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SURVIVAL OF LARVAL AND CYST STAGES OF GORDIIDS (NEMATOMORPHA) AFTER EXPOSURE TO FREEZING

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ABSTRACT: Hairworms infect terrestrial arthropods and are 1 of the most understudied groups of parasites. Recently, life cycles of 2 gordiids (*Paragordius varius* and *Paragordius obamai*) have been domesticated in the laboratory. We tested the viability of laboratory reared and post-frozen larval and cyst stages of the North American gordiid, *P. varius*, frozen at –80 C for 7 mo, and the viability of field collected and post-frozen cysts of the African (*P. obamai*) and North American (*P. varius*) gordiid frozen at –20 C for 2 mo. All snails exposed to post-frozen or control *P. varius* larvae became infected with cysts, and there was no significant difference in prevalence or mean intensity of cysts among control or experimental snail groups. As with larvae, no significant differences were observed in prevalence or mean intensity of emerging worms from crickets infected with post-frozen or control *P. obamai* or *P. varius* cysts. All female *P. obamai* and *P. varius* worms from control and post-frozen cyst infections laid eggs and larvae hatched from some of these eggs. Survival and cyst formation of *P. varius* larvae exposed to different combinations of drying and/or freezing temperatures indicated that gordiid larvae have the ability to survive drying and freezing, but survival significantly increases during freezing at lower temperatures. The major contribution of our study is the demonstration that gordiid larval and cyst stages can survive freezing temperatures to infect and develop in the next host.

The Nematomorpha represent 1 of 3 entirely parasitic animal phyla (Hanelt et al., 2005). Two classes exist within the phylum and include the marine Nectonematida and the freshwater Gordiida (Schmidt-Rhaesa, 1997). Nematomorphs, or hairworms, are 1 of the most understudied groups of animals (Poulin, 1998), but they have recently gone through a scientific renaissance (Hanelt et al., 2005). Within the last 15 yr, work on gordiids has focused on understanding their distribution (Schmidt-Rhaesa, 1997; Bolek, 2000), taxonomy (Schmidt-Rhaesa, 2002; Schmidt-Rhaesa et al., 2003, 2008; de Villalobos et al., 2009; Bolek et al., 2010; Chiu et al., 2011; Begay et al., 2012), life history (Hanelt and Janovy, 1999, 2003, 2004a, 2004b; Hanelt et al., 2012), and ecology (Bolek and Coggins, 2002; Hanelt, 2009; Sato et al., 2011). Emphasis has also been placed on using nematomorphs as model systems to study the biochemical mechanisms of host manipulation (Thomas et al., 2003; Biron et al., 2005) and as indicators of environmental stressors (Zanca et al., 2007; Achiorno, de Villalobos, and Ferrari, 2008; Achiorno, Ferrari, and de Villalobos, 2008; Achiorno et al., 2009, 2010).

The life cycles of 2 species of gordiids (*Paragordius varius* and *Paragordius obamai*) have been domesticated in the laboratory, allowing investigators to test hypotheses about gordiid host interactions, ecology, and evolution (Hanelt and Janovy, 2004a; Hanelt et al., 2012). The diecious North American *P. varius* has been maintained in culture for over a decade (Hanelt and Janovy, 2004a). More recently, the first parthenogenetic gordiid, *P. obamai*, was discovered in Kenya and is currently maintained in culture in our laboratories (Hanelt et al., 2012). Studies on these domesticated hairworms indicate that life cycles of gordiids involve 5 distinct life stages, including (1) egg strings, (2) free-living larvae, (3) parasitic cysts, (4) parasitic juveniles, and (5) diecious or parthenogenetic free-living adults (Hanelt and Janovy, 2004a, 2004b; Hanelt et al., 2012). Juvenile gordiids are obligatory parasites of terrestrial arthropods, whereas a variety of animals serve as paratenic hosts for the cyst stage (Hanelt et al., 2001; Bolek and Coggins, 2002; Hanelt and Janovy, 2003, 2004b). However, many questions still remain on how hairworm life cycles are maintained in nature. For example, we do not fully understand how gordiid larval and cyst stages survive winter in the environment for gordiid species distributed in geographical areas with a temperate climate. One thought is that larvae and/or cysts of these species must somehow overwinter by suspending their life cycles during cold periods and infect arthropod hosts during the spring, when temperatures are more suitable for their potential hosts.

Although we have made significant progress in maintaining gordiid life cycles in the laboratory, one major problem with continuous cultivation of gordiids in the laboratory is the logistical burden of maintaining colonies of numerous species of hosts for infections, especially when the appropriate numbers of these hosts are not available when infected stages of gordiids are available. One possible solution in overcoming these difficulties is to develop techniques in suspending life cycle stages of gordiids until colonies of the appropriate hosts are available for infection. Recent studies by Achiorno, Ferrari, and de Villalobos (2008) indicate that larvae of the Argentinean gordiid, *Chordodes nobilii,* remain infective to their paratenic hosts after exposure to temperatures of –3 C for 48 hr, suggesting that larvae and other gordiid life stages may be resistant to freezing. The purpose of this study was to determine if freezing larvae and/or cysts of 2 species of gordiid from North America and Africa for long periods of time could successfully produce infections in their respective paratenic and definitive hosts. Additionally, because a number of invertebrate taxa have been reported to survive drying and/or freezing (Stanley et al., 1994; Holmstrup et al., 2002), we wanted to determine if *P. varius* larvae were resistant to drying or freezing at low (–70 ± 2 C) or high temperatures (–30 ± 3 C) or a variety of these conditions. Simply placing gordiid larvae and cysts into a freezer does not allow differentiation between the effects of supercooling or internal freezing of the organism, and either or both of these effects may occur during the freezing treatment used in this study.

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MATERIALS AND METHODS

Gordian worm maintenance and original larva source

Physa gyrina snails infected with cysts of the North American diecious gordiid, *P. varius*, were collected with a dip-net from Millville Creek (40.9935°N, 96.5655°W), Lancaster County, Nebraska, stored in an 18.5-L bucket with stream water and a portable air pump, and brought back to Oklahoma State University. Snails were examined for the presence of cysts as previously described by Hanelt et al. (2001) within a week of collection. Briefly, shells were removed from each snail and the soft tissue was crushed between 2 slides. Each snail was infected naturally with 0–78 cysts (average = 16), determined by dissecting 10 snails. To isolate gordiid cysts from snails for cricket infections, the soft tissue was macerated with a razor blade. A small portion of the soft tissue from a single snail containing approximately 0–15 gordiid cysts was fed to a 24-hr-starved commercially reared cricket *Acheta domesticus* or laboratory reared crickets *Gryllus firmus*. The field isolated *P. varius* infections included 30 exposed crickets of each species. All crickets used were ultimate or penultimate developmental stage, and were maintained for 24 hr in exposure cages until they ingested their snail tissue. Postexposure crickets were maintained in groups of 15 in covered plastic shoeboxes (35 × 25 × 15 cm) with a paper-towel substrate and a 4-cm^2 egg carton for a hiding place. Crickets were watered and fed by a 50-mm plastic centrifuge tube filled with aged tap water with a cotton ball at the end, and ad libitum supply of Purina® Puppy Chow® dog food. After 30 days postexposure (DPE) all crickets exposed to *P. varius* were placed in 110 × 35–mm stender dishes partially filled with aged tap water and allowed to release worms. Worms were allowed to emerge; male and female *P. varius* worms were isolated as pairs in stender dishes with aerated aged tap water and allowed to mate. Female *P. varius* worms were allowed to release egg strings, and larvae were collected for infections (see below).

Infectivity of laboratory reared and post-frozen *Paragordius varius* larvae

Hatched larvae from 3 female worms were concentrated by swirling the stender dish water–larvae mixture. All hatched larvae were collected and placed in 1.5-dram vial filled with aged tap water. For larvae isolation the vial was gently turned 5 times in order to create homogenized slurry of larvae in aged tap water. By removing a drop of this suspension with a Pasteur pipette, approximately 100–200 larvae were pipetted into 48 1.5-ml well plates filled with 1 mm of aged tap water. To each well a single laboratory reared *P. gyrina* snail was added. These control snails were allowed to feed on the larvae mixture for 48 hr, then removed and maintained in 1.5-L jars filled with aerated aged tap water with a calcium gravel substrate. Snails were fed a diet of frozen lettuce and Tetra Min® fish food and examined for *P. varius* cysts over a period of 4 wk postexposure (WPE). The soft tissue of 10 randomly selected snails was examined for the presence of infection (prevalence) and number of formed cysts (intensity). To test the ability of *P. varius* larvae to survive being placed in a freezer and form cysts, hatched larvae from the same 3 female worms were concentrated and collected with a Pasteur pipette and placed in 5 1-ml plastic Eppendorf tubes filed with 0.75 ml of aged tap water. Eppendorf tubes were frozen at –80 ± 2 C for 7 mo. Frozen larvae from each of the 5 Eppendorf tubes were thawed at room temperature (25 ± 2 C), placed in a single 100 × 20–mm glass Petri dish with aged tap water and thoroughly mixed. Approximately 100–200 post-frozen larvae were pipetted into 48 1.5-ml well plates filled with 1 ml of aged tap water. To each well a single laboratory reared *P. gyrina* snail was added. Maintenance and examination of snails for the presence of cysts exposed to post-frozen *P. varius* larvae followed the same procedures as for control snail.

Infectivity of laboratory reared and post-frozen *Paragordius varius* cysts

Remaining control snails infected with cysts from non-frozen *P. varius* larvae were divided into 2 groups. Twenty snails were used for control infections of laboratory reared crickets, *G. firmus*, as previously described. To test the ability of *P. varius* cysts to survive being placed in a freezer and developing into adult worms in cricket hosts, a second group of 18 control snails infected with cysts from non-frozen *P. varius* larvae were divided into 2 groups of 9 snails each. Groups of 9 snails were then placed in 2 1-ml plastic Eppendorf tubes filed with 0.75 ml of aged tap water and frozen at –80 ± 2 C for 7 mo. Frozen snails from each of the 2 Eppendorf tubes were thawed at room temperature (25 ± 2 C) and the shells were removed. Cyst isolation and cricket infections followed the same procedures as for the original isolation of *P. varius*. Two groups of 70 *G. firmus* crickets were exposed to post-frozen and control cysts of *P. varius*, respectively, and exposed crickets were maintained as previously described.

Infectivity of field collected and post-frozen cysts of *Paragordius obamai* and *Paragordius varius*

In order to test if field collected cysts of gordiid species were viable after they were placed in a freezer at higher temperatures (–20 C), we collected snails infected with *P. obamai* and *P. varius* cysts from Africa and North America, respectively. *Biomphalaria pfeifferi* snails infected with the parthenogenetic *P. obamai* were collected from Kasabong stream, in Nyanza province, Kenya (0.1519°N, 34.3355°E). A sample (n = 10) of snails dissected revealed an average of 15 *Paragordius* cysts per snail (range: 0–42). Three field collected snails were used for control infections of commercially reared *A. domesticus*; 3 other field collected snails were frozen in a single Eppendorf tube at –20 ± 3 C for 2 mo. For cricket infections, frozen snails were thawed and processed as previously described. Two groups of 20 *A. domesticus* crickets were exposed to post-frozen or control cysts of *P. obamai* as previously described, and all crickets were maintained as previously described. Additionally, 15 *P. gyrina* snails were collected from Deer Creek, Payne County, Oklahoma (36.1165°N, 96.64767°W), and all snails were frozen in an Eppendorf tube at –20 ± 3 C for 2 mo. Field collected *P. gyrina* snails contained 19.8 *P. varius* cysts per snail (range: 0–74). For cricket infections, field collected and frozen snails were thawed and processed as previously described. Thirty *A. domesticus* crickets were exposed to post-frozen snail tissue collected from Oklahoma and all exposed crickets were maintained as previously described.

Thirty DPE all *A. domesticus* crickets exposed to post-frozen cysts of *P. varius*, and 55 DPE all *A. domesticus* crickets exposed to control or post-frozen cysts of *P. obamai* were placed in 110 × 35–mm stender dishes partially filled with aged tap water and allowed to release worms. All exposed crickets were placed in stender dishes daily for 5 min over a period of 3 wk until no more worms emerged or the cricket died. Worms were allowed to emerge, and the number of worms and worm sex was recorded. Presence of infection (prevalence) and number of emerging worms (intensity) was recorded for each species of control and experimental crickets. Male and female *P. varius* worms were allowed to mate, and individual female *P. varius* and *P. obamai* worms from control and/or post-frozen infections were observed for egg string deposition. Egg strings were allowed to develop in stender dishes with aerated aged tap water and the viability of the larvae for each species of worms from control and post-frozen cyst infections was confirmed based on larval ability to hatch and move.

Prevalence and mean intensity was calculated according to Bush et al. (1997). The chi-square test for independence was calculated to compare differences in prevalence among control and experimental groups of snails and crickets, and Student's *t*-test was used to compare differences in mean intensity among control and experimental groups of snails and crickets. Approximate *t*-tests were calculated when variances were heteroscedastic (Sokal and Rohlf, 1981).

Survival of laboratory reared *P. varius* larvae at different drying and freezing treatments

In order to determine if gordiid larvae were also resistant to drying, to being placed in a freezer at low temperatures, to higher temperatures, or to a variety of these conditions, larvae of *P. varius* maintained in culture at the University of New Mexico and originally isolated from snails from Millville Creek, Lancaster County, Nebraska, were exposed to 9 different drying and freezing treatment trials. A slurry suspension of hatched larvae in aged tap water collected from multiple *P. varius* female worms was concentrated as previously described. Ten to 11 replicates of approximately 250 ± 100 *P. varius* larvae were used per treatment group. For the control treatment group a suspension of *P. varius* larvae was pipetted into 10 1.5-ml well plates filled with 1 mm of aged tap water and to each well a single laboratory reared *P. gyrina* snail was added. For the freezing treatments, larvae in 2 treatment groups were suspended in 1 ml of aged

TABLE I. Prevalence and mean intensity ±1 SD values for cysts in *Physa gyrina* snails and emerged adult worms from *Gryllus firmus* cricket exposed to post-frozen or control (non-frozen) larvae or cysts of *Paragordius varius*.

		Post-frozen (no. infected/no. exposed survivors)	Control (no. infected/no. exposed survivors)	Statistic	*P*
Snail	Prevalence	100% (10/10)	100% (10/10)	$\chi^2 = 0.00$	= 0.99
	Mean intensity ±1 SD (range)	27 ± 28 (3–78)	61 ± 49 (5–180)	$t = -1.94$	= 0.07
Cricket	Prevalence	13% (7/55)	12% (8/65)	$\chi^2 = 0.005$	= 0.99
	Mean intensity ±1 SD (range)	3.3 ± 5.6 (1–16)	3.3 ± 1.8 (1–3)	$t = 0.02$	= 0.98

tap water in Eppendorf tubes and frozen at –30 ± 3 C for 15 or 30 days, whereas larvae in 2 additional treatment groups were suspended in 1 ml of aged tap water in Eppendorf tubes and frozen at –70 ± 2 C for 15 or 30 days. Larvae in 4 additional treatment groups were allowed to dry at room temperature. For the drying treatments, dry larvae in 2 treatment groups were frozen at –70 ± 2 C for 15 or 30 days, whereas dry larvae in 2 treatment groups were maintained at room temperature for 15 or 30 days. All dried larvae were resuspended in aged tap water, and all frozen larvae were thawed at room temperature 15 or 30 days post treatment, and placed in 1.5-ml well plates filled with 1 mm of aged tap water with an individual laboratory reared *P. gyrina* snail. Exposed snails were maintained and all surviving snails were processed for *P. varius* cysts as previously described.

We report prevalence, mean abundance ±1 standard deviation (SD), and percent of larval survival in the drying and freezing treatment groups compared to the control group. Because variances were heteroscedastic, the Kruskal–Wallis test and the Kolmogorov–Smirnov 2-sample tests were used to compare differences in mean abundance among control and treatment groups of exposed and surviving snails (Sokal and Rohlf, 1981).

RESULTS

Infectivity of laboratory reared and post-frozen *Paragordius varius* larvae

All snails exposed to post-frozen or control *P. varius* larvae became infected with cysts, and there was no significant difference in prevalence or mean intensity among control or experimental groups of snails (Table I). Encysted larvae from post-frozen and control infected snails were tightly folded and contained protruding spines on the pre-septum characteristic of the genus *Paragordius*. All cysts from post-frozen and control snail infections contained a clear halo around the tightly coiled larva typical of gordiid cysts (Fig. 1A), indicating that frozen larvae remained viable.

Infectivity of laboratory reared and post-frozen *Paragordius varius* cysts

No significant differences were observed in prevalence or mean intensities of emerging worms from surviving crickets infected with post-frozen or control *P. varius* cysts (Table I). Worms emerged from crickets 36–41 DPE. A total of 14 male and 16 female worms emerged from crickets exposed to post-frozen cysts, whereas 12 male and 13 female worms emerged from crickets exposed to control cysts. All worms emerged from their cricket hosts within 30 sec of placing an infected cricket into a stender dish filled with aged tap water. All female worms from crickets exposed to control or post-frozen cysts mated and began releasing egg strings within 1–3 days of copulating (Fig. 1B, C). Of those, egg strings from 9 female worms produced from post-frozen cysts and 6 female worms produced from control cysts developed viable larvae that hatched (Fig. 1D).

Infectivity of field collected and post-frozen cysts of *Paragordius obamai* and *Paragordius varius*

No significant differences were observed in prevalence or mean intensities of emerging worms from surviving crickets infected with post-frozen or control cysts of *P. obamai* ($\chi^2 = 3.75$, $P > 0.05$; $t' = 0.39$, $P > 0.05$). Of the 2 groups of *A. domesticus* crickets exposed to cysts of *P. obamai*, 10 control and 20 experimental crickets survived long enough for worms to emerge. Of those, 4 (40%) control crickets were infected with 5 female *P. obamai* (1.25 ± 0.5; range 1–2); whereas 2 (10%) experimental crickets were each infected with a single female *P. obamai*. Worms emerged from crickets 66–73 DPE. All female worms began producing egg strings within 12–48 hr of emergence from their cricket hosts. Three to 4 wk post oviposition, 2 female worms from control cricket infections produced larvae that developed and hatched; whereas all other developing egg strings from control and experimental cricket infections were destroyed by a fungus infection but all were in the process of developing. A total of 44 *P. varius* (15 males and 26 females) emerged from 27 of 30 *A. domesticus* crickets exposed to post-frozen cysts collected from Deer Creek, Payne County, Oklahoma. All female *P. varius* began releasing egg strings within 1 to 3 days of copulation and all egg strings produced viable larvae.

Survival of laboratory reared *P. varius* larvae at different drying and freezing treatments

Seven to 11 snails per treatment group survived long enough to be examined for *P. varius* cysts (Fig. 2). Prevalence ranged from a high of 100% for control larvae, larvae frozen in water at –30 C for 15 or 30 days, and larvae frozen in water at –70 C for 30 days, to a low of 22% for larvae dried at room temperature for 30 days (Fig. 2). Differences also existed in the percent of larval survival and cyst formation in snail hosts based on treatment group. Compared to control larvae, 86% of larvae frozen at –70 C for 15 days formed cysts in snails, whereas only 0.15% of larvae dried at room temperature for 30 days formed cysts in snails (Fig. 2). Mean abundance of *P. varius* cysts was highest in the control group (225 ± 85) and lowest in larvae dried at room temperature for 30 days (0.3 ± 0.7; Fig. 2). The Kruskal–Wallis analysis of variance revealed significant differences in mean abundance of *P. varius* cysts per treatment group (H corrected = 57.286; $P < 0.0001$). The Kolmogorov–Smirnov 2-sample tests did not show significant differences ($P > 0.05$) between the control larval treatment group and larvae frozen in water at –70 C for 15 or 30 days, or larvae frozen in water at –70 C for 15 or 30 days and larvae frozen in water at –30 C for 15 or 30 days. However,

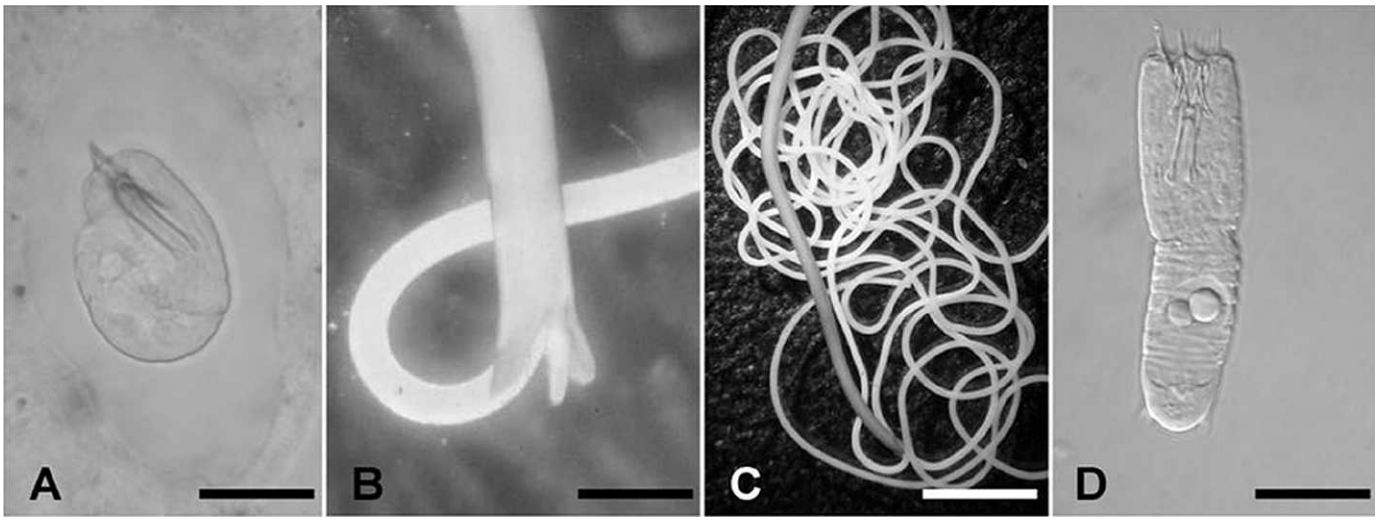

FIGURE 1. Life cycle stages of *Paragordius varius* recovered from experimentally infected snail and cricket hosts exposed to post-frozen larvae or cysts frozen for 7 mo at – 80 C. (**A**) Mature cyst in *Physa gyrina* infected with post-frozen larvae. Note the characteristic clear halo around the tightly folded larva and prominent spines on the pre-septum. Scale bar = 15 μm. (**B**) Posterior end of free living adult female *P. varius* in the process of releasing egg strings. Scale bar = 1 mm. (**C**) The same female *P. varius* with entire egg string released. Note the female is tan in color and the egg strings are white. Scale bar = 1 cm. (**D**) Hatched larva of *P. varius*. Note the characteristic granules of the pseudo-intestine. Scale bar = 20 μm.

significant differences existed ($P < 0.03$) among treatment groups for other combinations (Fig. 2).

DISCUSSION

The major contribution of our study is the demonstration of the survival of larvae and cyst stages of gordiid species to supercooling and/or freezing at low temperatures and the ability of these life stages to infect and develop in the next host. Two recent studies have examined the effects of temperature on gordiid species (Achiorno, Ferrari, and de Villalobos, 2008; Zanca et al., 2007). Zanca et al. (2007) demonstrated that eggs of *C. nobilii* were capable of embryonic development at a temperature of 5 C. In their study, developing larvae in eggs maintained at a temperature of 5 C developed slowly and remained in the egg, whereas developing eggs maintained at a temperature of 22 C developed much more quickly and hatched. These 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. More recently, Achiorno, Ferrari, and de Villalobos (2008) examined egg development and egg, larval, and adult survival of *C. nobilii* in response to extreme temperatures. In their study Achiorno and colleagues 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 hr survived freezing. Their study showed that mosquito larvae, *Aedes aegypti*, exposed to post-frozen *C. nobilii* larvae and examined 72 hr PE contained gordiid larvae. They indicated that cysts never develop in their mosquito gordiid system. However, studies on 5 other species of gordiids indicate that 5–14 DPE are required for cysts to form after larvae are ingested by snail and other invertebrate paratenic host (Hanelt and Janovy, 2003, 2004b; Bolek et al., 2010; Hanelt et al., 2012). Our study is the first to demonstrate that post-frozen gordiid larvae are capable of producing cyst stages in their paratenic hosts. In addition, our study is the first to demonstrate that post-frozen cysts are capable of producing adult worms that are capable of mating and producing viable larvae. Taken together, these observations indicate that supercooling and/or freezing larval and cyst stages of gordiids but not their egg strings or adults is a reliable technique of maintaining a typical gordiid life cycle in the laboratory.

Field studies on some species of gordiids in North and South America indicate that female worms oviposit during times of the year when egg, larval, and/or cyst stages have the opportunity to experience temperatures of –4 to –10 C (Bolek and Coggins, 2002; Zanca et al., 2007; Achiorno, Ferrari, and de Villalobos, 2008). However, it is unlikely that in nature larval and cyst stages of the African gordiid, *P. obamai*, and the North American gordiid, *P. varius*, experience such rapid cooling and/or freezing temperatures (–20 to –80 C) and for such lengths of time (2–7 mo) as in our study. Unfortunately, we have no comparative developmental worm data for crickets exposed to post-frozen cysts at –80 C for the African *P. obamai* and the North American *P. varius* because all our crickets exposed to control and post-frozen cysts of *P. obamai* at –80 C died. However, examination of a single cricket 47 DPE to *P. obamai* post-frozen cysts at frozen at –80 C for 7 mo indicated that the cricket was infected with a single immature female worm. These data suggest that both North American and African gordiid species can survive rapid cooling and/or freezing at low temperatures. One possible explanation for the ability of cyst stages of both North American and African gordiids to survive freezing for long periods of time could indicate an evolved response of gordiids to glacial periods. Because gordiids have a worldwide distribution and fossil records of gordiids have been reported dating back to 100–110 million yr from the Early Cretaceous (Poinar and Buckley, 2006), it is not unreasonable to suspect that some species of gordiids have experienced glacial events during their evolutionary history. However, we must be cautious about such conclusions, because in our study we did not differentiate if

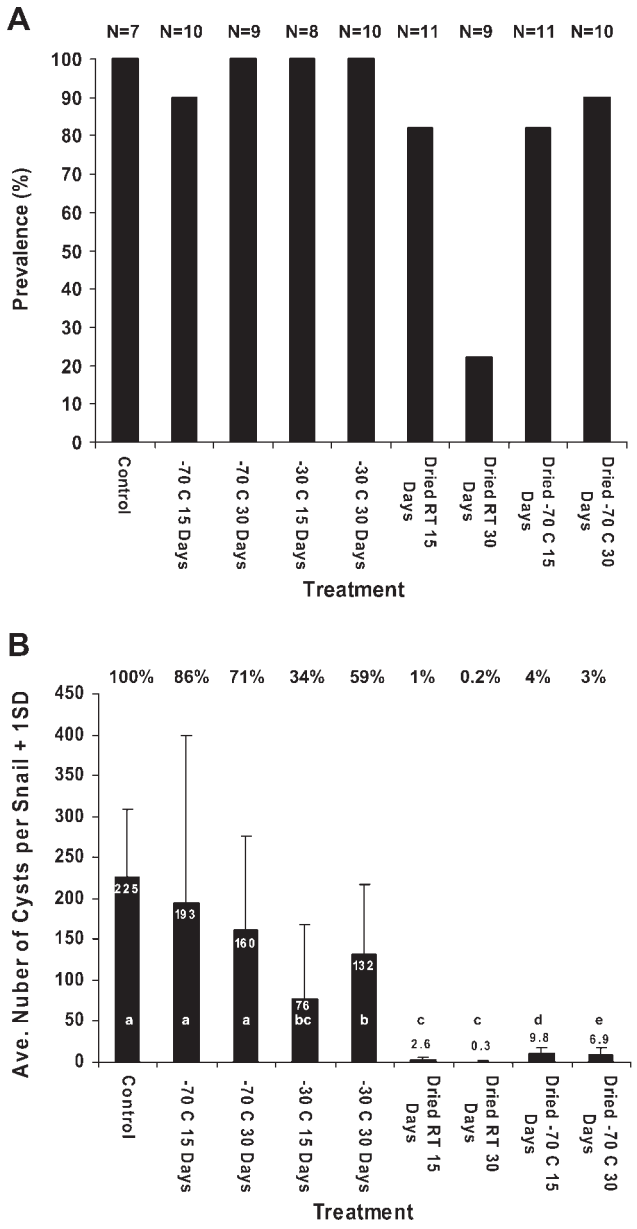

FIGURE 2. The ability of *Paragordius varius* larvae to form cysts in snail hosts after exposure to different combinations of drying and freezing conditions. (**A**) Prevalence of *P. varius* cysts in *Physa gyrina* snails exposed to *P. varius* larvae from 9 different treatments. N = number of surviving and examined snails per treatment group; RT = room temperature. (**B**) Mean abundance +1 SD of *P. varius* cysts in *P. gyrina* snails exposed to *P. varius* larvae from 9 different treatments. % = percent of larval survival and cyst formation compared to control infections. RT = room temperature. Lower case letters represent significant differences among group combinations ($P < 0.03$) for all significant differences.

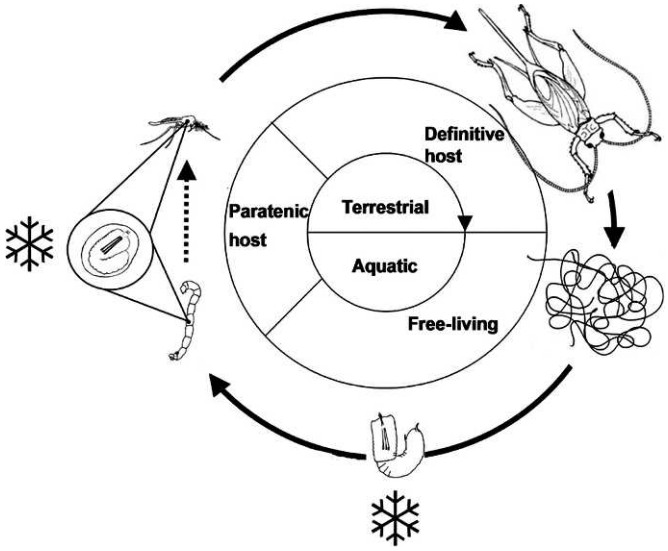

FIGURE 3. General gordiid life cycle. Snowflake symbols indicate gordiid life cycle stages that remain viable and survive super cooling and/or freezing.

gordiid larvae and cysts were exposed to just sub-zero temperatures or whether ice formed internally in these non-adult stages, as has been demonstrated for a number of insect and nematode taxa (see Lee, 2010; Wharton, 2011). Importantly, our study on the survival of *P. varius* larvae exposed to different combinations of drying and/or freezing temperatures indicate that gordiid larvae have the ability to survive both drying and freezing conditions, but survival dramatically increases during rapid freezing at low temperatures. In our study, survival and cyst formation of *P. varius* larvae rapidly frozen at low temperatures of –70 C was not significantly different from the control group, whereas larvae frozen at higher temperatures (–30 C), dried and frozen, or dried had a significant lower survival and cyst formation than control larvae and/or larvae frozen at –70 C. These observations suggest that rapid cooling and not necessarily the ability of gordiid larvae to freeze increases the survival of gordiid larvae.

Our discovery of the ability of gordiid larval and cyst stages to survive supercooling and/or freezing for long periods of time will allow us the flexibility to maintain gordiid life cycles continuously in the laboratory (see Fig. 3). More importantly, our discovery of the ability of gordiid cysts from field collected snails to survive freezing and produce viable adult worms suggests that this technique will enable researchers to establish other nematomorph life cycles from around the world in the laboratory. Recent studies on the distribution of gordiids by using cysts indicate that the nematomorph cyst stage is much more commonly encountered in the environment than previously thought. In a 3-yr study of 50 streams in a 2,000-km^2 prairie ecosystem in Nebraska, free-living adult gordiids were found at 1 stream, whereas cysts were recovered from aquatic snails at 35 streams, indicating that cysts are the most encountered gordiid stages in nature (Hanelt et al., 2001). Additional studies by Hanelt and Janovy (2002), Bolek and Coggins (2002), and Bolek et al. (2010) on cyst morphology of 5 North American and African species of gordiids from 3 genera suggest that cyst stages of gordiids may be useful in generic separation. Based on these observations Hanelt et al. (2012) collected *B. pfeifferi* snails infected with cysts of an unidentified *Paragordius* sp. from Kenya, a country for which no gordiid records exist. After genus level identification of the cyst, and exposing the appropriate group of laboratory reared arthropods, the first parthenogenetic species of gordiid, *P. obamai*, was discovered. Taken together, the ability of genus level identification of gordiid cysts, the ease

of collecting gordiid cysts in nature, and the ability of these cysts to survive freezing for a relatively long period of time will make it easier for nematomorph researchers to culture colonies of appropriate hosts and establish other gordiid life cycles in the laboratory from around the world.

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