

SEASONAL OCCURRENCE, MORPHOLOGY, AND OBSERVATIONS ON THE LIFE HISTORY OF *GORDIUS DIFFICILIS* (NEMATOMORPHA: GORDIOIDEA) FROM SOUTHEASTERN WISCONSIN, UNITED STATES

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ABSTRACT: A total of 584 adult nematomorphs, *Gordius difficilis*, was collected from 2 man-made ponds and their overflow stream in southeastern Wisconsin. Ponds were surveyed throughout the year, but all free-living worms were found during July–August of 1996, July–September of 1997, and June–August of 1998. Overall sex ratio was male biased; however, sex ratio was variable during different months. Observations during 1998–2000 indicated that worms mated within 24–48 hr of emergence from their hosts and began laying eggs by mid-August, continuing until mid-October. Eggs with well-developed larvae were recovered during October and November. Encysted larval nematomorphs were recovered from aquatic and semiaquatic invertebrates (gastropods, earthworms, and insects), whereas developing hairworms were found in terrestrial European ground beetles *Pterostichus melanarius*. It is hypothesized that semiaquatic invertebrates may serve as intermediate/paratenic hosts in this system and are preyed upon by terrestrial carabid beetles, thus completing the life cycle. In addition, scanning electron microscopy observations of *G. difficilis* add previously unreported observations on intraspecific variation in body length, cuticle morphology, and gametes of this species. This is the first report of *G. difficilis* from Wisconsin as well as the first report of this species from *P. melanarius* and aquatic and semiaquatic invertebrates.

In 1996, a large population of a rare North American hairworm, *Gordius difficilis* (Montgomery, 1898) Smith (1994), was located in southeastern Wisconsin. The natural history and ecology of nematomorphs, also known as gordian or horsehair worms, is poorly known (Chandler, 1985; Poinar, 1991a, 1991b; Schmidt-Rhaesa, 1997a). Larvae are most commonly reported as obligate parasites of orthopteran and coleopteran insects, whereas adults occur in aquatic habitats and do not feed (May, 1919; Schmidt-Rhaesa, 1997a). Adult worms often occur in small numbers; little information exists on their general biology and on variation in morphology among populations.

Gordius difficilis was initially described from a single male from North Carolina as *Gordius aquaticus difficilis* (Montgomery, 1898). Montgomery (1907) later suggested that this taxon was a form of *Gordius robustus* Leidy 1859. Smith (1994) recently redescribed this species using scanning electron microscopy (SEM) from a collection of 16 male and female specimens from Massachusetts. This species differs from *G. robustus* in having an iridescent cuticle, white in females and yellowish-brown in males and, in general, is smaller in size and thickness than *G. robustus*. Males possess a parabolic ridge anterior to the cloaca containing a discrete row of hairlike structures. Both sexes lack a dark ring behind the head as seen on *G. robustus* and possess distinct areoles. Recently, Hanelt and Janovy (2000) reported *G. difficilis* from a vivid metallic ground beetle *Chlaenius prasinus* from western Nebraska. No other information is available on its basic biology; the present study provides some of this information. In this paper, the seasonal occurrence and observations on the life history of *G. difficilis* from southeastern Wisconsin are reported. Also provided are previously unreported observations on cuticle morphology, gametes, and hosts of this species.

MATERIALS AND METHODS

Horsehair worms were collected during July and August 1996 at 2 man-made ponds in a woodland lot in Waukesha County, Wisconsin (42°11.78'N, 88°21.65'W). Four ponds and their small (10.4 m by 1.4 m) overflow streams were then surveyed for free-living nematomorphs every 2–4 wk from June to December of 1996 and March to December of 1997 and 1998. Once worms were observed, ponds and streams were searched 1–3 times per week in 1997 and daily in 1998. The overflow stream and adjacent ponds were searched for up to an hour or until no additional worms could be found. In 1996–1998, worms were taken to the laboratory, washed, placed in distilled water along with leaves and sticks collected from the stream, and observed for courtship behavior. Worms collected in September 2000 were placed in stream water along with leaves and sticks and observed for egg deposition. Placing worms in distilled water along with leaves and sticks in 1996–1998 decreased fungal growth on the animals but did not appear to cause any behavioral differences compared with worms maintained in stream water in 2000. Hairworms were stored at room temperature in a 75.7-L aquarium or in bottles in the refrigerator at 4°C. Once observations were completed, worms were separated, killed, and preserved in 95% ethanol. All worms from 1996 and 1997 collections were sexed under a stereomicroscope and the length measured to the nearest 1 mm. Worms from the 1998 collection were sexed and allowed to release eggs and, therefore, were not measured.

For light microscopy studies, the anterior, middle, and posterior 5–10 mm sections of cuticle from male and female worms were cut, dehydrated through ethanol, cleared in xylene, mounted in Canada balsam, and keyed to species according to Montgomery (1898). Additionally, adult specimens of male and female *G. difficilis* were compared with male and female *G. robustus* collected from Indiana (Bolek, 2000). For SEM studies, the midbody cuticle and anterior and posterior extremities of 7 males and 4 females were cut, fixed in 2% gluteraldehyde in 0.2 M Sorenson's phosphate buffer (pH 7.4) and 2% osmium tetroxide, dehydrated in a graded series of ethanol, critical-point dried using liquid CO₂, mounted on aluminum stubs, coated with gold palladium, and examined with a Hitachi S-570 Scanning Electron Microscope.

Aquatic and terrestrial insects were sampled by dip net and fall traps and collected by hand under boards during May and October of 1997 and May–September of 1998. Insects were fixed in 10% formalin or 95% ethanol, keyed to family or species, and examined for developing larval hairworms. In 1999 and 2000, field observations were made on the occurrence of adult worms and hosts and on egg deposition by this species. Egg strings were located visually and by sifting sediments and detritus around live and decomposing adult worms. Additionally, during 2000, random samples of aquatic and semiaquatic invertebrates were collected and examined for encysted larval nematomorphs using wet mount and histological techniques. Encysted larvae were measured with the aid of an ocular micrometer. Adult male and female *G. difficilis*

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TABLE I. Sex ratio of *Gordius difficilis* individuals collected during July and August 1996, July, August, and September 1997, and June, July, and August 1998. Chi-square value significant ($P < 0.025$) for July 1996 and ($P < 0.005$) for August 1997, July 1998, and total collection.

Date	Undeter-			Sex ratio of males to females	χ^2
	Males	Females	mined		
July 1996	3	12	1	1.00:4.00	5.40
August 1996	0	6	0	0.00:6.00	NC*
July 1997	61	65	3	1.00:1.07	0.12
August 1997	146	41	3	3.56:1.00	58.96
September 1997	4	1	0	4.00:1.00	NC
June 1998	44	55	0	1.00:1.25	1.22
July 1998	98	36	0	2.72:1.00	28.68
August 1998	4	1	0	4.00:1.00	NC
Total	360	217	7	1.65:1.00	35.44

* Not calculated because of small sample size.

specimens as well as snails, tipulids, and oligochaetes were deposited in the Harold W. Manter Laboratory collection, University of Nebraska State Museum, Lincoln, Nebraska. (HWML 16560, 16561, 16562, 16563, 16564). The European ground beetle *Pterostichus melanarius* has been deposited and cross-referenced with the nematomorph specimens in the Entomology section of the University of Nebraska State Museum, Lincoln, Nebraska.

RESULTS

Seasonal occurrence and morphology

A total of 581 free-living adult worms was collected during this study, 19 in 1996, 324 in 1997, and 238 in 1998. Additionally, 3 worms were removed from the intestines of 2 green frogs *Rana clamitans* collected from a third, downstream pond in 1996. Although ponds and streams were surveyed throughout the year (except winter), free-living adults were found only during July and August of 1996, July, August, and September of 1997, and June, July, and August of 1998. Field observations in 1999 revealed that free-living worms appeared on 31 May and persisted until 18 November. Of the 4 ponds and adjacent stream sampled throughout 1996–1998, most worms, 558 (96%), were collected from the stream, whereas 15, 7, and 3 were collected from 2 small ponds and from 2 green frogs collected downstream, respectively. Overall male-to-female sex ratio was male biased (1.65:1.00); however, sex ratio was variable during different months (Table I). More females were observed during July 1996, July 1997, and June 1998 than during August 1996, August and September 1997, and July and August 1998. More males were present during August 1997 and July 1998 than during July or September 1997 and June and August 1998.

A statistically significant difference existed between mean lengths of male and female worms (1-tailed *t*-test, $P < 0.05$). On average, female worms were longer ($n = 119$, mean \pm SD 245 ± 66 mm, range 105–405 mm) than males ($n = 212$, 188

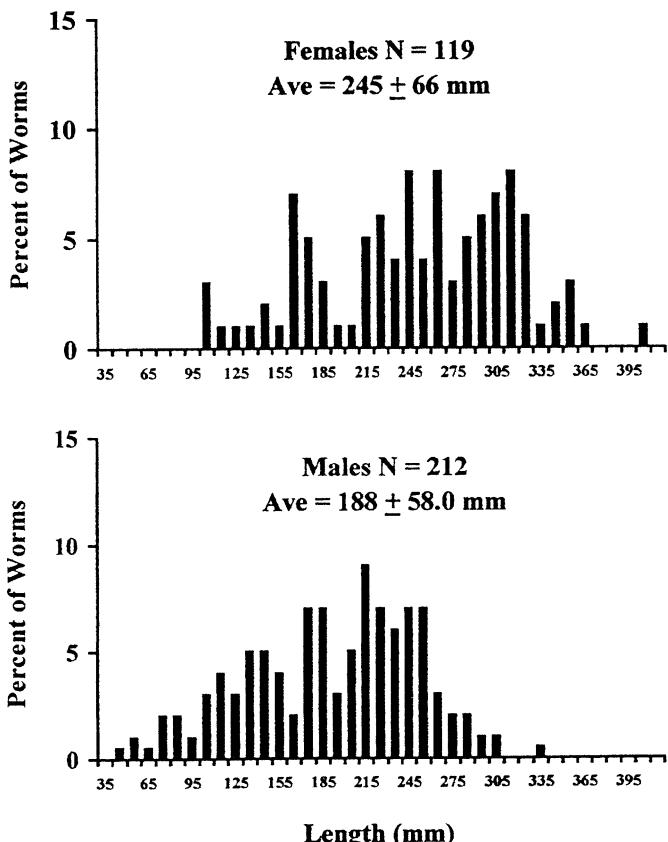
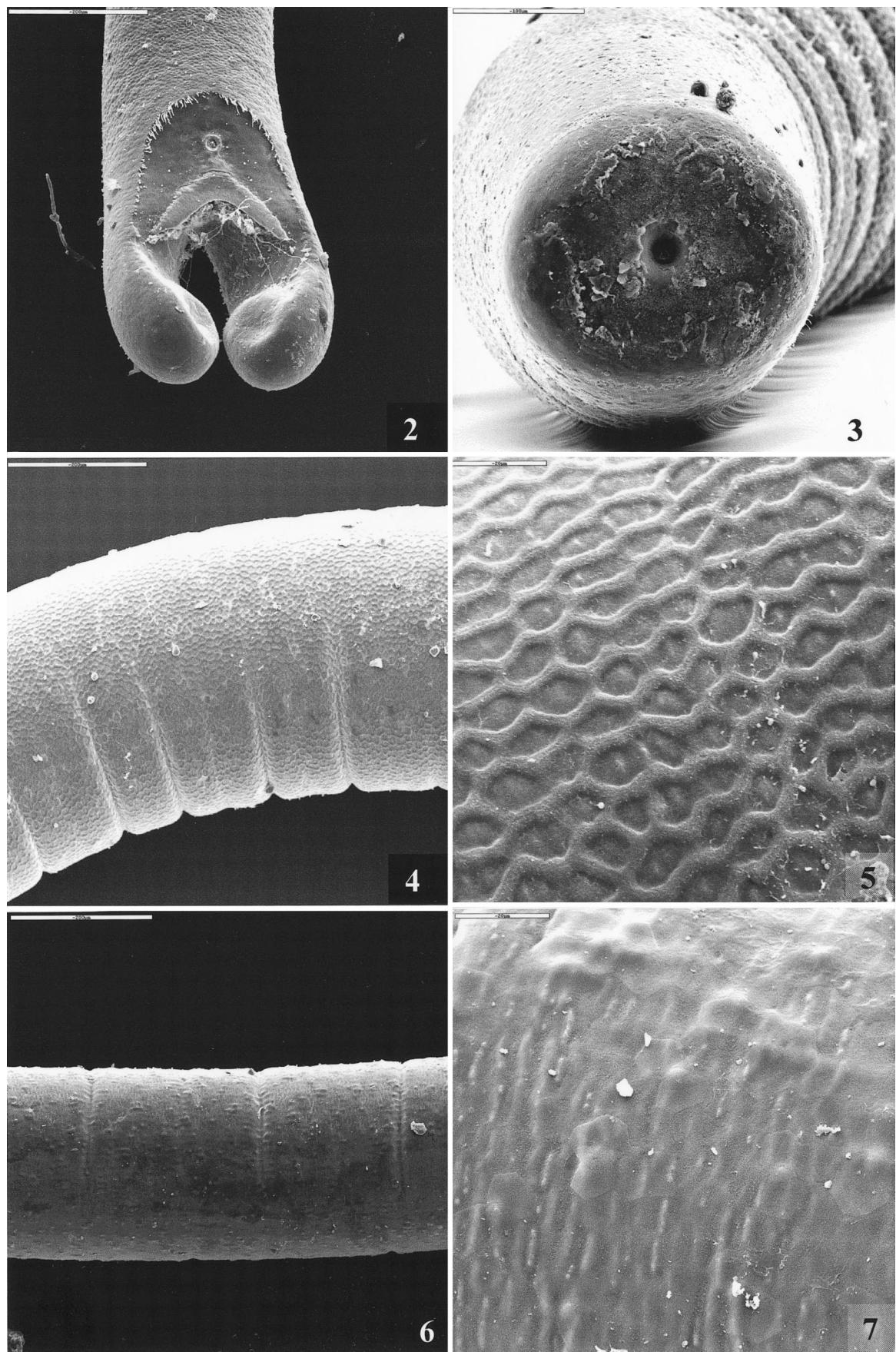
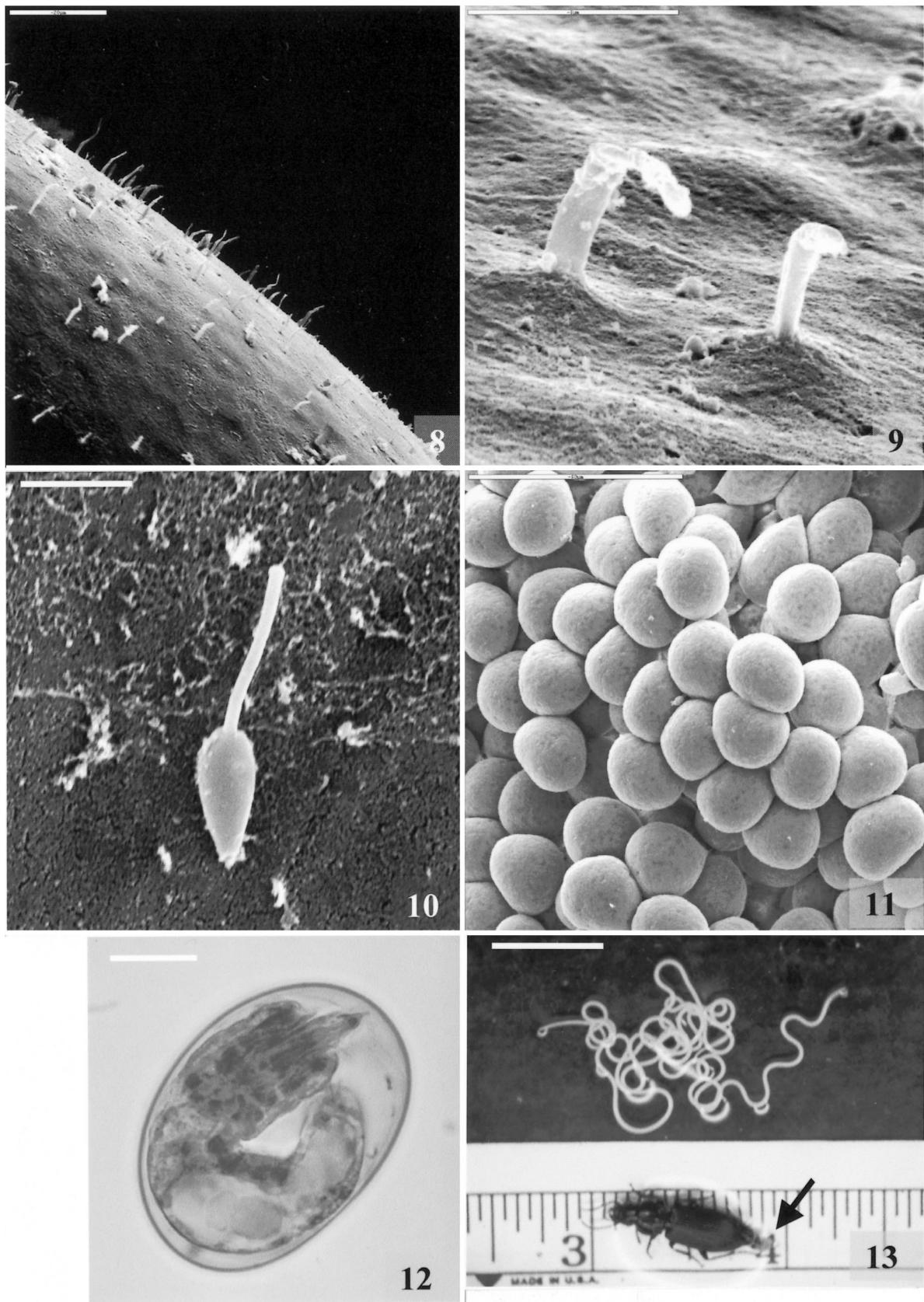


FIGURE 1. Frequency distribution of lengths of adult male and female *Gordius difficilis* collected from southeastern Wisconsin.

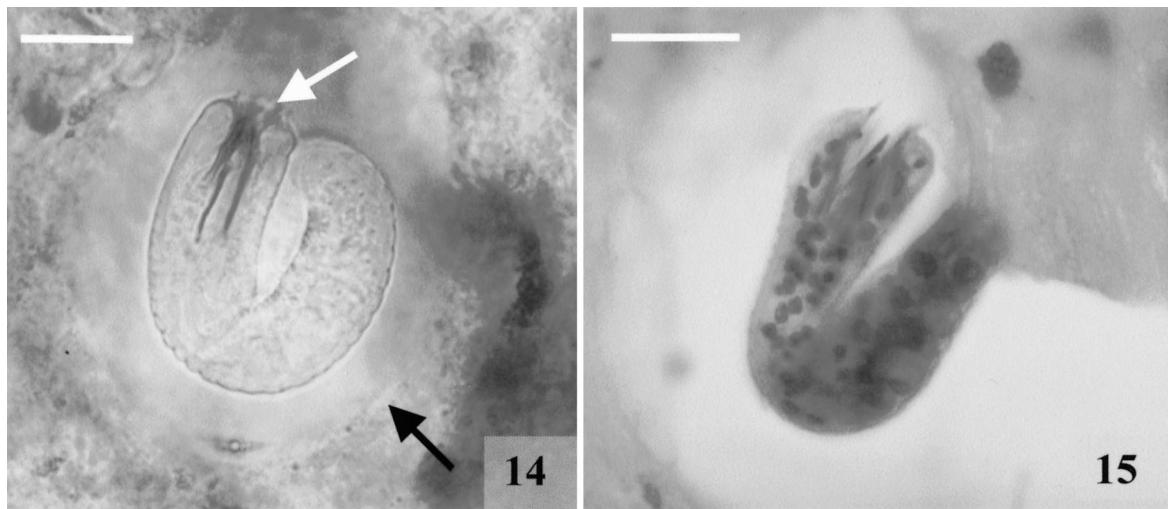
\pm 58 mm, 45–335; Fig. 1). Female worms were white in color, whereas males were white to yellowish-brown. No dark collar was present on the anterior region of the specimens; the only dark pigment observed was on the posterior region of the females' cloaca and on the postcloacal crescent of males. SEM revealed that males possessed 2 terminal lobes and a postcloacal crescent. The cloacal opening was situated in a broad nonareolar field bordered by an inverted U-shaped ridge containing hairlike structures (Fig. 2). Females possessed a round posterior end, cylindrical in shape, with a terminal cloaca (Fig. 3). Sexual dimorphism also existed in midbody cuticle of male and female worms. Females were thicker than males and possessed well-formed, raised midbody areoles (Figs. 4, 5). In male specimens, areoles were variable, with some males having poorly developed areoles in the midbody region (Figs. 6, 7). Additionally, patches of hairlike structures occurred on the anterior and posterior regions of male and female worms (Fig. 8). These hairlike structures were variable in distribution, being clumped or occurring singly (Fig. 9).

FIGURES 2–7. Scanning electron micrographs of the cuticle of *Gordius difficilis*. 2. Posterior region of male; note inverted U-shaped ridge containing hairlike structures. Scale bar = 200 μ m. 3. Posterior region of female, cylindrical in shape with a terminal cloaca. Scale bar = 100 μ m. 4. Midbody cuticle of female. Scale bar = 200 μ m. 5. Enlargement of female showing details of well-developed areoles. Scale bar = 20 μ m. 6. Midbody cuticle of male. Scale bar = 200 μ m. 7. Enlargement of male showing details of poorly developed areoles. Scale bar = 20 μ m.





FIGURES 8–13. Light and scanning electron micrographs of the cuticle, gametes, and host of *Gordius difficilis*. **8.** Posterior region of female containing hairlike structures. Scale bar = 20 μm . **9.** Enlargement showing individual hairlike structures. Scale bar = 5 μm . **10.** Spermatozoa on the surface of a female cuticle. Scale bar = 2 μm . **11.** Eggs in a sectioned female worm. Scale bar = 50 μm . **12.** Egg containing a well-developed larva with stylet. Scale bar = 12.5 μm . **13.** Recently emerged female worm from *Pterostichus melanarius*; note the white color of the female and the distended abdomen of beetle (black arrow). Scale bar = 12 mm.



FIGURES 14–15. Light photomicrographs of encysted larva of *Gordius difficilis*. **14.** Encysted larva in the musculature of a physid snail; note the well-developed stylet (white arrow) and clear halolike cyst wall (black arrow). Scale bar = 15 μm . **15.** Cross section of an encysted larva in the musculature of the body cavity of an oligochaete; no host reaction to the cyst is seen. Scale bar = 15 μm .

Mating behavior and egg deposition

When male and female worms were placed together they immediately formed copulatory tangles. Males were observed to move up and down the female's body with their coiled posterior end. Once its bifurcated tail was in proximity with the female cloaca, the male deposited a mass of sperm that remained on the female for up to 2 wk. Spermatozoa released by males were rod-shaped and lacked a flagellum. Spermatozoa deposited on females possessed a round end and a rod-shaped end and were 5–6 μm in length (Fig. 10). In daily collections in 1998, 84 of 92 females (91%) contained a mass of sperm on the posterior end. In 1997, when worms were placed in large aggregations of 50 or more individuals, males were observed to deposit a mass of sperm on both the posterior and anterior ends of the females. This was only observed on 1 individual in the 1998 field collection, with most worms being found singly or in aggregations of 2–3 individuals.

In the laboratory, only 1 female from the 1998 collection deposited eggs over a period of 4 wk, whereas 35 worms collected during September 2000 continued releasing eggs for up to 4 wk, until observations were discontinued. Eggs were released in strings approximately 0.5 cm in length. SEM revealed that eggs inside female worms were smooth and spherical in shape, 18–20 μm by 15–16 μm in size (Fig. 11). Deposited eggs were $47 \pm 2.90 \mu\text{m}$ (range 38–52.5, $n = 20$) by $31.78 \pm 2.14 \mu\text{m}$ (range 27.5–37.5, $n = 20$) and had a thin smooth shell but did not show development. Field observations in 1999 and 2000 indicated that female and male worms buried and congregated 1–5 cm down in the gravel and detritus layers of the stream. Females began laying eggs in the gravel by the second week of August 1999 and September 2000 and continued until mid-October, when spent females with a clear flattened body were commonly found. Egg strings collected during October 1999 and November 1999 and 2000 contained developed larvae (Fig. 12). No free-living adult worms were found after 18 November 1999 and 23 November 2000.

Hosts and encysted larval nematomorphs

A total of 188 aquatic and terrestrial insects from 4 orders and 5 families was collected. Included were 33 Trichoptera (Limnephilidae), 22 Diptera (Tipulidae), 131 Coleoptera (9 Dytiscidae and 122 Carabidae), and 2 Orthoptera (Gryllacrididae). Only carabid coleopterans were infected with developing larval hairworms. Three species of carabids were collected, *Poecilus lucublandus* ($n = 2$) and *Patrobus longicornis* ($n = 6$), both native species, and *P. melanarius* ($n = 114$), an introduced European species. Of these, 4 female and 1 male *P. melanarius* were infected with a single *G. difficilis* each, whereas 1 female beetle was infected with 2 worms (mean intensity = 1.2 ± 0.41). Hairworms in the process of emerging from *P. melanarius* were observed on 2 occasions; in both cases, worms emerged through the terminal segment of the abdomen (Fig. 13). Additionally, the 2 green frogs collected had carabid beetles, which had nematomorphs in their stomach contents.

Random samples of semiaquatic and aquatic invertebrates were collected from the ponds and stream and examined for encysted larval nematomorphs during March–July of 2000. Included were 50 Trichoptera (Limnephilidae), 28 Diptera (25 Chironomidae, 3 Tipulidae), 25 Gastropoda (Physidae), 20 Amphipoda (Gammaridae), 20 Isopoda (Asellidae), 20 oligochaetes (Lumbricidae), 10 Coleoptera (9 Dytiscidae, 1 Hydrophylidae), and 1 Megaloptera (Sialidae). Of these, 24 of 25 gastropods, with a mean intensity of 13.75 ± 10.28 (range 2–35), 12 of 20 oligochaetes, with a mean intensity of 27.1 ± 29.55 (range 1–76), and 1 of 3 tipulids, with an intensity of 1, were infected with encysted larval nematomorphs. Encysted larvae were tightly coiled, with an average length of $36.68 \pm 3.05 \mu\text{m}$ and width $21.50 \pm 1.26 \mu\text{m}$ (range 32.11–41.99 by 20.00–22.50, $n = 20$; Fig. 14). Histological examination revealed that all encysted larvae were located in the musculature and hemocoel of the tipulid and musculature, body cavity, and gut of the gastropods and earthworms (Fig. 15). Additionally, no host reactions to the cyst were observed in any of the invertebrates examined (Figs. 14, 15).

DISCUSSION

Although a number of nematomorph species have been reported from Wisconsin (Watermolen and Haen, 1994), *G. difficilis* is reported here for the first time in Wisconsin. It appears to be a species that is collected from small woodland streams during June–September and has been reported from 4 states including Massachusetts, Nebraska, North Carolina, and now, Wisconsin (Montgomery, 1898; Smith, 1994; Hanelt and Janovy, 2000).

Sex ratios of horsehair worms in field studies are often skewed from the expected 1:1 ratio (Pennak, 1989; Watermolen and Haen, 1994; Poulin, 1996), but few studies exist on the temporal sex ratio changes of these worms. In a seasonal study of a New Zealand species, *Gordius dimorphus*, Poulin (1996) found that the sex ratio shifted from a male-biased collection during November to a female-biased collection during December. He gave the following hypotheses for the temporal change in sex ratio observed: (1) male nematomorphs do not survive as long as females, and females accumulate in the stream as summer progresses; and (2) length of time for development and emergence of male and female worms may differ in the definitive host. In his study, average length of male worms was greater than that of females. The present observations on the temporal sex ratio and sexual size dimorphism of *G. difficilis* differ from those of Poulin (1996). The present collections became predominantly male-biased over the emergence period of the worms, and female worms on average were longer than males, both sexes being larger than previously reported by Smith (1994) and Hanelt and Janovy (2000). Because the present collections occurred 1–3 times per week in 1996 and 1997 and daily in 1998, it is believed these data represent true emergence periods from the host. It remains unclear why nematomorphs exhibit skewed sex ratios, but the present field observations in 1999 and 2000 indicate that as the season progressed, more female worms were found in the detritus and gravel layers of the stream. It may be that female worms are present at the study site but are more cryptic during the later part of the year. These observations suggest that the sex ratio of free-living adults may be variable over time and space and, therefore, single collections may not be reliable predictors of the overall sex ratio of nematomorph populations.

Little is known about the intraspecific variation of characters in most nematomorph species (Schmidt-Rhaesa, 2001). In species of *Gordius*, the presence and distribution of areoles and hairlike structures is poorly known (Smith, 1994; Schmidt-Rhaesa, 1997a) even though a number of studies base species identification on the presence and distribution or absence of these structures (Smith, 1994; Schmidt-Rhaesa et al., 2000). These data confirmed sexual dimorphism in size and midbody cuticle morphology of *G. difficilis*, with females being larger and having well-formed and elevated areoles. Female-biased size dimorphism is characteristic of most nematomorphs that have been examined in large numbers (Watermolen and Haen, 1994), but too little is known about most species to make any general conclusions. More importantly, these data suggest that overall size, areole structure, and the presence of supracuticular structures may be variable within a species and among populations. In an SEM study of *G. difficilis*, Smith (1994) noted

very tightly packed areoles in the midbody region of males. However, in the Wisconsin specimens, areoles were variable, with some males having poorly developed areoles in the midbody region. Additionally, in the present study, patches of hairlike structures occurred on the anterior and posterior regions of male and female worms. In the original description of females and redescription of males (Smith, 1994), no supracuticular structures were present on female worms, whereas only males possessed tubercles.

The present observations on the mating behavior and gamete morphology of *G. difficilis* support previous reports published on nematomorphs (May, 1919; Dorier, 1930; Valvassori et al., 1988; Schmidt-Rhaesa, 1997b). In 1998, when worms were collected daily, 91% of all females possessed a sperm drop on the posterior region of the body. Therefore, these worms most probably copulate during or within 24–48 hr after emergence from the host. Also, consistent with the study of Poulin (1996) on *G. dimorphus*, field observations in the present study indicate that most *G. difficilis* occurred singly, with a few worms being found in groups of 2–3 individuals, and no worms were observed copulating in the field. Because of the unequal sex ratio observed in most field studies and the poor swimming ability of adult worms, females may be a limiting resource to males during certain periods, and thus it may be advantageous for worms to begin mating during or as soon as they emerge from the host. Interestingly, these observations indicate that female worms deposited egg strings buried 1–5 cm deep in the detritus and gravel layer of the stream, which may be important in the transmission dynamics of the aquatic larvae. Earthworms, tipulid larvae, and aquatic snails that contained encysted larval nematomorphs were also commonly found in the detritus layers and around the edges of the stream where egg strings were commonly collected.

In general, hosts and host specificity of developing nematomorphs for most species are poorly known, but, in the majority of cases, arthropods are commonly reported for developing larval nematomorphs (Cappucci, 1976; Poulin, 1995; Schmidt-Rhaesa, 1997a).

Only terrestrial European ground beetles *P. melanarius* were infected with developing larvae of *G. difficilis* at the study site in Wisconsin. Although some of the sample sizes were too small to detect the presence of *G. difficilis* in these potential hosts, they do represent the relative abundance of these insects present at this study site. These results are similar to those of Hanelt and Janovy (2000), who reported *G. difficilis* emerging from vivid metallic ground beetles *C. prasinus* in western Nebraska, but from no other invertebrates collected at their site.

Gordian worms are commonly recovered from carabid beetles, but most records of *Pterostichus* spp. infected with nematomorphs are from Europe (Cappucci, 1976; Schmidt-Rhaesa, 1997a; de Villalobos et al., 1999). The only report from North America of *P. melanarius* infected with nematomorphs is from Ontario, Canada, by an unidentified nematomorph species (Tomlin, 1975). Therefore, the current report is a new host record for *G. difficilis*. The low prevalence and intensities observed at our study site are characteristic of field studies of population structure of other nematomorphs infecting terrestrial hosts (Poulin, 1995).

Until recently, studies on nematomorph life cycles and transmission of aquatic free-living larval hairworms to terrestrial

insect hosts have been in a state of confusion (Pennak, 1989). A study by Hanelt and Janovy (1999) on *G. robustus* indicates that these hairworms must pass through an encysted stage in an invertebrate host before developing to an adult stage in an orthopteran host. Most studies commonly refer to invertebrates that contain encysted nematomorphs as intermediate hosts. However, life cycles, including developmental changes of encysted nematomorphs, for most species are unknown, and it is unclear if these may serve as intermediate, paratenic, or both types of hosts at particular locations. We therefore refer to invertebrates with encysted larvae as intermediate/paratenic hosts, until these life cycle stages are elucidated.

Currently, no information exists on how terrestrial carabid beetles become infected with aquatic larval stages of hairworms, but it is hypothesized that semiaquatic invertebrates may serve as hosts that bridge an ecological gap between an aquatic environment and a terrestrial environment (Schmidt-Rhaesa, 1997a). Several investigations on the ecology of *P. melanarius* have shown that these beetles are generalist feeders, being primarily carnivorous, with more than 38 different prey groups being reported in their diet (Pollet and Desender, 1985). Of the invertebrates sampled at the Wisconsin study site, aquatic snails were the ones most commonly infected with encysted larval nematomorph stages. The use of aquatic snails as intermediate/paratenic hosts infecting terrestrial beetles is questionable, but the possibility exists that semiaquatic tipulids and earthworms or another intermediate/paratenic host may serve this role. Infected earthworms, tipulids, and *P. melanarius* specimens were collected in the detritus layer along the margins of the stream and, therefore, overlap ecologically. Both earthworms and tipulids have been reported as prey items in the diet of larvae and adult *P. melanarius* (Pollet and Desender, 1985). More recently, studies on the diet of *P. melanarius* in Europe indicate that up to 36% of field-collected beetles contained earthworm remains (Symondson et al., 2000). Also, population studies in Canada indicate that there is a relationship between *P. melanarius* and earthworm abundance (Niemela et al., 1997), indicating that earthworms may be an important prey item in the diet of these beetles. These observations suggest that earthworms may play a role as intermediate/paratenic hosts for *G. difficilis* using *P. melanarius* for development.

Finally, our removal of most of the reproductive free-living *G. difficilis* specimens from this population during 1996–1998 did not appear to cause a decline in adult worms emerging during 1999–2001 (M.G.B., unpubl. data). In a recent study on physid snails as sentinels of freshwater nematomorphs, Hanelt et al. (2001) reported that the occurrence of gordiids, on the basis of the cyst stage, was much more common than finding adult worms suggested. They indicated that 70% of 50 sites sampled contained encysted nematomorphs, compared with finding adults at only 1 of these sites sampled over a period of 3 yr. We hypothesize that relatively long-lived (1–2 yr) aquatic and semiaquatic invertebrates such as snails, oligochaetes, and tipulids that do not show any host reaction to the nematomorph cyst (Figs. 14, 15) may serve as reservoirs for nematomorph populations. Nematomorph cysts in intermediate/paratenic hosts may have a relatively longer life span than the adult worms; therefore, the removal of most if not all the adult worms from our population over a period of 3 yr had no apparent effect on adult worms emerging in subsequent years.

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