

Cold hardiness in two helminth parasites of the freeze-tolerant wood frog, *Rana sylvatica*

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Abstract: Wood frogs, *Rana sylvatica*, tolerate the freezing of their body tissues as an overwintering adaptation. Various parasites infect wood frogs of northern populations, but nothing is known about their strategies for surviving within a frozen host. We examined winter-conditioned wood frogs that were experimentally exposed to 0°C (nonfrozen) or -4°C (frozen) to determine whether endoparasites survive the freezing of their host. We found no differences in the prevalence or intensity of adult lungworms *Rhabdias ranae* (Nematoda) or of larvae of an unidentified species of digenetic trematode between these groups. Live individuals of both species were observed in hosts that recovered from experimental freezing at -4°C. Within the host, *R. ranae* also tolerated exposure to -5°C, a temperature near the lower limit of survival of the wood frog. Cryostage experiments showed that, like its host, *R. ranae* was highly susceptible to inoculative freezing and tolerant of the freezing of its tissues. *Rhabdias ranae* frozen in vitro in the presence or absence of 250 mM glucose, the cryoprotectant used by wood frogs, recovered from a 10-h exposure to -4°C. The mechanism of cold tolerance used by larval trematodes was not investigated; however, we hypothesize that freeze avoidance by supercooling may be important in this species. Freeze-tolerant anurans, such as the wood frog, are useful subjects in the study of coevolution of thermal tolerance in parasites and their host.

Résumé : La Grenouille des bois, *Rana sylvatica*, tolère le gel de ses tissus, une adaptation qui lui permet de survivre à l'hiver. Divers organismes parasitent les grenouilles des populations nordiques, mais les stratégies qu'ils utilisent pour survivre chez un hôte gelé n'ont jamais été déterminées. Nous avons suivi des grenouilles des bois acclimatées à l'hiver et exposées expérimentalement à des températures de 0°C (non gelées) et -4°C (gelées) pour déterminer si leurs endoparasites survivent au gel. Nous n'avons pas constaté de différences entre les groupes quant à la fréquence ou la gravité des infections causées par des nématodes adultes parasites du poumon *Rhabdias ranae* ou des larves d'une espèce non identifiée de trématode digène. Des individus vivants des deux espèces ont été trouvés chez des hôtes qui ont récupéré après un gel expérimental à -4°C. Chez l'hôte, *R. ranae* s'est avéré également tolérant à une exposition à -5°C, près du seuil inférieur de survie de la Grenouille des bois. Des expériences de gel ont démontré que, comme son hôte, *R. ranae* est très sensible à un gel provoqué par inoculation, et tolérant au gel de ses tissus. Des *R. ranae* surgelés in vitro en présence ou en l'absence de 250 mM de glucose, la substance cryoprotectrice utilisée par les grenouilles, ont récupéré après une exposition de 10 h à -4°C. Nous n'avons pas cherché à identifier le mécanisme qui permet la tolérance au froid chez ces grenouilles, mais nous posons en hypothèse que le recours à la surfusion pour échapper au gel est un phénomène important chez cette espèce. Les anoures qui sont tolérants au froid, tels la Grenouille des bois, représentent des sujets expérimentaux d'une grande utilité pour l'étude de la coévolution de la tolérance thermique chez les parasites et chez leurs hôtes.

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Introduction

Wood frogs (*Rana sylvatica*) range in North America from northern Georgia to within the arctic circle, in Alaska

(Tyning 1990). Commonly found in deciduous and coniferous woodlands, their habitats also include grasslands and tundra. Wood frogs have a life-span of at least 3 years and breed explosively in ephemeral ponds, bogs, or streams in late winter (Tyning 1990; Harding and Holman 1992). Little is known of their life history outside of the breeding season. However, some reports indicate that wood frogs overwinter in shallow depressions in the forest floor, overlain by stones or organic detritus, and possibly snow (Kirton 1974; Licht 1991). Observations of these frogs hibernating in such exposed sites have fostered considerable interest in the cold hardiness of this species.

Wood frogs survive freezing episodes during which the body temperature falls as low as -6°C and up to 65% of the water in their body freezes (Storey and Storey 1988). Wood frogs can recover fully after remaining frozen for more than a month (Layne et al. 1998). Physiological responses pro-

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moting freeze tolerance in the wood frog, and in other freeze-tolerant anurans, include the production of cryoprotectant and the redistribution of water within the body (see Costanzo et al. 1993 and references therein). During the early stages of freezing, glucose is synthesized from glycogen reserves in the liver and circulated throughout the body. Concentrations of glucose in the blood and other tissues increase rapidly and may ultimately exceed 300 mM. Concurrently, up to 50% of the bulk water in organs is translocated to and freezes within the coelom and lymphatic spaces. This protective dehydration of organs concentrates glucose and reduces the amount of ice forming within tissues. Both cryoprotective responses are triggered coincident with the onset of freezing.

Although it is clear that wood frogs are adapted to survive exposure to subzero temperatures, nothing is known about the cold hardiness of their parasites. It is possible that some parasites are eliminated by cold exposure of the host. For example, chilling of bumblebees (*Bombus terrestris*) parasitized by conopid flies delays development and may prevent pupation of the parasitoid larvae (Muller and Schmid-Hempel 1993). Alternatively, parasites of freeze-tolerant anurans may have evolved mechanisms for coping with the extreme conditions within the frozen host. However, little is known about the cold tolerance of even the most common parasites, and very few studies have investigated the cold hardiness mechanisms of parasites in living hosts (Wharton 1999). Presumably, these mechanisms include freeze tolerance or freeze avoidance via supercooling, a phenomenon in which the body fluid remains liquid at temperatures below its equilibrium freezing point (Humble and Ring 1985; Hance and Boivin 1993; Tyrell et al. 1994).

We investigated the effect of host freezing on the prevalence (infection rate) and intensity (number of parasites in individual frogs) of two species of helminth parasites, the nematode lungworm *Rhabdias ranae* and a digenetic trematode, which commonly infect wood frogs (Muzzall and Peebles 1991; Yoder and Coggins 1996). We also used cryomicroscopic techniques to investigate the possible mechanisms by which *R. ranae* might survive within the frozen host.

Materials and methods

Study animals

Eighteen wood frogs (*R. sylvatica*), including juveniles and adults of both sexes, were collected in Clinton County, southern Michigan, in mid-October 1998. They were shipped under refrigeration by express courier to Miami University, randomly assigned to one of three treatment groups, and placed in transparent plastic boxes containing damp moss. We conditioned the frogs for overwintering by exposing them to a 12:4°C thermoperiod and corresponding 10 h light : 14 h dark photoperiod inside a programmable environmental chamber (1–35X, Percival, Boone, Iowa). Three weeks later, frogs were exposed to 4°C in darkness to acclimate them to winter conditions. Frogs were kept in this manner until midwinter and then examined for parasites or used in experiments.

We also studied adult male wood frogs ($N = 9$) indigenous to Adams County, southern Ohio. These frogs were collected from breeding pools in late February 1998 and kept over the summer in an outdoor enclosure erected in a wooded area at Miami University's Ecology Research Center, Butler County, southwestern Ohio. Their diet consisted of native invertebrates supplemented three times per week with crickets coated with a powdered multivitamin.

The frogs ceased feeding and became quiescent in early November. At this time they were transferred to the laboratory and placed in opaque plastic boxes containing damp moss. Frogs were held at 4°C, in darkness, until they were used in experiments in mid-February, just prior to the usual time of emergence of frogs in this Ohio population.

Freezing exposure of wood frogs

We compared the prevalence and intensity of parasitic infections in Michigan frogs subjected to freezing at -4°C ($N = 7$) or chilling at 0°C ($N = 7$), a temperature slightly above the equilibrium freezing point of -0.4°C of frog tissues (Costanzo et al. 1993). These frogs were subjected to cold exposure in early January. At this time, baseline levels of parasitic infection were determined using four additional (control) frogs taken directly from their holding boxes at 4°C .

Experimental freezing or chilling was initiated by resetting the environmental chambers in which the frogs were housed to the desired target temperature (-4 or 0°C , respectively). During cooling, temperature adjacent to the dormant frogs was monitored using single-channel temperature loggers (StowAway XTI, Onset Computer). Frogs in the -4°C chamber began to freeze at a temperature near -0.5°C , probably because they were inoculated by frozen moss (see Costanzo et al. 1999). Cooling rate during freezing was $0.16^{\circ}\text{C}/\text{h}$. Frogs were kept frozen at -4°C for 4 d and then held at 0°C for 3 d. Thereafter, these frogs, and the frogs chilled to 0°C , were exposed to 4°C inside the environmental chamber. Most of these frogs were held under these conditions for ca. 10 weeks and ultimately examined for parasitic infections in late March, the usual time of emergence in this Michigan population. However, one frog that failed to recover from freezing was examined for parasites shortly after thawing, and three frogs chilled to 0°C were euthanized 5–10 d after the treatment to provide *R. ranae* for experimentation.

We conducted an additional experiment to determine whether *R. ranae* can withstand freezing of the host at a near-lethal temperature. Ohio frogs ($N = 7$) were frozen to -5°C in mid-February following a protocol similar to that described above, except that frogs were cooled inside individual plastic cups containing damp moss. By permitting frogs to cool very slowly ($0.07^{\circ}\text{C}/\text{h}$) this arrangement promoted freezing survival at this critical temperature (Costanzo et al. 1993). After remaining frozen for 72 h, these frogs were thawed at 0°C and then held at 4°C . Frogs were euthanized 3 d after thawing and examined for *R. ranae* infections.

Assessing parasitic infections

Wood frogs were euthanized by double-pithing and examined for the presence of ectoparasites on the skin and buccal surfaces. We dissected the frogs and searched their coelom, mesenteries, and organs for cysts and free endoparasites. We focused our search on the alimentary canal (rectum, duodenum, and stomach), urinary bladder, heart and pericardium, liver and gall bladder, lungs, and kidneys. Nematodes were deemed alive if they exhibited spontaneous undulatory movement. They were fixed in acetic acid, stored in 70% ethyl alcohol, cleared in glycerol, and examined on temporary mounts. Kidneys infested with encysted trematodes were fixed with acetic acid or AFA (ethyl alcohol, formalin, acetic acid) solution and stored in 70% ethyl alcohol. We made microscopic examinations of some of the transparent cysts, prior to fixation, to determine whether the trematodes were motile.

Supercooling and inoculative freezing of *R. ranae*

We used cryomicroscopic techniques to investigate the capacity for supercooling and susceptibility to inoculative freezing of *R. ranae*. These parameters were determined from the temperatures of crystallization (T_c) of *R. ranae* cooled in the absence and presence, respectively, of external ice. Both experiments were performed on

a temperature-controlled cryostage (Linkam, BCS 192) that was mounted on a compound microscope (Olympus, BH-2). Fifteen *R. ranae* were isolated from the lungs of Michigan ($N = 1$) and Ohio ($N = 2$) wood frogs and stored at 4°C in amphibian phosphate saline (APS; 6.1 g/L NaCl, 0.15 g/L KCl, 0.88 g/L Na₂HPO₄, 0.15 g/L KH₂PO₄; 230 mosmol/kg) for up to 12 h before use in the experiments. In pilot experiments, *R. ranae* tolerated storage, without apparent ill effects, for at least 36 h.

Rhabdias ranae were prepared for use in supercooling trials by removing them from the APS bath and submerging them in mineral oil. This procedure displaced from their surface moisture that otherwise might freeze and inoculate them during cooling and also kept them from desiccating during the trial. *Rhabdias ranae* were then placed individually in a small droplet (ca. 10 µL) of mineral oil held within a quartz crucible. A coverslip was applied and the crucible was placed on the prechilled cryostage adjacent to a surface-mounted thermistor. We allowed the crucible to attain thermoequilibrium at 0°C before initiating the trial.

Supercooling trials were conducted by cooling the cryostage (1°C/min) until each specimen froze. We recorded images of *R. ranae* during cooling and freezing using an S-VHS cassette recorder (Panasonic, AG-1960) that received input from a closed-circuit camera (Panasonic, WV-CL700) mounted on the microscope. Digital readings from the thermistor were displayed by the video output; these values generally were within 0.3°C of the temperature of the specimen. Freezing of the body fluids was readily discerned as "flashing," which is the sudden darkening that occurs when ice forms internally (see Lee et al. 1993). The T_c of each specimen was taken as the lowest temperature recorded before the onset of flashing.

Inoculation trials were conducted in a similar manner except that *R. ranae* were cooled in the presence of external ice crystals intended to seed the freezing of body fluids. We placed specimens on a thin film of APS and cooled them on the cryostage until each froze. Because this film, like all small volumes of aqueous solutions, tended to supercool, we added a potent ice-nucleating agent to the APS used in this experiment. The nucleator was a killed, lyophilized preparation of the ice-nucleating bacterium, *Pseudomonas syringae* (Genencor, Rochester, N.Y.), which induces water to freeze at temperatures near -3°C. We thus ensured that *R. ranae* were in contact with seed crystals during cooling.

Tolerance of *R. ranae* to in vitro freezing

At the conclusion of each supercooling or inoculative freezing trial the cryostage was programmed so that the frozen specimen was held, for 5 min, at a temperature 1.5–2.0°C below its T_c . Subsequently, it was warmed (1°C/min) to 0°C, removed from the cryostage, and held in chilled APS for 2–6 h. We assessed the viability of *R. ranae* by warming them to room temperature and, after a 30-min period, determining whether they undulated spontaneously or could be induced to undulate with gentle mechanical stimulation.

We also investigated the ability of *R. ranae* to tolerate freezing under more naturalistic physiological conditions (i.e., slow cooling and exposure to moderate subzero temperature, elevated glucose levels, and relatively high osmotic pressure). Four *R. ranae* isolated from a (nonfrozen) Michigan wood frog in late January were placed in a microcentrifuge tube containing 1.0 mL chilled APS ($N = 2$) or APS containing 250 mM glucose ($N = 2$). These vessels were inserted into glass test tubes submerged in a refrigerated alcohol bath. Specimens were equilibrated at 0°C and then cooled (0.5°C/h) until they attained the target temperature of -4°C. The solutions were inoculated with small ice crystals to initiate freezing at ca. -1.0°C. Temperatures were recorded during cooling using copper/constantan thermocouples connected to a temperature logger (Omega Electronics, RD3752).

Rhabdias ranae were kept in the frozen solutions at -4°C for 10 h. They were then warmed to 0°C, removed from the refrigerated bath, placed in petri dishes along with their respective APS solutions, and permitted to warm to room temperature. After 30 min, we assessed viability of the specimens as described above.

Animal care and experimental procedures were approved by Miami University's Animal Care and Use Committee in accordance with guidelines established by the U.S. Public Health Service and the Canadian Council on Animal Care.

Results

Parasitic infections of wood frogs

Michigan wood frogs harbored various endoparasites but were most commonly infected by the nematode lungworm *R. ranae* and the metacercaria of an unidentified species of digenetic trematode. We found several juvenile *R. ranae* in the coelom and mesenteries of five frogs; however, most of the *R. ranae* were adults (many of which were gravid) and located in the lungs. The larval trematodes were found exclusively in the kidneys and were clustered along the ventrolateral aspect of the renal cortex. We also found the nematode *Oswaldocruzia* sp. in the alimentary tracts of four frogs. Clusters of white opaque cysts occurred in the coelomic cavities of four frogs. Some of these cysts were dissected and found to contain larval tetrathyridia (Cestoidea: Cyclophylloidea), possibly of the genus *Mesocestoides*. Unidentified protozoans were found in the gut contents of nine frogs. No parasites were found on the skin or buccal surfaces, heart or pericardial sac, liver, gall bladder, or urinary bladder.

Wood frogs showed a high prevalence and intensity of infection by *R. ranae* (Table 1). Each of the four control frogs examined in early January harbored adult *R. ranae* in their lungs (mean intensity = 17.8 lungworms/frog). Five of the seven (71%) frogs chilled to 0°C were infected with 1–114 *R. ranae* (Table 1). Live *R. ranae* were also found in the lungs of three of the seven (43%) frogs subjected to freezing at -4°C. No difference was found in the prevalence (Fisher's exact test; $P = 0.30$) or intensity (Mann-Whitney U test; $U = 14.0$, $P = 0.21$) of *R. ranae* in unfrozen and frozen frogs.

Metacercarial trematodes were found in the kidneys of all Michigan frogs examined. Intensities were estimated in eight cases because the cysts could not be accurately enumerated (Table 1). Mean (\pm SE) intensities were 87 ± 33 , 90 ± 23 , and 69 ± 16 cysts/frog for control, nonfrozen, and frozen frogs, respectively. These values did not differ statistically (ANOVA; $F_{[2,15]} = 0.23$, $P = 0.80$). Microscopic examination of some of the cysts isolated from several freezing-exposed frogs revealed that the larva were alive, as judged from their sporadic undulatory movements.

Ohio wood frogs were examined for *R. ranae* infections shortly after they recovered from freezing at -5°C. Lungs of three of the seven (43%) frogs harbored mature live *R. ranae*.

Supercooling and inoculative freezing of *R. ranae*

Cryomicroscopy experiments showed that *R. ranae* immersed in oil supercooled extensively ($T_c = -21.3 \pm 0.2^\circ\text{C}$ (mean \pm SE); range = -22.1 to -20.7°C; $N = 6$). In contrast, specimens coated with saline nucleated at markedly higher temperatures ($T_c = -3.2 \pm 0.1^\circ\text{C}$; range = -3.7 to -2.9°C; $N = 9$). This difference was highly significant (Student's t

Table 1. Host characteristics and numbers of lungworms (*Rhabdias ranae*) and digenetic trematode metacercaria found in Michigan wood frogs kept in artificial hibernation and exposed to 4 (control), 0 (nonfrozen), or -4°C (frozen).

Treatment group	Examination date	Sex	Age-class	Body mass (g)	No. of <i>R. ranae</i>	No. of metacercaria
Control	Jan. 7	F	Juvenile	2.5	31	~200
	Jan. 8	F	Adult	5.0	6	~110
	Jan. 8	F	Juvenile	3.4	11	23
	Jan. 8	F	Adult	6.6	23	15
Nonfrozen	Jan. 20 ^a	F	Juvenile	3.3	1	57
	Jan. 20 ^a	F	Adult	7.2	4	60
	Jan. 25 ^a	M	Adult	3.2	2	~115
	Mar. 26	F	Adult	8.0	0	~195
	Mar. 26	F	Adult	8.0	114	46
	Mar. 26	F	Juvenile	2.0	0	~135
	Mar. 26	F	Juvenile	2.8	32	21
Frozen to -4°C	Jan. 14 ^b	M	Adult	3.5	7	13
	Mar. 26	M	Adult	5.9	1	47
	Mar. 26	F	Adult	6.1	1	~135
	Mar. 26	M	Adult	4.1	0	~75
	Mar. 27	F	Adult	6.1	0	~115
	Mar. 27	F	Adult	7.4	0	55
	Mar. 27	F	Adult	3.4	0	42

^aFrog was euthanized 5–10 d after chilling to 0°C to provide *R. ranae* for experimentation.

^bFrog did not fully recover from freezing and was examined for parasites shortly after thawing.

test; $t = 88.0$, $P < 0.0001$). Freezing of the saline film invariably commenced at a temperature ($T_c = -3.0 \pm 0.05^{\circ}\text{C}$; $N = 9$) slightly higher than that at which the specimen froze; thus, *R. ranae* were likely inoculated by external ice.

Image analysis revealed that *R. ranae* were inoculated shortly after ice spicules in the saline film contacted their cuticles (Figs. 1A, 1B). Internal freezing was signaled by a characteristic "flashing," which is a manifestation of the increased optical density associated with crystallization of body fluids (Fig. 1C). The exact sites of inoculation could not be visualized; however, ice fronts apparently originated near the posterior or anterior ends, or less frequently near the mid-point of the body. Occasionally, freezing was initiated more or less simultaneously at both ends. There was no consistency in the direction (anterior or posterior) moved by the ice front within the body. Tissues throughout the body crystallized within several seconds of the inoculation event (Fig. 1D).

Tolerance of *R. ranae* to in vitro freezing

Rhabdias ranae used in supercooling and inoculation trials showed variable tolerance to freezing, depending on the T_c of the individual. None of the *R. ranae* that supercooled extensively and ultimately nucleated at temperatures near -21°C recovered, whereas all specimens inoculated by external ice and exposed to minimum temperatures near -3°C survived. The latter results corroborated findings of our more naturalistic tests of freeze tolerance. *Rhabdias ranae* incubated in APS, or APS containing 250 mM glucose, recovered from a 10-h exposure to -4°C . Unfortunately, we could not confirm that the specimens themselves were frozen during these tests. However, given the high susceptibility to inoculative freezing we observed in the cryomicroscopy ex-

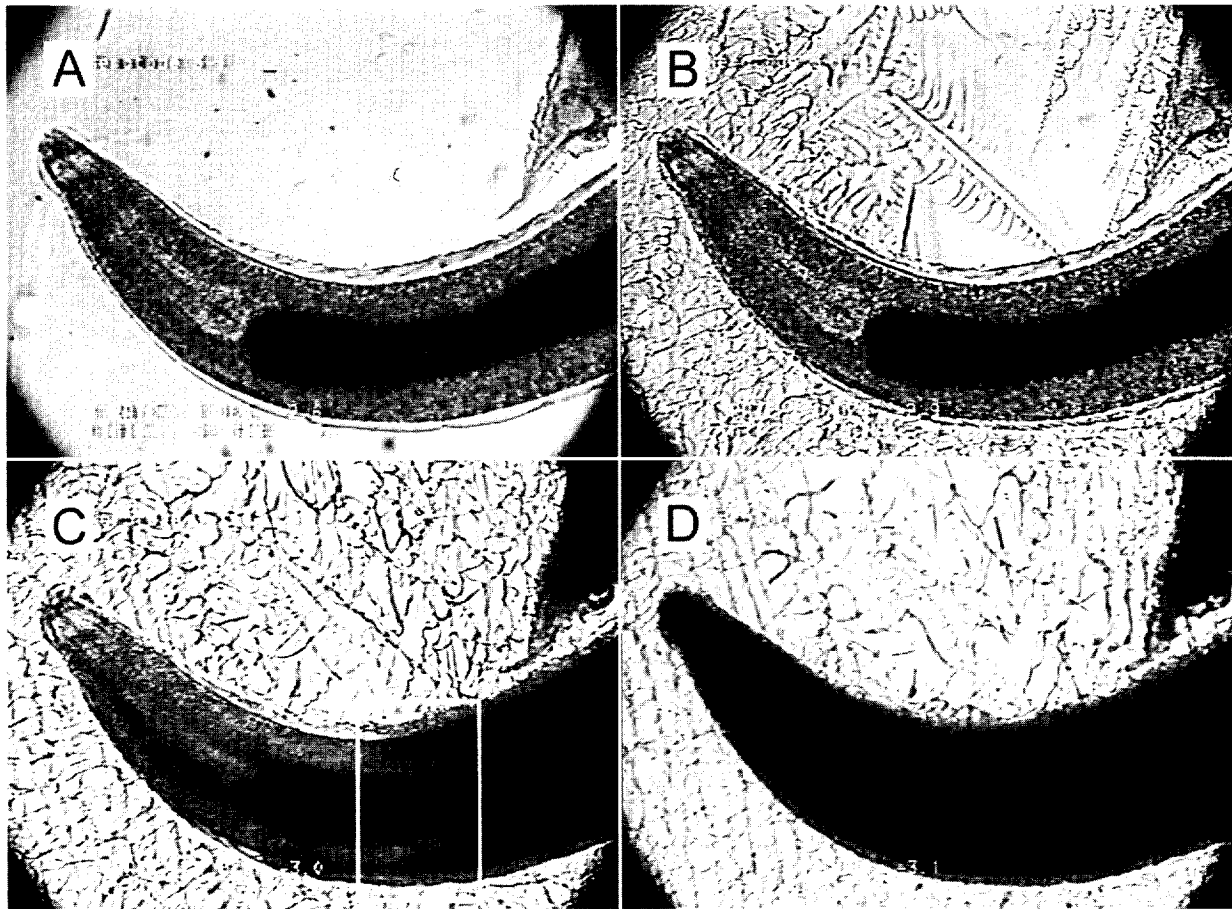
periments (i.e., T_c in the presence of ice was ca. -3°C), this was likely the case.

Discussion

Our investigation focused on the cold hardiness of two helminths, the nematode *R. ranae* and an unidentified digenetic trematode that parasitize wood frogs. *Rhabdias ranae* commonly infects the wood frog and other species of freeze-tolerant frogs (e.g., *Hyla versicolor*, *Pseudacris triseriata*, *Pseudacris crucifer*), as well as freeze-intolerant amphibians, including *Rana pipiens*, *Rana clamitans*, *Bufo americanus*, and *Plethodon cinereus* (Muzzall and Peebles 1991; Yoder and Coggins 1996; McAlpine and Burt 1998). Digenetic trematodes occur most commonly in aquatic amphibians (McAlpine and Burt 1998) but are known to infect wood frogs (Muzzall and Peebles 1991; Yoder and Coggins 1996). In addition to these helminths, our wood frogs harbored *Oswaldocruzia* sp. (Nematoda), tetrathyridian cestodes possibly of the genus *Mesocestoides*, as well as several unidentified protozoans. *Oswaldocruzia* sp. is a common parasite of amphibians, including wood frogs (Muzzall and Peebles 1991; Yoder and Coggins 1996). Cestode prevalences are generally low in North American frogs (McAlpine and Burt 1998), although tetrathyridia (e.g., *Mesocestoides* sp.) are found in various anurans (McAllister and Conn 1990) and were recently reported in the wood frog (McAllister et al. 1995). Amphibians commonly harbor nonpathogenic, gastrointestinal protozoans (Faeh et al. 1998). Collectively, our observations indicate that wood frogs may be infected with various parasites during hibernation.

Baker (1979) studied seasonal variation in prevalence and intensity of *R. ranae* in wood frogs collected near Guelph,

Fig 1. Ice inoculation and progression of internal freezing in a lungworm (*Rhabdias ranae*) cooled on a cryostage in contact with freezing saline. (A) Anterior end of *R. ranae* bathed in supercooled saline (-2.6°C). (B) Initiation of freezing of the saline medium and contact of *R. ranae* by external ice (-2.9°C). (C) Inoculation of *R. ranae* and posterior–anterior progression of the ice front, which is indicated by the darkened region (-3.0°C). (D) Fully frozen *R. ranae* (-3.1°C). Note that in each panel the caudal extremity is in view in the upper right corner of the field. The specimen revived after thawing. Total magnification, 20.874 \times .



Ontario. Transmission, presumably by skin penetration, occurred during summer and autumn, and the subadults inhabited the coelomic cavity before maturing and translocating to the lungs. Because adults oviposit in the spring, survival of *R. ranae* depends on successful overwintering within lungs of the host. Baker (1979) found no change in the prevalence (ca. 75–80%) or intensity (ca. 6–10 lungworms/frog) of infection between October and the following April, suggesting that winter mortality of *R. ranae* is low and that this species is adapted to survive inside the dormant host. Similarly, we found a high prevalence and generally low intensity of infection with *R. ranae* in our wood frogs (Table 1). However, in our study both the prevalence and intensity of infection with *R. ranae* in frogs frozen to -4°C appeared slightly lower than in frogs in the control and nonfrozen groups, raising the possibility that some *R. ranae* were eliminated by freezing of the host. Unfortunately, resolution of this question is confounded by both the small size of the samples and the variation in ages of the frogs used in our experiments. For example, this outcome may reflect the fact that the frozen group by chance consisted of relatively large frogs. Baker (1979) reported lower prevalence and intensity of infection with *R. ranae* in frogs smaller than 31 mm or larger than

51 mm. Nevertheless, our data indicate that *R. ranae* can successfully overwinter within the lungs of wood frogs, even those exposed to freezing temperatures.

We found the kidneys of wood frogs infested with metacercaria of a digenetic trematode. Intensity of the infection was usually high (>50 cysts/frog) and unchanged by exposure of the host to freezing. Many digeneans have a complex life cycle that involves an aquatic mollusc as the first intermediate host and a larval or adult amphibian as the second intermediate host. Development is completed after the latter is ingested by the avian or mammalian definitive host (Martin and Conn 1990). In the wood frog, a terrestrial species, transmission of the aquatic cercarial larvae likely occurs during the tadpole stage or possibly during the breeding season, when adult frogs become concentrated in vernal pools.

Mechanisms of cold tolerance in helminth parasites of wood frogs

Although little is known about the cold hardiness of parasites (Wharton 1999), they likely exhibit the same adaptations for surviving cold exposure as are used by other cold-hardy animals (Storey and Storey 1988). Nematodes have received more attention than any other group; however, this

work has focused on free-living species and off-host stages (Wharton 1995). Freeze avoidance via supercooling is one adaptation that is commonly used by animals that remain free of surface moisture. Supercooling probably is of little importance among nematodes as they are restricted to moist microenvironments (Wharton 1999). Soil-dwelling nematodes may avoid freezing by undergoing a protective desiccation that concentrates osmolytes in the hemolymph and reduces its equilibrium freezing point (Forge and MacGuidwin 1992). Some nematodes shed virtually all of their bulk water, becoming anhydrobiotic and increasingly tolerant to cold and other environmental stresses; others are susceptible to inoculative freezing and tolerate the freezing of their body tissues (Wharton 1995, 1999; Brown and Gaugler 1998).

There are few reports of parasites that tolerate freezing within living hosts (Humble and Ring 1985; Hance and Boivin 1993; Tyrell et al. 1994). Tyrell et al. (1994) discovered freeze tolerance in an enteral parasite of the freeze-tolerant alpine weta (*Hemideina maori*), an orthopteran endemic to New Zealand. This nematode, *Wetanema* sp., reportedly survives freezing at temperatures (to -61°C) substantially lower than its host. Cryostage experiments showed that *Wetanema* sp. is readily inoculated by external ice through a single site near its posterior end, such as the anus (Tyrell et al. 1994). We found that *R. ranae* also is susceptible to inoculative freezing; however, there was no distinct pattern of ice propagation within the body, suggesting that inoculation may occur at any of several sites. Given its high susceptibility to inoculative freezing, which perhaps is a manifestation of its thin body cuticle (Baker 1978), it is unlikely that *R. ranae* can remain supercooled within the frozen host.

In our experiments, *R. ranae*, like *Wetanema* sp., tolerated freezing on the cryostage and, quite likely, within its host. We exposed wood frogs to -4 or -5°C because these temperatures approach the lower limit to freezing survival (Storey and Storey 1988). Whether *R. ranae* tolerates temperatures markedly lower than its host, as does *Wetanema* sp., remains to be determined. However, our present results suggest that their capacity for freeze tolerance is at least as good as that of the wood frog.

Physiological mechanisms promoting cold hardiness in parasites are as yet unknown (Wharton 1999). Cryoprotective responses, including accumulation of polyols or other carbohydrates, may be important in these animals as they are in other organisms (Storey and Storey 1988). It is possible that *R. ranae* uses some of the cryoprotectant mobilized during freezing of the wood frog. However, this species survived in vitro freezing both in the absence or presence of exogenous glucose. Additional work is needed to test this hypothesis more rigorously and to identify the physiological mechanisms promoting freeze tolerance in *R. ranae*.

Our study provides evidence of cold hardiness in a metacercarial trematode that parasitizes hibernating wood frogs. Cysts in the kidneys of frogs exposed to freezing appeared similar to those of nonfrozen frogs and contained live larvae, indicating that this species can survive exposure to subzero temperatures inside its frozen host. It is probable that the survival strategy employed by this parasite, unlike that of *R. ranae*, involves supercooling. Supercooling capacity is promoted both by small body size and by an ability to resist inoculative freezing (Lee and Costanzo 1998). Assuming that

the cyst wall is a barrier to ice transmission (Wharton 1999), it is possible that these larvae supercool to very low temperatures. Generally, encysted stages of many parasites tolerate environmental stresses, including exposure to subzero temperatures (Fan 1998; Kapel et al. 1999).

Implications of parasite cold hardiness

Tyrell et al. (1994) questioned whether the parasitic nematode *Wetanema* sp. might serve as a nucleus and catalyze the freezing of host tissues. Because nucleation at high temperatures promotes freezing survival (Storey and Storey 1988), this result would benefit both the parasite and its freeze-tolerant host. However, these investigators found no difference in the T_{cs} of infected and uninfected hosts. It is doubtful that parasites of the wood frog are important in this regard because in amphibians freezing is initiated by contact with ice nuclei in the winter microenvironment (Costanzo et al. 1999). Our cryomicroscopy studies rather suggest that ice within tissues of the host triggers the freezing of *R. ranae*.

Parasitic infection may compromise aspects of organismal performance, including tolerance to environmental stresses such as desiccation and hypoxia (Jensen et al. 1996; Sousa and Gleason 1989). Toads (*Bufo bufo*) infected with the lungworm *Rhabdias bufonis* may incur reduced growth rate, locomotory performance, dietary intake, and survival (Goater and Ward 1992; Goater et al. 1993). Heavy infection of the kidneys with echinostomatid metacercariae, similar to those found in our wood frogs, may critically displace tissue and impair organ function (Martin and Conn 1990). Whether parasitic infection diminishes winter survival of *R. sylvatica* (or other freeze-tolerant anurans), perhaps by compromising the cryoprotectant system, remains to be determined. Prolonged exposure of anurans to cold may depress immunological function and susceptibility to disease (Manerio and Carey 1997), a result that may compound the effects of parasitic infection. This relationship merits investigation particularly in view of the putative role of parasitism in the decline of amphibian populations (Carey et al. 1999; Johnson et al. 1999).

Results of our study suggest that at least two species of helminths are adapted to overwinter in tissues of the wood frog, a host that tolerates somatic freezing. Whether adverse conditions prevailing in the host during cold exposure may eliminate less hardy species of endoparasites remains to be determined (see Muller and Schmid-Hempel 1993). Nevertheless, the wood frog and other freeze-tolerant anurans are useful subjects in the study of coevolution of thermal tolerance in parasites and their host.

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