around the female and glide along her body toward the posterior end (Figure 15.9). 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 15.9). Field observations on *G. difficilis* indicated that sperm drops can remain on the posterior region of females for at least a week (Figure 15.9; Bolek and Coggins, 2002).

The spermatozoa of gordiids are unique in form and change their shape during sperm transfer between male and female worms (Schmidt-Rhaesa, 1997). Spermatozoa released by males are rod shaped and lack a flagellum, whereas spermatozoa deposited on a female's posterior end possess a round end and a rod shaped end (Figure 15.10). Ultrastructural work on spermatozoa indicates that nematomorph spermatozoa contain a nucleus and compartments, which have been named the acrosomal tube, acrosomal sheath, and multivesicular complex. Once inside the

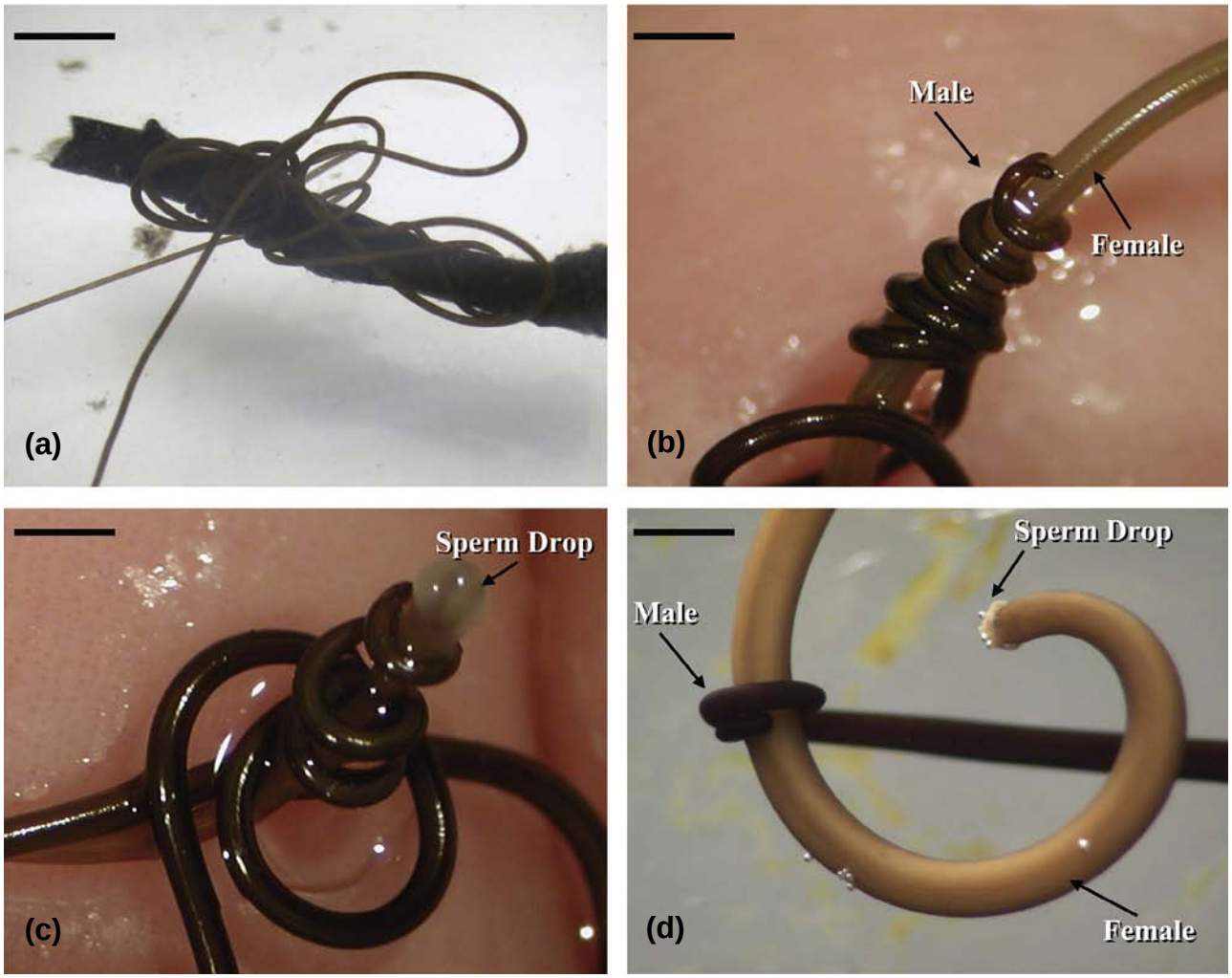

FIGURE 15.9 Gordiids in the process of mating. (a) Numerous individuals of *C. morgani* forming a Gordian knot while attached to a stick. Scale bar = 1.0 cm. (b) A male *G.* cf. *robustus* in the process of gliding along a female's body. Note the spread tail lobes of the male. (c) A sperm drop deposited by a male *G.* cf. *robustus* on the cloacal region of a female. Scale bars in B and C = 2 mm. (d) A male *Neochordodes* sp. wrapped around a female and after depositing a sperm drop on the female's cloaca. Scale bar = 2 mm.

female's seminal receptacle, spermatozoa are very slender and elongate in shape (Schmidt-Rhaesa, 2012). It is unclear if and how these spermatozoa move because they lack a flagellum or pseudopods (Schmidt-Rhaesa, 2012).

Egg Laying

After mating in dioecious species and emergence from the host in parthenogenetic species, females produce up to 8 million eggs during their short (2 weeks–2 months) free-living phase (Bolek and Coggins, 2002; Hanelt, 2009a). Females of *N. 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 (Figure 15.11). In contrast, species of *Gordius* deposit short pieces of egg strings approximately 1–2 cm in length on the substrate or while within 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 (Figure 15.11; Matthew Bolek, personal observations; Szmygiel et al.(2014)). Field observations on *G. difficilis* indicate that worms emerge from their hosts during June–August, females mate within a day of host emergence, and female and male worms bury and congregate 1–5 cm down in the gravel and detritus layers of streams. Females begin laying eggs in the gravel during late August and early September and continue until mid-October. At that time, females that have released all their eggs may contain a clear flattened body (Bolek and Coggins, 2002). Once laid, gordiid egg strings develop within 2–3 weeks into larvae that hatch and remain in the vicinity of the egg strings (Figure 15.11; Hanelt and Janovy, 2004a; Bolek et al., 2010; Hanelt et al., 2012; Bolek et al., 2013a).

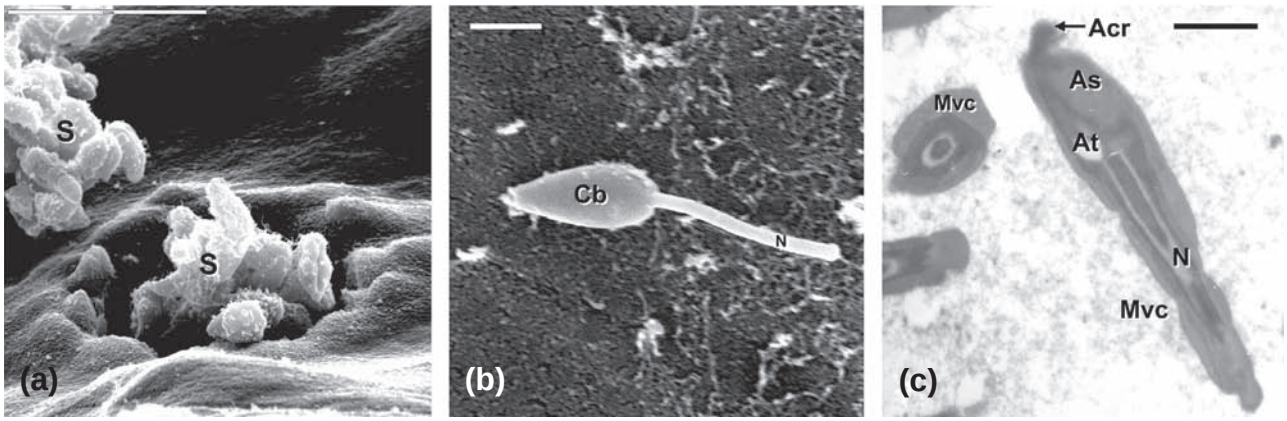

FIGURE 15.10 Scanning and transmission electron micrographs of gordiid spermatozoa. (a) Sperm (S) emerging from the cloacal opening of a male *G. difficilis*. Note the rod shape of the spermatozoa. Scale bar = 10 µm. (b) A single sperm on the posterior region of the cloaca of a female *G. difficilis* from Bolek and Coggins (2002). Note the round end (Cb) and rod shaped end (N) where part of the nucleus is located. Scale bar = 1.5 µm. (c) Cross and longitudinal sections of a spermatozoon from the reproductive system of a *Gordius* sp. Note the numerous compartments and organelles, including Arc = acrosome, As = acrosomal sheath, At = acrosomal tube, Mvc = multivesicular complex and N = nucleus. Scale bar = 1.5 µm.

Larvae, Cysts, and Hosts

Free-living gordiid larvae are semi-sessile and not capable of moving great distances. The few descriptions of gordiid larvae that exist in the literature indicate that larvae of most species are very uniform in morphology. They are 60–100 µm long and 14–30 µm wide. Larvae are cylindrical in shape and superficially annulated; their bodies are divided by a septum into two regions, the pre-septum and the post-septum (Figure 15.12). The pre-septum contains three rings of cuticular hooks and an eversible proboscis, which is supported by three internal stylets and various sets of muscles (Müller et al., 2004). The outer cuticular ring contains six hooks, one of which is positioned ventrally and is bifurcated, whereas the middle and inner rings contain six hooks, none of which are bifurcated (Figure 15.12; Szmygiel et al.(2014)). Internally, the post-septum contains the pseudo-intestine, 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 presumed to have a glandular function and is emptied during cyst formation. Externally, the post-septum contains one to four terminal spines among some gordiid genera (Szmygiel et al.(2014)).

Although superficially similar, three distinct larval types have been reported in the Gordiida (Figure 15.12). Larvae of *Gordius* have an elongated oval pseudo-intestine subdivided into unequal portions, a post-septum approximately three times the length of the pre-septum, and a single terminal spine on the post-septum. In contrast, larvae of *Paragordius* are shorter, with a post-septum 1.3 times the length of the pre-septum, an elongate pseudo-intestine with a pair of anterior granules and a large posterior mass, two pairs of spines on the post-septum, and very long hooks on the outer ring of the pre-septum. Finally, larvae of *Chordodes* and *N. occidentalis* contain a v-shaped pseudo-intestine, a post-septum approximately equal to the length of the pre-septum, and four terminal spines on the post-septum (Inoue, 1958; Poinar and Doelman, 1974; Hanelt and Janovy, 2002; Bolek et al., 2010; Chiu et al., 2011; Bolek et al., 2013a; Szmygiel et al.(2014)). Additionally, distinct differences exist in the proboscis morphology and proboscis spine orientation among the three larval types (Figure 15.12; Szmygiel et al.(2014)). The proboscis is dorsoventrally compressed in *Gordius* and *Paragordius* and laterally compressed in *Chordodes* and *N. occidentalis*. *Gordius* contains spines on both lateral sides and the dorsal side of the proboscis, whereas *Paragordius* has spines on both lateral sides and the ventral side of the proboscis. In contrast, larvae of *Chordodes* and *N. occidentalis* contain spines on the dorsal, ventral, and left lateral sides of the proboscis (Figure 15.12).

Three transmission mechanisms have been proposed for aquatic gordiid larvae to reach their terrestrial arthropod hosts: (1) larvae are consumed directly by the definitive hosts while drinking water; (2) larvae encyst in water and/or on vegetation and are ingested accidently while the definitive host drinks water or consumes vegetation; and (3) larvae enter and encyst within a paratenic hosts, which are preyed on or scavenged by the definitive hosts (Hanelt et al., 2005; Schmidt-Rhaesa, 2012). Several experimental studies indicated that when definitive arthropod hosts drink suspended larvae in water they become infected (May, 1919; Inoue, 1962; Hanelt and Janovy, 2004a). However, comparative studies by Inoue (1962) and Hanelt and Janovy (2004a) clearly showed that prevalence and intensities of these infections in definitive hosts are much lower compared to when definitive hosts are exposed to gordiid cysts in paratenic hosts. Other studies by Dorier (1930) demonstrated that gordiid larvae of the European *G. aquaticus* can encyst freely in the absence of a host on the surface of water

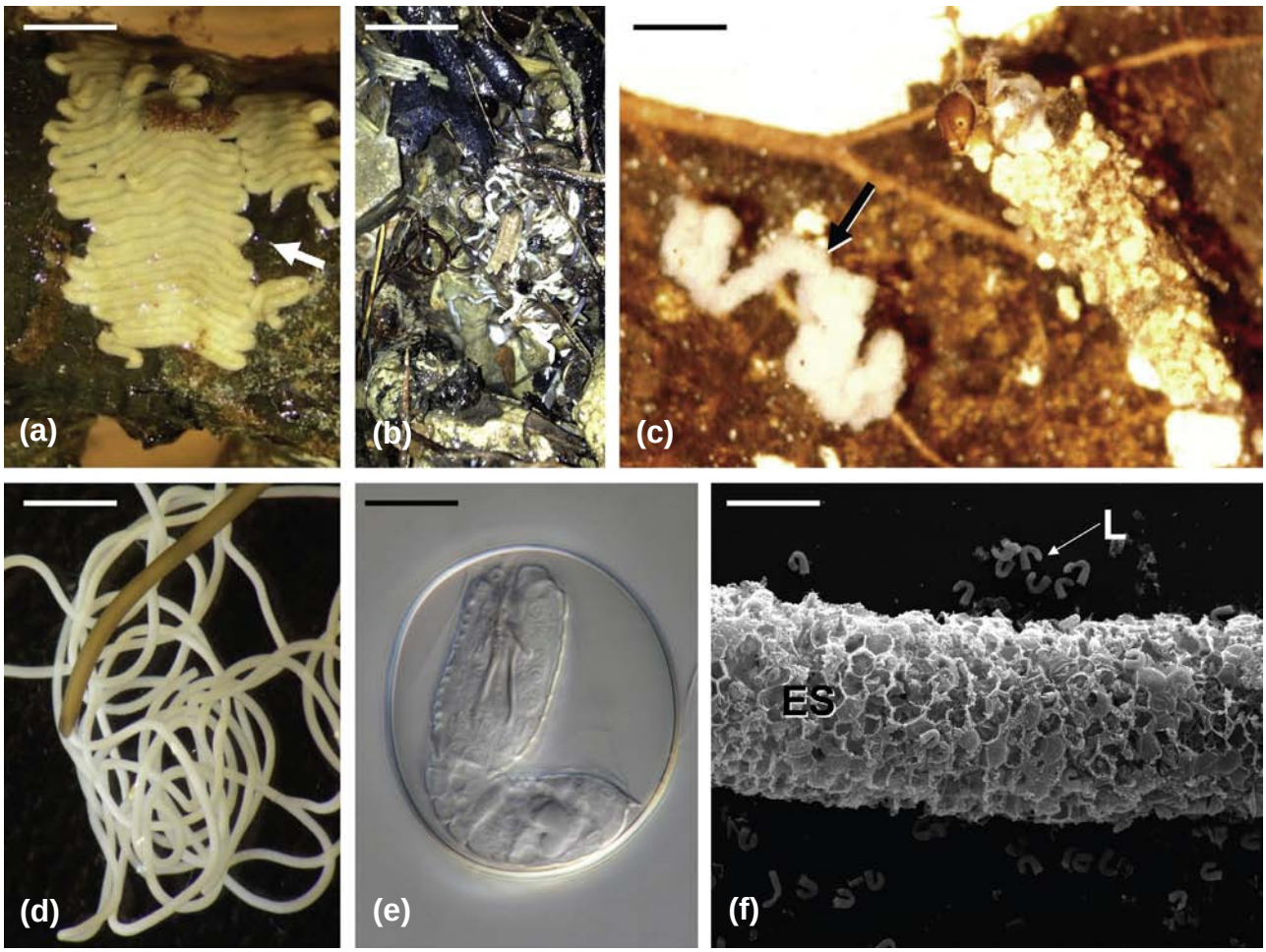

FIGURE 15.11 Examples of gordiid egg strings and eggs. (a) Egg string of *C. morgani* deposited on a branch in a zig-zag pattern. Scale bar = 5.0 mm. (b) A Gordian knot containing numerous individuals of *G.* cf. *robustus* (brown) in the process of depositing short pieces of egg strings (white). Scale bar = 2.0 cm. (c) A piece of *G. difficilis* egg string next to a tricopteran larva in a stream. Scale bar = 1.0 mm. (d) Posterior end of a female *P. varius* (tan) in the process of depositing a single and very long egg string (white). Scale bar = 5.0 mm. (e) A fully developed larva of *N. occidentalis* inside an egg. Scale bar = 20.0 µm. (f) A scanning electron micrograph of a partial egg string (ES) of *P. varius*. Note the numerous hatched larvae (L) scattered around the periphery of the egg string. Scale bar = 100.0 µm.

or on aquatic vegetation, suggesting that terrestrial arthropods can be infected with these cysts when they ingest aquatic vegetation or drink water. Since Dorier's original studies, these observations have not been confirmed. In our observations on cyst formation, we occasionally find a few larvae of *G.* cf. *robustus* forming cysts in empty egg strings (Figure 15.13), but the role of these cysts in transmission is unclear. Although both of these transmission mechanisms are plausible, gordiid larvae reside in the benthos and cannot swim. Therefore, it is unlikely that these transmission mechanisms play a major role in the natural life cycle of gordiids (Hanelt and Janovy, 2003).

Other studies on larvae of numerous gordiid species indicate that gordiid larvae never encyst in air or water (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, gordiid cysts have been reported from most aquatic metazoan animals including, molluscs, annelids, arthropods, fish, and amphibians. More importantly, experimental studies by Hanelt and Janovy (2004b) demonstrated that three phylogenetically distinct species of gordiids (*Chordodes morgani*, *G.* cf. *robustus*, and *P. varius*) indiscriminately infected and formed cysts in a variety of aquatic invertebrates and fish. Hanelt and Janovy (2004b) also demonstrated that within paratenic hosts, gordiid cyst survived metamorphosis of 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 in surviving insect metamorphosis, and their ability to infect terrestrial arthropod hosts, supports the paratenic host strategy in the life cycles of gordiids.

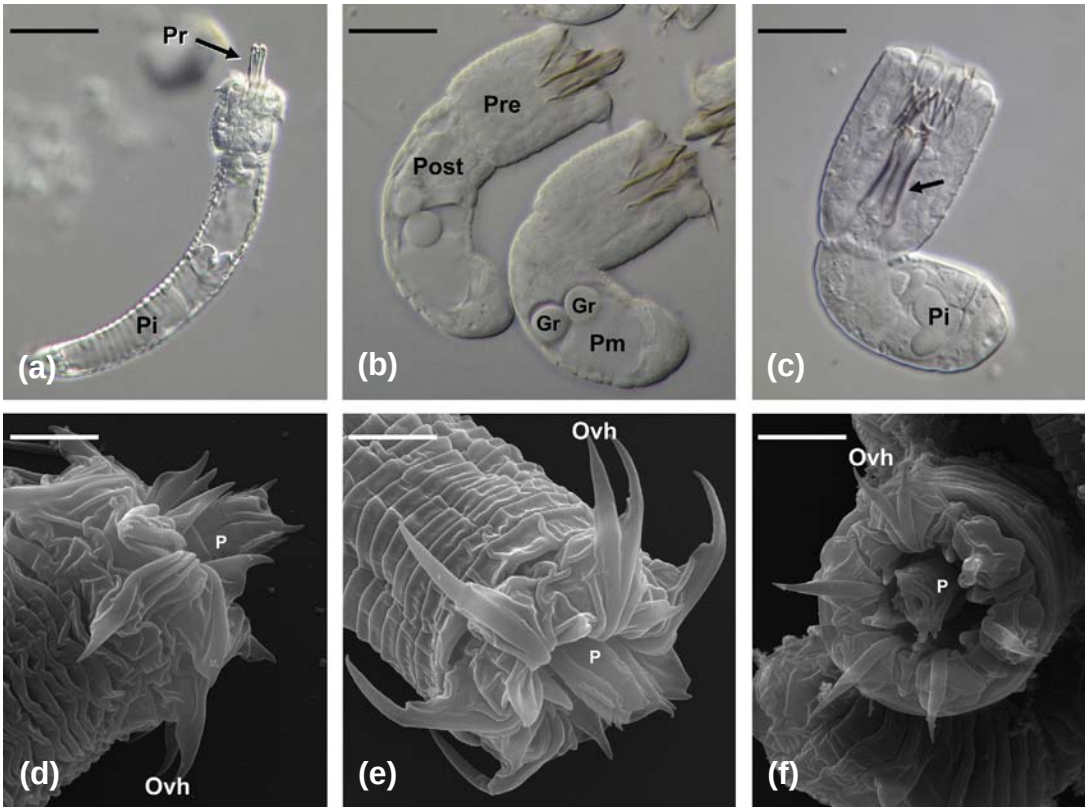

FIGURE 15.12 Examples of three types of gordiid larvae. (a) A typical *Gordius* larva. Note everted proboscis (Pr), the pseudo-intestine (Pi), and very long post-septum. Scale bar = 20.0 µm. (b) Larvae of *P. varius*. Note the relative size of the pre-septum (Pre) and post-septum (Post), and complex structure of the pseudo-intestine containing two anterior granules (Gr) and posterior mass (Pm). Scale bar = 10.0 µm. (c) Larva of *N. occidentalis*. Note the three internal stylets (black arrow), the v-shaped pseudo-intestine (Pi) and relative size of the pre-septum to post-septum. Scale bar = 10.0 µm. (d) Scanning electron micrograph of the anterior end of a *Gordius* larva. Note the orientation of the proboscis (P) to the outer ventral hooks (Ovh). Scale bar = 5.0 µm. (e) Scanning electron micrograph of the anterior end of a *P. varius* larva. Note the orientation of the proboscis (P) to the very large ventral outer hooks (Voh). Scale bar = 5.0 µm. (f) Scanning electron micrograph of the anterior end of a *C. morgani* larva. Note the laterally compressed proboscis (P) in relationship to the ventral outer hooks (Voh). Scale bar = 10.0 µm.

Gordiid larvae are viable and infective to their paratenic hosts for a few days to a few weeks (Poinar and Doelman, 1974; Hanelt et al., 2005; Bolek et al., 2010). From these studies, it is clear that larvae of gordiids are incapable of movement after a few weeks. However, our unpublished observations indicate that nonmotile larvae of *C. morgani*, *G.* cf. *robustus*, *N. occidentalis*, and *P. varius* are infective to paratenic hosts for at least an additional month after they stop moving (Matthew Bolek, personal observations). The most likely and well supported mechanism for gordiid larvae infecting paratenic hosts is direct uptake through ingestion of larvae by paratenic hosts (Hanelt and Janovy, 2003). Once ingested, larvae use their proboscis and cuticular rings of hooks on the pre-septum to penetrate the intestinal epithelial tissue of the gut of paratenic hosts (Inoue, 1960, 1962; Poinar and Doelman, 1974; De Villalobos and Ronderos, 2003; Hanelt and Janovy, 2003). After boring through the intestinal wall, most larvae encyst as cysts on the outside of the host's gut (Figure 15.13). However, some larvae continue to migrate throughout the body cavity of their host and can encyst in any tissue. Laboratory infections show that if large numbers of larvae are ingested by paratenic hosts, the host dies most likely because of tissue damage during larval migration (Poinar and Doelman, 1974).

Although it is unclear whether hairworm larvae have a significant impact on mortality of paratenic hosts in nature, several laboratory studies and field observations indicate that insect paratenic hosts mount an immune reaction to hairworm larvae and cysts (Poinar and Doelman, 1974; De Villalobos and Ronderos, 2003; Hanelt and Janovy, 2003). Host reactions usually involve humoral melanization of larvae (Figure 15.13) and/or cysts. Melanization of gordiid larvae and cysts have been reported in a variety of aquatic larval insects including mosquitos, chironomids, caddisflies, mayflies, stoneflies, as well as larval beetles (Poinar and Doelman, 1974; Poinar, 1991; Hanelt and Janovy, 2003; Figure 15.13).

During cyst formation, larvae empty the contents of their pseudo-intestine. Laboratory observations by Dorier (1930), Poinar and Doelman (1974), Hanelt and Janovy (2003), Hanelt et al. (2012) and Bolek et al. (2010; 2013a,b)

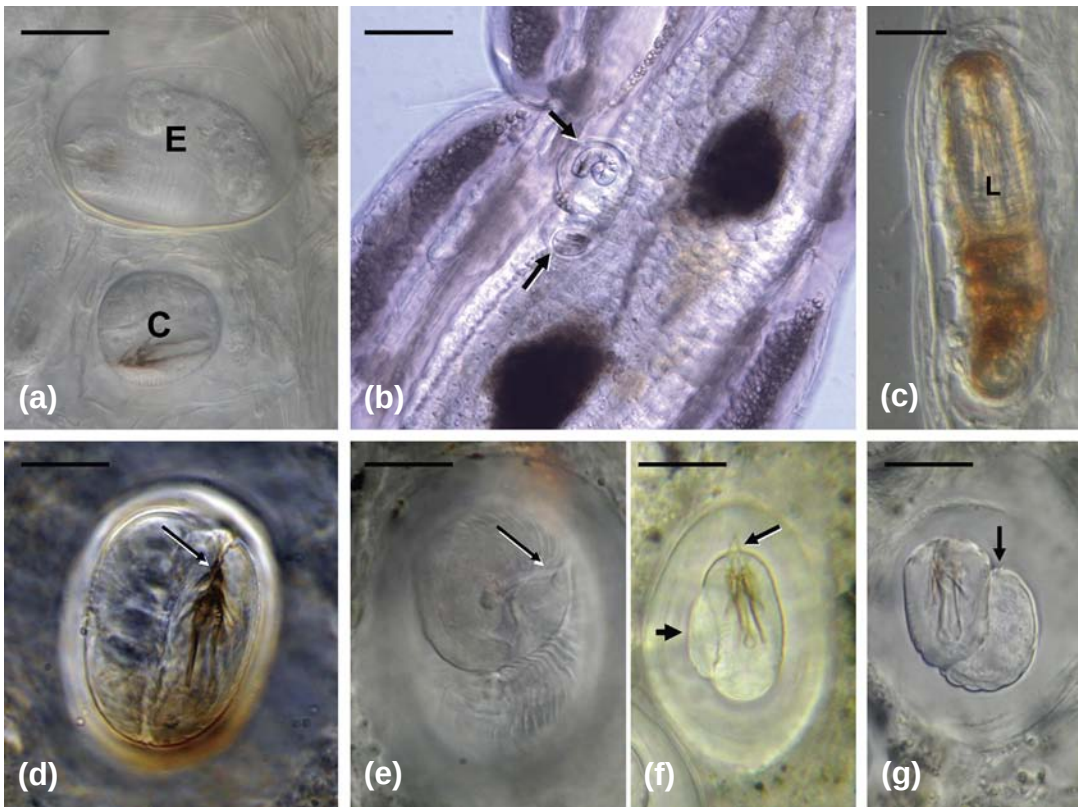

FIGURE 15.13 Representative cysts and larvae of gordiids. (a) A *G.* cf. *robustus* cyst (C) next to an egg (E) within an egg string. Scale bar = 20.0 µm. (b) Two types of gordiid cysts (arrows) encysted on the outside of the gut of a larva chironomid. Scale bar = 50.0 µm. (c) A *Chordodes* like larva (L) on the outside gut wall of an aquatic beetle larva in the process of being melanized (orange brown pigment). Scale bar = 15.0 µm. (d and e) Typical folding pattern of a *Gordius* larva within a cyst photographed at two focal planes. Note clear cyst wall (E), no protruding spines (arrow in D) and the double folded post-septum with its posterior end reaching the posterior end of the pre-septum (arrow in E). Scale bars = 10.0 µm. (f) Typical folding pattern of a *Paragordius* larva within a cyst. Note the clear cyst wall, distinct spines on the pre-septum (black thin arrows), and the position of the posterior end of the post-septum (short thick arrow). Scale bar = 20.0 µm. (g) A cyst of *N. occidentalis*. Note the clear cyst wall, reduced content of the pseudo-intestine and the position of the posterior region of the post-septum (arrow). Scale bar = 20.0 µm.

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 15.13). 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 et al., 2003). More importantly, if a paratenic host is ingested by another animal other than a definitive host, the cysts are digested out and larvae re-penetrate into the second paratenic hosts and re-encysts (De Villalobos et al., 2003; Hanelt and Janovy, 2003).

As with larvae, three types of cyst folding patterns have been reported in the Gordiida (Hanelt and Janovy, 2002; Szmygiel et al.(2014)). Work by Szmygiel et al.(2014) indicated that morphological differences in the three types of cyst folding patterns are correlated to the three types of gordiid larval morphologies. Larvae of *Gordius* and *Paragordius* species have a post-septum, which is significantly longer than the pre-septum. As a result, larvae within cysts of both of these genera are always folded twice. However, the post-septum of *Gordius* species is about three times as long as the pre-septum and, consequently, the posterior end of the post-septum always reaches the posterior end of the pre-septum (Figure 15.13). In contrast, the post-septum of *Paragordius* species is only 1.3 times as long as the pre-septum, and, as a result, the posterior end of the post-septum never reaches the posterior end of the pre-septum (Figure 15.13). Additionally, larvae of *Paragordius* contain distinctly longer hooks on the outer ring of the larval pre-septum, and these are clearly visible as large spines in the cyst stages (Figures 15.12 and 15.13). 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 (Figure 15.13).

Most definitive hosts for gordiids are predaceous or omnivorous arthropods, which capture infected paratenic arthropod hosts after they metamorphosed from an aquatic habitat or scavenge on dead infected paratenic hosts (Figure 15.7; Inoue, 1962). A few herbivorous orthopteran hosts are

also known as definitive hosts for gordiids, but it is unclear how these hosts become infected in nature (Schmidt-Rhaesa, 2012). In the laboratory, the maturation of gordiids within the definitive host takes several months and can range from as long as 8 months for *Gordius tolosanus*, (Svábeník, 1925), 2–3 months for *Chordodes japonensis*, *C. kenyaensis* and *P. obamai* (Inoue, 1962; Hanelt et al., 2012; Bolek et al., 2013a), and as short as 1 month for *P. varius* (Hanelt and Janovy, 2004a). Some field studies indicate that definitive hosts appear to show a high degree of parasite induced pathology. A few reports 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). Only one report found that naturally infected female hosts may be capable of reproducing (Poulin, 1995). More recently, a study by Biron et al. (2005b) using naturally infected crickets showed that female crickets were capable of producing eggs only after they release 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 hairworms and did not regain the ability to produce sperm after they released worms.

GENERAL ECOLOGY AND BEHAVIOR

Host Specificity

The ecology of Nematomorphs is closely associated with their terrestrial hosts and the aquatic habitat of the free-living adult worms. In the Nearctic region, few studies have sampled for free-living adult worms throughout the year and even fewer studies have examined multiple arthropod species for gordiid infections (Bolek and Coggins, 2002; Poinar and Weissman, 2004; Looney et al., 2012). As a result, very little information is available on the ecology of freshwater gordiids in the Nearctic. The few worldwide studies that exist on these topics suggest that, depending on the gordiid species, hairworms vary in their definitive host specificity, free-living adults are seasonal and usually in nature gordiid populations have skewed sex ratios.

Knowledge on host specificity for most gordiid species is poor, and almost all host records are based on field observations (Schmidt-Rhaesa, 1997; Schmidt-Rhaesa et al., 2003, 2008; Schmidt-Rhaesa, 2012). Definitive arthropod host data are only available for eight of the 22 described Nearctic species (Schmidt-Rhaesa et al., 2003; Poinar and Chandler, 2004; Schmidt-Rhaesa et al., 2009). Field studies indicate that some, but not all, gordiid species appear to be host specific at the definitive host level (Poinar, 1991; Bolek and Coggins, 2002; Schmidt-Rhaesa et al., 2003, 2005; Chiu et al., 2011; Looney et al., 2012). In the Nearctic region, *C. morgani* has been reported from as many as four phylogenetically distinct orthopteran and cockroach species, suggesting that some hairworms are generalists at the definitive host level. In contrast, other species, particularly in the *G. 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 (Schmidt-Rhaesa et al., 2003; Ben Hanelt, personal observations). Similar host specificity observations have been reported for European gordiids. For example, in a study in France, *Paragordius tricuspidatus* was reported from only one orthopteran species, whereas *Spinochordodes tellinii* was found to infect nine phylogenetically distinct arthropod species (Schmidt-Rhaesa et al., 2005).

Standard measures of parasitism of hairworm infections in arthropod populations indicate that prevalence (percent of arthropods infected), intensity (number of worms per host), and species richness (number of species of gordiids infecting a hosts) is generally low. Although Thorne (1940) reported that 99% of Mormon crickets (*Anabrus simplex*) were infected with *G. robustus* in Utah, most field studies report a prevalence ranging from less than 1% to as high as 28% in different arthropod populations (Schmidt-Rhaesa, 2012). Field data on intensities of worms within definitive hosts indicate that intensities of up to seven worms per host can occur. However, more commonly, single worm infections are present in a majority of infected arthropods at a given location (Assmuss, 1858; Von Linstow, 1891b; Baker, 1985; Valvassori et al., 1988; Zervos, 1989; Studier et al., 1991; Poulin, 1995; Hanelt and Janovy, 2000; Bolek and Coggins, 2002; Poinar and Weissman, 2004; Chiu et al., 2011). Finally, most field studies report only a single gordiid species infecting a single arthropod individual (Bolek and Coggins, 2002; Poinar and Weissman, 2004; Chiu et al., 2011).

It is unclear if the low species richness of gordiids in arthropod hosts is due to host specificity, worm biogeography, low transmission rates, gordiid species interactions, or a combination of these factors. However, in the Nearctic region, a number of studies have reported different hairworm species from the same arthropod genus and/or species indicating that multiple species infections within single arthropod hosts are possible (Bolek and Coggins, 2002; Schmidt-Rhaesa et al., 2003; Poinar and Weissman, 2004; Poinar et al., 2004). The only study to report on the interactions between two species of hairworms in single host individuals was presented by Bolek et al. (2013a). Bolek et al. (2013a) exposed individual laboratory reared crickets to snail tissue containing cysts of *P. obamai*, *C. kenyaensis*, or both species. They found that all individuals of *C. kenyaensis* recovered from crickets concurrently infected with *P. obamai* appeared deformed compared to *C. kenyaensis* recovered from crickets infected with

only *C. kenyaensis*. Deformed individuals of *C. kenyaensis* were smaller and thicker in size than worms from single species infections. Additionally, these worms had difficulty emerging from their cricket hosts and could not swim or mate. In contrast, individuals of *P. obamai* appeared not to be affected by the presence or absence of *C. kenyaensis* in the cricket host.

Studies on adult free-living worms in nature indicate that the occurrence of worms is seasonal (Bolek and Coggins, 2002; Schmidt-Rhaesa et al., 2005; Salas et al., 2011). Bolek and Coggins (2002) reported the occurrence of free-living individuals of *G. difficilis* in Wisconsin (United States) from June to October; whereas Inoue (1958) reported *C. japonensis* from Japan during September and October. Additionally, over a period of 3 years, Schmidt-Rhaesa et al. (2005) captured hosts and recently emerged adult hairworms of two species around a swimming pool in Southern, France. Their data showed 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. The authors hypothesized that these differences in the seasonal occurrence of the two gordiid species were dependent on the occurrence of their hosts. At their study site, both gordiid species infected different species of definitive hosts and their occurrence was correlated with the abundance of these hosts. More recently, Salas et al. (2011) examined the seasonal occurrence of free-living individuals of four species of sympatric gordiids over a 1-year period from the El Simbolar stream in Argentina. In their study, free-living worms of all four species occurred in the stream during the fall, winter, and spring. However, three of the species (*Noteochorododes cymatium*, *Noteochorododes talensis*, and *Pseudochordodes dugesi*) were most abundant during the winter and spring and their occurrence was correlated with water temperature. In contrast, the fourth species (*Chordodes brasiliensis*) was most abundant during the fall and the occurrence of *C. brasiliensis* was correlated with stream flow rate and pH.

Sex Ratios

Finally, field observations on the sex ratio of gordiids rarely show equal sex ratios of free-living male and female worms. Usually, field collections of free-living worms are biased toward males (Salas et al., 2011). Cochran et al. (2004) reported that of 1391 individuals of *G. difficilis* collected during a 32-year period in six Midwestern states of the United States, 1205 were males. Similarly, Thomas et al. (1999), studying *Euchordodes nigromaculatus* in New Zealand, collected only 61 males and no females. However, in some collections, females are more common than males. For example, Watermolen and Haen (1994) report 67 individuals of *G. robustus* from Wisconsin, of which 66 were females. Additionally, a few other studies indicate that males and females are collected in equal numbers (De Villalobos and Camino, 1999; Valvassori et al., 1988). Some of these skewed sex ratio observations in field studies are in contrast to laboratory life cycle studies on dioecious hairworm species. Both Hanelt and Janovy (2004a) and Bolek et al. (2013a) found no statistically significant differences in the sex ratios of *P. varius* or *C. kenyaensis* emerging from laboratory reared and infected cricket definitive hosts.

Two field studies involving multiple sampling from single localities at different times of the year shed light on the observed discrepancies in sex ratios of laboratory cultured and field collected gordiids. Bolek and Coggins (2002) examined the seasonal occurrence of *G. difficilis* in Wisconsin by conducting a worm removal experiment over a 3-year period. Their study indicated that, although the total free-living worm collection was male-biased, the population sex ratio of free-living worms changed from being female-biased early in the season to being male-biased late in the season. In a similar study, Poulin (1996) collected *Gordius dimorphus* from New Zealand's South Island during spring and summer of a single year. In his study, the sex ratio was female-biased during the spring and male-biased during the summer. These authors hypothesized that the observed differences in sex ratios of field collected gordiids could be due to: (1) time differences in the life span of male and female worms; (2) differences in males and females developmental times in the definitive hosts; and/or (3) differences in habitat selection by male and female worms.

More recently, Hanelt et al. (2012) infected laboratory reared crickets with field collected cysts of an African *Paragordius* species and discovered a parthenogenetic species of gordiid (*P. obamai*) from Kenya. *P. obamai* represents the first and only known species within this phylum to reproduce asexually. However, evidence from the literature suggests that *Paragordius* may contain other species with a similar reproductive strategy. In fact, of the 18 known *Paragordius* species, males have only been described from seven (39%) of these species (Hanelt et al., 2012). This is in contrast to two other widely distributed gordiid genera. Of the 82 valid species of *Gordius*, males have been documented in 88% of these species (Schmidt-Rhaesa, 2010); whereas males have been documented in 65% of the 56 valid species in *Chordodes* (Schmidt-Rhaesa et al., 2008; Bolek et al., 2010; Chiu et al., 2011; Bolek et al., 2013a). These observations on *P. obamai* and the lack of male specimens from most species in *Paragordius* raise the intriguing possibility that parthenogenesis is much more common in this group than previously recognized, and must be confirmed with additional field sampling and establishment of life cycles in the laboratory.

FIGURE 15.14 Typical stream habitats for gordiids in North America. (a) Cedar Creek, a first order stream in Keith County, Nebraska. (b) A typical second order stream in Payne County, Oklahoma. (c) A first order stream in the Huachuca Mountains, Cochise County, Arizona.

Habitat Selection

Adult free-living worms can be found in a variety of aquatic habitats ranging from puddles, small streams (Figure 15.14), ponds, and lakes, to large rivers (Hanelt et al., 2005; Schmidt-Rhaesa, 2012). Additionally, in the Nearctic region, there are a number of reports of gordiids collected in water sources in caves (Dearolf, 1953; Reddell, 1965; Holsinger and Peck, 1971; McDaniel and Smith, 1976; Conn, 1981; Goggin et al., 1989; Reeves, 2000). Within aquatic habitats, adult free-living worms can occur in the sediment, among moist fallen leaves, under rocks around the shore, in algal mats, on aquatic vegetation, and small branches within bodies of water (Bolek and Coggins, 2002; Schmidt-Rhaesa, 2012). In the Nearctic region, single individuals and aggregations of worms in Gordian knots are commonly recovered from slow moving water in spring-fed habitats (Bolek and Coggins, 2002; Cochran et al., 2004).

Studies on Gordian knots within streams indicate that knots are more likely to be attached to floating rather than immobile substrates (Daoust et al., 2012). Observations on Gordian knots in natural habitats have been recorded for the Nearctic species *G. difficilis* and the European *P. tricuspidatus*. These studies indicate that Gordian knots are dominated or are entirely composed of males. In contrast, Bolek and Coggins (2002) never observed Gordian knots of *G. difficilis* in a small stream in southeastern Wisconsin. Over a 3-year collecting period, they found individual female worms on the sediment of the stream during their emergence period from their hosts (June–July), and male and female worms congregated 1–4 cm bellow the stream sediment where females deposited egg strings during August–September. These studies suggest that female and male worms utilize different habitats during their short free-living phase. It is clear from laboratory studies and unpublished field observations that females of some genera (*Chordodes*, *Euchordodes*, and *Neochordodes*) attach their egg strings on sticks and/or rocks, where other genera, such as *Gordius* and *Paragordius*, deposit unattached egg strings in and/or on the sediment or in algal mats (Bolek and Coggins, 2002; Hanelt and Janovy, 2002; Poinar, 2010; Szmygiel et al.(2014); Matthew Bolek, personal observations).

Finally, a recent study from Japan on *Gordionus chinensis* suggests that anthropogenic disturbances on ecosystems can alter nematomorph distribution and abundance (Sato et al., 2014). These authors examined the abundance of *G. chinensis* and their terrestrial camel cricket hosts (*Diestrammena* spp.) in eight watersheds along a forest recovery gradient after clear-cut logging (3–48 years) of second growth forest. They found that *G. chinensis* suffered local extinction immediately after clear-cut logging and took much longer to recover (>50 years) compared to their terrestrial camel cricket hosts (30 years) and benthic invertebrate paratenic hosts (40 years). After local extinction, gordiid abundance increased linearly with forest age, however, forest age explained a relatively a small portion (23%) of the variation. The authors hypothesized that effects of other factors, such as forest management history and their interactions with forest age, should be examined in future studies. Currently, no clear explanation is available for the slow

recovery process of gordiids compared to their hosts in this ecosystem (Sato et al., 2014).

Physiological Constraints

Very little information is available on the physical constraints of gordiids and the few studies that are available concentrate on the free-living adult and the free-living larvae and cyst stages. Bolek et al. (2013a) indicated that in laboratory cultures of the African *C. kenyaensis*, adult free-living worms die within 24 h if they emerge from their cricket hosts in cages without a water source, suggesting that free-living worms cannot survive drying for extended periods of time. Observations on field collected and laboratory cultured worms indicate that over a 24 h photograph period, free-living worms are more active during the night and less active during daylight suggesting that Nematomorphs can detect light (Matthew Bolek, personal observations). However, the importance of this behavior is unclear and no studies have been conducted on phototaxis behavior in hairworms.

A few studies have examined the effects of temperature on gordiid species (Zanca et al., 2007; Achiorno et al., 2008; Bolek et al., 2013b). Zanca et al. (2007) demonstrated that eggs of the South American *Chordodes nobilii* could develop at 5 °C. In their study, developing larvae in egg strings maintained at 5 °C developed slowly and remained in the egg, whereas developing egg strings maintained at 22 °C developed much more quickly and hatched. The authors suggested that this delayed larval development and hatching at low temperatures was a survival strategy of gordiid species that commonly deposit their egg strings during seasons when temperatures are cold. Additionally, Achiorno et al. (2008) examined egg development and egg, larval, and adult survival of *C. nobilii* in response to extreme temperatures. In their study, Achiorno et al. (2008) demonstrated that all eggs, most larvae, and all adult gordiids died at a high temperature of 40.5 °C, and all eggs and most adult worms (89%) died at a low temperature of −3 °C. In contrast, their study indicated that a high proportion of gordiid larvae frozen at −3 °C for 48 h survived freezing and were capable of infecting mosquito larva paratenic hosts. More recently, Bolek et al. (2013b) showed that both larvae and cysts of North American and African gordiids in the genus *Paragordius* can survive super cooling and/or freezing (−20 to −80 °C) for at least 7 months. More importantly, postfrozen 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 of time.

Effects of Pesticides

A few studies exist on the effects of pesticides on gordiids. Both glyphosate, a broad-spectrum systemic herbicide used to kill weeds, and malathion, an organophosphate insecticide, did not affect larval development of *C. nobilii* at ecologically relevant concentrations. However, both pesticides affected larval infectivity of paratenic hosts and survival of free-living adults (Achiorno et al., 2009). Additionally, acute exposure with three standard toxicants, sodium dodecyl sulfate, cadmium chloride, and potassium dichromate, were tested on the sensitivity of the pre-parasitic stages of *C. nobilii* by Achiorno et al. (2010). At environmentally relevant concentrations, egg development was not affected. However, compared to unexposed controls, larval survival and infectivity of paratenic hosts were reduced even at the lowest concentrations for all three toxicants assayed.

Feeding Behavior

There is no evidence that adult Nematomorphs ever feed in the free-living stage. However, adults are relatively long lived (several weeks to several months in cold water) during the free-living and non-feeding phase of their life cycle (Hanelt et al., 2005). These observations suggest that they must store some sort of energy source to be used for basic metabolic function. Parenchymal cells in free-living adults contain vacuoles filled with lipids and glycogen and these may serve as an energy source during the non-feeding free-living stage (Reutter, 1972). Nutrients are most likely absorbed through the juvenile cuticle from the host's tissue and fluids during the relatively long 1–8 month developmental period in the definitive host (Hanelt and Janovy, 2004a; Hanelt et al., 2012; Bolek et al., 2013a). Intestinal cells appear to be active in nutrient uptake during the parasitic phase but are not active in the free-living stage (Schmidt-Rhaesa, 2005). It is unknown if larvae can feed on paratenic hosts during cyst development or if cysts absorb nutriment while in paratenic hosts.

Predation, Parasitism, and Commensals

Because of their secretive habits, few reports exist on the predators of hairworms, and most reports come from stomach content analyses of aquatic vertebrates, with one observation of a predation attempt on a gordiid by a crayfish (Cochran et al., 1999). Many studies report single or partial worms in the stomach contents of fish and frogs, but a few studies have found entire Gordian knots in the stomach contents of aquatic vertebrates (Forbes, 1883; Evermann and Clark, 1920; Clemens, 1928; Riggs, 1952; Probst and Cooper, 1954; Kempinger, 1996; Cochran et al., 1999; Bolek and Coggins, 2002; Kinziger et al., 2002; De Villalobos et al., 2008). In the Nearctic region, these reports include finding entire or partially digested worms in the stomachs and/or digestive tracts of lake sturgeon (*Acipenser fulvescens*), a number of species of sunfish (*Lepomis* spp.), salmonids including Lake Whitefish (*Coregonus clupeaformis*), brook

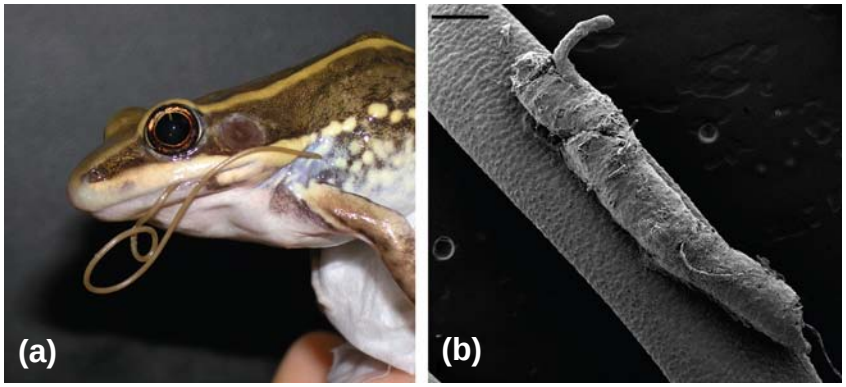

FIGURE 15.15 Predators and epibionts of gordiids. (a) A free-living *Paragordius tricuspidatus* escaping from the mouth of a frog (*Rana erythraea*), which ingested an infected cricket. *(Photograph courtesy of Frédéric Thomas)* (b) A chironomid pupa on the midbody region of the cuticle of a *Neochordodes* sp. Scale bar = 300.0 µm.

trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*), a single species of minnow (the creek chub, *Semotilus atromaculatus*), and green frogs (*Rana clamitans*).

From these reports, it is unclear if fish and/or frogs feed directly on free-living hairworms or on their infected hosts. However, Cochran et al. (1999) partially tested this hypothesis by providing free-living adult worms to five species of fish. Their study showed that most fish rejected free-living worms, indicating that fish likely feed on infected arthropods and not directly on free-living worms. More recently, studies from Japan by Sato et al. (2008, 2011) indicated that gordiids are only found in the stomachs of Japanese trout (*Salvelinus leucomaenis japonicus*) that also consumed camel crickets (*Diestrammena* sp.). Based on these observations, these authors hypothesized that hairworm infections and their manipulations of terrestrial arthropod hosts can drastically change energy inputs into aquatic ecosystems. Using estimates of seasonal prey abundance, they argued that, during peak worm emergence times, infected orthopterans account for 60% of the annual energy intake of Japanese trout (Sato et al., 2011). Other studies by Ponton et al. (2006a,b) indicate that when infected orthopterans are consumed by aquatic vertebrates, a considerable number of hairworms (18–35%) can escape the predators of their hosts. Worms usually escape from fish predators through the mouth, nose, or gills and through the mouth from frog predators (Figure 15.15).

Other reports of potential vertebrate predators of hairworm and/or their hosts include birds. Poinar (2010) reported observing a tapaculo (*Scleorchious rubecula*) in Chile bringing an adult *Gordius* to its nestlings. However, it is unclear if the hairworm was ingested by these birds. Additionally, Fair et al. (2010) collected eight individuals of *G.* cf. *robustus* from seven nests within nest boxes occupied by western bluebirds (*Sialia mexicana*) in New Mexico, United States. All of the nest boxes containing worms were less than 100 m from stagnant or slow-flowing streams. The authors suggested that the most likely explanation for the presence of hairworms in these nest boxes was that gordiids engaged in antipredator avoidance after their insect hosts were collected by the parent birds and before being digested by the nestling birds. Finally, an unusual case of hairworms associated with birds was reported by Notman and Yeates (1992) from New Zealand. A partial skeleton of a tomtit (*Petronica macrocephala*) was found hanging from a branch. Upon closer inspection, it was discovered that a gordiid coiled around the bird's foot and the branch where the bird was apparently perching. As in the blue bird example, it is likely that the tomtit was also eating an infected insect from which an adult worm emerged, the worm then tied itself around the bird's foot and the branch ultimately causing the bird to starve to death.

Compared to predators of gordiids, even fewer studies exist on symbionts, commensals, and/or parasites of freshwater hairworms. De Villalobos et al. (2010) reported a number of epibionts on the surface of the cuticle of *Chordodes nobilli* from Argentina. These included diatoms (*Metrichia* sp.) and larvae and pupae of tricopterans and elmid beetles. Our SEM studies commonly reveal protists and pupae of dipterans on the surface of gordiids as can be seen in the recently collected *Neochordodes* species from Mexico (Figure 15.15). Investigations on prokaryotes, such as *Wolbachia* species, which are commonly associated with arthropods and nematodes infecting arthropods, have not been reported in Nematomorphs (Duron and Gavotte, 2007; Hudson and Floate, 2009; Hanelt et al., 2012). This is particularly interesting in the parthenogenetic species *P. obamai* because *Wolbachia* species associated with their arthropod and nematode hosts are known to manipulate the sex ratio of their hosts. Hanelt et al. (2012) ruled out the involvement of reproduction manipulating endosymbionts by use of next generation sequencing data, thus suggesting that parthenogenesis is determined genetically in this gordiid species and may have evolved as a means to assure reproduction. Additionally, Duron and Gavotte (2007), Hudson and Floate (2009), and Hanelt et al. (2012) found

no internal (symbiotic or nonsymbiotic) prokaryotes in four species of gordiids examined to date. Finally, gordiid cysts have even been reported as hyperparasites in trematodes infecting molluscs, fish, and newts (Cort, 1915; Fischthal, 1942; Hanelt, 2009b).

COLLECTING, CULTURING, AND PREPARING SPECIMENS

Collecting

Until recently, no special sampling techniques have been developed for collecting hairworms, and most specimens have been collected visually by finding adult worms or netting individuals with dip-nets or seines (Bolek and Coggins, 2002; Salas et al., 2011). The lack of knowledge on the diversity of hairworm species exists because studies have been hindered by: (1) the lack of reliable ways to collect adult free-living hairworms over large geographical areas; and (2) the relatively short life span of the free-living adults, making them difficult to collect. However, recent studies suggest that non-adult stages of gordiids, such as cysts, may be the most commonly encountered life stages of gordiids in the environment (Hanelt et al., 2001; Szmygiel, 2012; Bolek et al., 2013a). Both Hanelt et al. (2001) and Szmygiel (2012) sampled for adult and cyst stages of gordiids in 50 and 46 streams in a single county in Nebraska and Oklahoma (United States), respectively. Free-living adult gordiids were found in only one stream in Nebraska, and none in Oklahoma. In contrast, cysts of gordiids infected aquatic snails in 70% of the streams sampled from Nebraska and Oklahoma. These and other studies indicate that cysts are the most encountered gordiid stages in nature and may be useful for sampling large geographic areas for Nematomorph biodiversity studies (Hanelt et al., 2001, 2012; Szmygiel, 2012; Bolek et al., 2013a).

Until recently, one major difficulty in utilizing gordiid cyst stages for hairworm biodiversity studies was the lack of knowledge of the morphology of cysts for most hairworm species and genera. The lack of descriptions of non-adult stages of gordiids stems from the fact that the life-cycles for most species of gordiids were unknown. However, recent advances in our understanding of gordiid life-cycles in combination with new culturing and domestication techniques have made these life stages more accessible for morphological studies (Hanelt and Janovy, 1999, 2002, 2004a,b; Hanelt et al., 2012; Bolek et al., 2010, 2013a,b). Using these techniques, Szmygiel et al. (2014) examined cyst morphology for nine species of gordiids from two continents. Their study indicated that some gordiid genera, such as *Gordius* and *Paragordius*, can be identified to genus based on the morphology of the cyst and are distinct from cysts of closely related genera, such as *Chordodes* and *Neochordodes* (Szmygiel et al. (2014)). Using this information, Hanelt et al. (2012) and Bolek et al. (2013a) collected snails infected with two types of gordiid cysts from Kenya, a country for which no gordiid records existed. After exposing the appropriate group of laboratory reared arthropods, two new species of gordiids were discovered.

Culturing

Culturing gordiids in the laboratory from field collected cysts is one solution for obtaining difficult-to-find, free-living adult worms however, it raises a number of difficulties. First, there are logistical and timing issues of returning live cysts for arthropod infections to the laboratory. However, recent work on North American and African gordiids indicates that gordiid cysts can survive super cooling and/or freezing for up to 7 months (Bolek et al., 2013b). The ability of gordiid cysts from field collected snails to survive rapid cooling and/or freezing temperatures and produce viable adult worms when fed to laboratory reared hosts provides a technique, which will allow the establishment of other and novel nematomorph life cycles in the laboratory. Second, until recently, most species of definitive arthropod hosts for gordiids were not commonly available for laboratory infections of gordiids. However, over the last few years, cultures of numerous species of beetles, roaches, mantids, crickets, and millipedes have been established for the exotic arthropods pet trade (McMonigle and Willis, 2000; McMonigle, 2008, 2011, 2012a,b). This recent commercial availability of diverse arthropod host species will allow for the establishment of other novel life cycles of nematomorph species in the laboratory.

To establish hairworm cultures in the laboratory, freshwater snails in the genus *Physa* should be collected with a dip-net from small (first and second order) streams. Snails can be examined for the presence of gordiid cysts by removing their shells. To aid in removing the snail body from the shell, snails should be frozen in a small container of water. Once thawed, the body of the snail can then be easily removed from the shell with forceps. Next, the soft tissue of each snail should be flattened to a few millimeters in thickness between two microscope slides, one of the microscope slides is than removed, the snail tissue is covered with a cover slip and examined with a compound microscope at 100–1000× total magnification for the presence of cysts. If present, cysts should be identified to genus and or clade based on size, folding pattern, and the presence or absence of visible spines, according to Szmygiel et al. (2014).

To isolate gordiid cysts from snails for arthropod infections, the soft tissue should be macerated with a razor blade. Small portions of the soft tissue from a single snail containing gordiid cysts can then be fed to laboratory or commercially reared arthropods of the appropriate species that have been starved for 24 h. Once exposed to the Nematomorphs,

the arthropods should be maintained in species-specific cages with an appropriate substrate and hiding places and provided species-specific food and water ad libitum. Starting at 4 weeks postexposure, individual arthropods should be placed in containers of water daily to allow matured worms to exit. In most cases, mature worms will exit the arthropod host within 30–60 s of the arthropod being placed in water (Hanelt and Janovy, 2004a; Hanelt et al., 2012; Bolek et al., 2013a). Arthropods should not be left in water for more than a few minutes; and if no worms emerge on a particular day, individual arthropods should be returned to their cages and re-submerged on following days until no more worms emerge. Once emerged, adult worms should be separated by sex and maintained in Stender dishes or plastic bottles partially filled with aged tap water and an aerator. Individual male and female worms should be placed together and allowed to mate. Once males deposit a sperm drop on the posterior end of the female individual males should be removed. Depending on the gordiid species, females will deposit egg strings in the water column or on air hoses within days to weeks of mating. To prevent fungal growth, egg strings should be rinsed in a dilute bleach solution and then isolated in aged tap water in Stender dish or plastic bottles partially filled with aged tap water and aerated (Hanelt and Janovy, 1999). Over a period of 2–4 weeks, the egg strings will change in color from white to brown as larvae develop (Hanelt and Janovy, 2004a; Bolek et al., 2013a). Hatched larvae will congregate on the bottom of containers.

To isolate gordiid larvae for paratenic host infections, hatched larvae can be concentrated by swirling the Stender dish or plastic bottle water-larvae mixture, collecting the larvae with a Pasture pipette, and placing them in vials partially filled with aged tap water and capped. Within days to weeks of larva hatching, a suspension of approximately 100–200 larvae can be then pipetted into 1.5-mL well plates filled with 1 mm of aged tap water. To each well, a single laboratory reared snail or another type of paratenic host is then added. Snails should be exposed to the larvae mixture for 48 h, then removed and maintained in 1.5 L jars filled with aerated aged tap water with a calcium gravel substrate. Snails should be fed a diet of frozen lettuce and Tetra Mint® fish food ad libitum and examined for gordiid cysts over a period of 4 weeks postexposure. Fully developed cysts will contain a tightly folded larva surrounded by a clear 'halo like' cyst wall. Once developed, cysts in snail tissue can be frozen or used fresh to infect arthropod definitive hosts.

Specimen Preparation

For hairworm identification, adult free-living worms can be preserved in 70% ethanol for morphological work and 95–100% ethanol for molecular work (Schmidt-Rhaesa, 2002b; Bolek et al., 2010; Hanelt et al., 2012). For proper morphological identification, SEM is preferred (Schmidt-Rhaesa, 2002b). However, morphological characteristics can be observed using light and DIC microscopy using oil

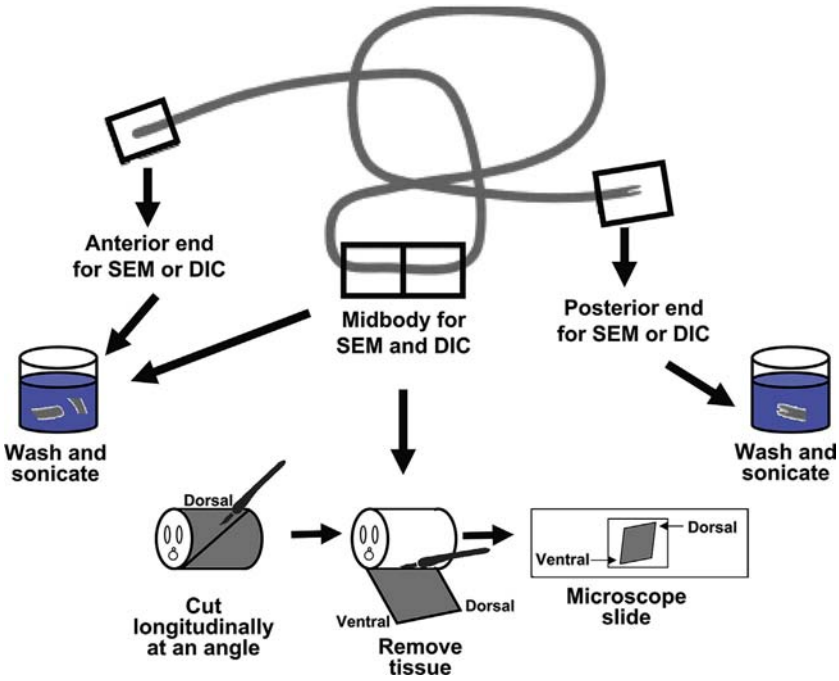

FIGURE 15.16 Protocol for the preparation of adult free-living gordiids for scanning electron microscopy (SEM) and differential interference contrast (DIC) microscopy. *Modified after Schmidt-Rhaesa, 2002b and Salas et al. (2011).*

immersion objectives (1000×) if SEM is not available. Before processing for morphological work, specimens should be washed and cleaned ultrasonically for a few minutes in water mixed with a few drops of Clinique rinse-off eye makeup solvent (Clinique Laboratories, New York, USA) followed by rinsing with distilled water (Salas et al., 2011). Once worms are washed, the anterior, posterior, and midbody sections are cut with a scalpel (Figure 15.16). Approximately 5–10 mm sections of the anterior and posterior regions should be cut and prepared for SEM or DIC. Males of some genera, such as *Gordionus*, possess specialized cuticular structures called adhesive warts, which are used for species identification and are located some distance anteriorly to the cloacal opening (Begay et al., 2012). Therefore, it is critical that long enough segments of the posterior region are cut to observe these structures. For the midbody regions, two 5–10 mm long pieces are cut, one for SEM observations and the other for light or DIC microscopy. For SEM, all pieces are dehydrated in a graded ethanol series, then critically point dried, sputter coated, and examined using standard SEM protocols (Bolek et al., 2010, 2013a). To obtain information about the dorsal and ventral side of the cuticle for light and DIC microscopy, the midbody section is observed with a dissecting microscope. Usually, on one or both of the cut sides, the ventral nerve cord, the intestine, and/or gonads are visible, which allows for easy identification of the dorsal and ventral side. Once the dorsal and ventral sides are determined, the cuticle on the midbody region is cut diagonally along the lateral side with a scalpel (Figure 15.16). The internal tissue is then removed using a scalpel with a curved blade or a razorblade. The internal tissue should be saved in 95–100% ethanol for molecular work. The remaining and clean cuticle is then placed on a microscope slide and the position of dorsal and ventral sides can be reconstructed on the spread cuticle. A few drops of glycerol or glycerin are added and the preparation is covered with a cover slip for light or DIC observations. To aid in observing areoles and other cuticular structures, a portion of the cuticle may be folded before glycerol is added. Permanent slides can be prepared by transferring the cuticle into glycerol jelly, covering it with a covers slip, and sealing it with Canada balsam, Permount™, or clear fingernail polish. Voucher specimens and/or types should be deposited in museum collections and any tissues preserved in 95–100% ethanol for molecular work or resulting sequences should be deposited in appropriate museums and/or GenBank. Additionally, vouchers of any field collected and/or laboratory infected paratenic and/or definitive hosts should be deposited in museum collections.

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