Osmotic and freezing tolerance in spermatozoa of freeze-tolerant and -intolerant frogs

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Costanzo, Jon P., John A. Mugnano, Heidi M. Wehrheim, and Richard E. Lee, Jr. Osmotic and freezing tolerance in spermatozoa of freeze-tolerant and -intolerant frogs. *Am. J. Physiol.* 275 (*Regulatory Integrative Comp. Physiol.* 44): R713–R719, 1998.—The wood frog (*Rana sylvatica*) is a freeze-tolerant species that encounters subzero temperatures during its winter breeding season, whereas the leopard frog (*R. pipiens*) is freeze intolerant and breeds in spring. Osmotic and freezing tolerances of spermatozoa from these species were inferred from spermolysis rate, integrity of the plasma membrane as judged using vital dye assay, and motility rate. Sperm of *R. sylvatica* became motile in hypotonic media (≤220 mosmol/kg) and tolerated in vitro exposure to osmotic concentrations spanning nearly three orders of magnitude. Relative to sperm from *R. sylvatica*, which were unaffected by freezing at temperatures of −4°C or greater, *R. pipiens* sperm were more susceptible to osmotic damage and cryoinjury. These differences likely reflect cellular adaptations to somatic freezing in *R. sylvatica*. Unprotected sperm from both species were extensively damaged by freezing at −8°C, but the presence of glucose, the cryoprotectant used by *R. sylvatica*, or the permeant glycerol markedly diminished cryoinjury. These data suggest the feasibility of developing gamete cryopreservation protocols to aid efforts in conserving amphibian populations.

cryoprotection; reproduction; amphibian; anuran

AMONG THE FEW SPECIES of amphibians and reptiles for which freeze tolerance has been described, the best studied is the wood frog, *Rana sylvatica*, a species that overwinters beneath woodland duff within the frost zone (for reviews, see Refs. 8, 19, 32). During somatic freezing, hepatic glycogen reserves of *R. sylvatica* are rapidly converted to glucose, which then accumulates in various tissues (31); glucose is the cryoprotectant promoting freezing survival (9). Another primary adaptation for freeze tolerance is the massive redistribution of bulk water within the body in which >50% of organ water is translocated to the coelom and lymph spaces, where it ultimately freezes. This protective dehydration of tissues and organs concentrates cryoprotectant within the remaining cytosol and protects against physical damage due to ice forming excessively within tissues (20). Depending upon season, physiological condition, and geographic origin, *R. sylvatica* can survive the freezing of 65–70% of its body water at temperatures as low as −3 to −6°C and can tolerate freezing episodes lasting >4 wk (8, 19, 32).

Breeding of *R. sylvatica* occurs during a brief, late-winter thaw, much earlier than most sympatric anurans. Exposure of frogs to subzero temperatures migrating to breeding pools may hamper reproductive success. Male *R. sylvatica* appear normal after thawing but nevertheless exhibit reduced mate-searching effort, make fewer mating attempts, and compete poorly for females (6). Given that spawning occurs within minutes or hours after frogs arrive at the breeding pool, and that little time would be available for the repair or replacement of gametes, we questioned whether freezing might also compromise fertility.

Unfortunately, aside from a few brief reports (26, 27) there is little known about the tolerance of amphibian sperm to exposure to high subzero temperatures. In this study, we compared osmotic and freezing tolerances of spermatozoa from *R. sylvatica* and the leopard frog, *R. pipiens*. These species are well suited to comparative studies because they recently diverged from a common ancestor, they share various morphological and physiological attributes, and their geographic ranges widely overlap (5). However, *R. pipiens* overwinters underwater, breeds in spring, and lacks freeze tolerance (10, 18, 28).

MATERIALS AND METHODS

Animals and sperm preparations. Male *R. sylvatica* were collected on 27 February 1995 within 12 h of arriving en masse at a traditional breeding pond in southern Ohio. In the laboratory, frogs were housed on damp moss, fasted, and kept at 4°C in darkness. Male *R. pipiens* indigenous to northern United States were obtained during March 1995 (West Jersey Biological Supply) and kept at 4°C in darkness within cages containing water in one end. All frogs were acclimated 1–3 wk before use.

Testes were isolated from euthanized (double pithed) frogs, weighed to the nearest 0.1 mg, and macerated with Teflon-coated forceps in a volume (15 mg/ml) of suspension buffer (SB), an isotonic phosphate-free amphibian saline (in g/l: 6.50 NaCl, 0.25 KCl, 0.15 $CaCl_2$, 0.10 $NaHCO_3$, 0.54 *D*-glucose; 220 mosmol/kg, pH 7.4 at 23°C). Tissue homogenates were centrifuged (7 *g*, 3 min) and supernatant containing sperm was stored on ice in 0.5-ml polyethylene microcentrifuge tubes until used.

Cell measurements. Samples of mature sperm from *R. sylvatica* ($n = 3$) and *R. pipiens* ($n = 3$) were viewed at ×900 total magnification. Head length and width measurements were made on 12 randomly selected cells from each sample using Linkham VTO 232 videographic analysis software.

Sperm viability. Spermolysis induced by osmotic or freeze/thaw stress was inferred from decreases in counts of intact cells in the treated suspensions. Viability of these intact cells was based on two aspects of functional capacity: selective permeability of the plasma membrane (vital dye assay) and motility.

Cell counts and motility determinations were made in duplicate on 10-µl aliquots of suspension using a Levy hemocytometer (improved Neubauer, 0.10 mm depth). Sperm were observed at ×40 using Nomarski differential-interfer-

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ence contrast illumination. The number of sperm per microliter of suspension was determined using standard counting techniques (21), and percentage of motile sperm (i.e., cell body undulating and/or under forward propulsion by the flagellum) was determined for a random sample of 400 cells.

Sperm viability was assessed using our modification of a dual fluorochrome vital dye procedure (FertiLight, Molecular Probes, Eugene, OR). This procedure distinguished functionally dead cells, which permit entry of red dye, from those with discriminating plasma membranes (12). A 200-µl aliquot of sperm suspension was dyed with 2 µl SYBR 14 (20 µM). After incubation at 23°C for 15–20 min, 2 µl propidium iodide solution (1.2 mM) was added and the suspension was again incubated. The proportion of viable cells in a 10-µl aliquot of dyed suspension was based on ~400 cells observed in randomly selected fields using fluorescence microscopy (excitation wavelength, 490 nm).

Osmotic tolerance. We investigated the effect of hyposmotic exposure on spermolysis and motility. Sperm from *R. sylvatica* ($n = 3$) were prepared in (undiluted) SB, apportioned into 100-µl aliquots and gently pelleted by low-speed centrifugation (67 *g*, 3 min). The supernatant was discarded and replaced with 100 µl SB in 100 (control), 75, 50, 25, or 2.5% formulations (corresponding osmotic concentrations: 220, 165, 110, 55, and 5.5 mosmol/kg, respectively); sperm were resuspended using reflux aspiration, incubated at 23°C for 10 min, and used in determinations of cell count and motility.

Hyperosmotic tolerance of sperm from *R. sylvatica* ($n = 3$) and *R. pipiens* ($n = 3$) was studied using samples prepared as above, except that cells were resuspended in 100 µl SB (control) or SB fortified with NaCl to a total osmotic concentration of 1.0, 2.0, or 3.0 osmol/kg. Sperm were not returned to isotonic as this procedure may have substantially increased cell damage (11).

Freeze tolerance. Tolerance to freezing/thawing was studied in motile sperm from *R. sylvatica* ($n = 5$) and *R. pipiens* ($n = 5$) prepared in 50% SB, apportioned into 250-µl aliquots, and chilled at 4°C (control) or immersed and thermoequilibrated to 0°C in an ethanol bath. The latter samples were then cooled (about −0.2°C/min) to an ultimate incubation temperature of −2, −4, −6, or −8°C. Sample temperature was inferred from an additional replicate outfitted with a copper-constantan thermocouple connected to an OM500 Omega Engineering (Stamford, CT) data logger. Ice nucleation of the supercooled suspensions was initiated at −1.7°C (~1.5°C below the equilibrium freezing/melting point) by briefly applying aerosol coolant to the outside of the tube. Samples were kept in the bath 10 min after attaining thermoequilibrium at the target incubation temperature, thawed and/or warmed during a 20-min incubation at 23°C, and then used in determinations of cell count, viability, and motility.

Cryoprotection. Sperm from *R. sylvatica* ($n = 5$) were frozen in suspensions containing glucose or glycerol to assess the cryoprotective efficacy of these agents. Sperm prepared in 50% SB (which contains 1.5 mM glucose) were gently pelleted by centrifugation (67 *g*, 3 min) and resuspended in 50% SB augmented with 0, 15, or 150 mM glucose or 150 mM glycerol. Samples of the motile sperm were either kept at 4°C (control) or frozen to −8°C, thawed and/or warmed as above, and then used in determinations of cell count, viability, and motility.

Viability of sperm from frozen testes. One intact testis from each of three frogs was wrapped in a small piece of damp filter paper (moistened with SB) and placed in a microcentrifuge tube. The remaining organ was used to prepare a motile sperm suspension in 50% SB, a portion of which was kept at 4°C (unfrozen control). The remaining portion, together with the intact testis, was frozen and incubated at −8°C and subsequently thawed, as above. Determinations of cell count and motility were then made on the unfrozen control suspension, the suspension that was frozen, and a suspension prepared from the testis (15 mg/ml, based on prefreeze organ mass), which had been frozen.

Statistical evaluation. Means representing two treatment groups were compared using Student's *t*-tests for independent samples. Comparisons of means from three or more treatment groups were made using one- or two-factor ANOVA followed by Fisher's protected least-significant difference. Bivariate data were used in linear regression and correlation analyses. Analyses of percentage data were performed using angularly transformed values. Values are means ± SE. Significance was judged at $P \leq 0.05$.

RESULTS

Catastrophic damage resulting in spermolysis generally was reflected by decreases in counts of intact sperm in treated samples relative to control samples. Functional viability of the remaining cells was assessed by vital dye assay, which gave the proportion of intact cells retaining discriminating plasma membranes. Sperm motility, a more rigorous test of cell function, was also used. Although interindividual variation in motility was high, even among control samples, loss of motility provided a useful and corroborative indicator of cell damage.

Species differences. Correlation analysis of the combined data for *R. sylvatica* ($n = 17$–26) and *R. pipiens* ($n = 8$) revealed that testis mass was directly related to body mass [$r^2 = 0.80$, degrees of freedom (df) = 34, $F = 130.0$, $P < 0.0001$] and that larger testes contained more sperm ($r^2 = 0.83$, df = 24, $F = 111.1$, $P < 0.0001$), the complement of which was computed from cell count and testis mass (Fig. 1). On a mass-specific basis, the

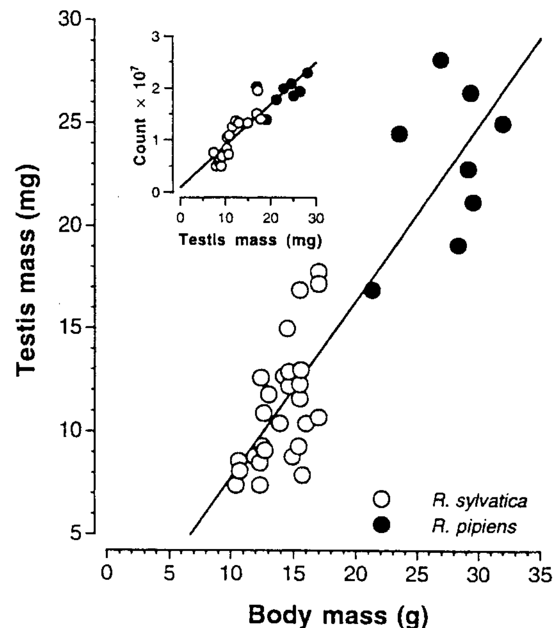

Fig. 1. Correlations of body mass and testis mass, and of testis mass and testicular sperm complement (*inset*), for *Rana sylvatica* ($n = 17$–26) and *R. pipiens* ($n = 8$). Lines of best fit: $y = -0.715 + 0.854x$; $y = -0.096 + 0.080x$, respectively.

testicular sperm count was similar between species (Table 1).

Variability in sperm size occurred within samples from individuals, as well as within and between species. Cell body length varied by up to 40% among sperm from a given animal. Interindividual differences occurred in the mean length of sperm from *R. sylvatica* ($F = 8.3$, $P = 0.001$) and the mean length ($F = 15.8$, $P < 0.001$) and width ($F = 4.0$, $P = 0.029$) of sperm from *R. pipiens*. Sperm from *R. sylvatica* were longer and narrower than those of *R. pipiens* (Table 1), but other differences in morphology were not observed.

Osmotic tolerance. The osmotic concentration of SB strongly influenced sperm count ($F = 23.8$, $P < 0.0001$). Densities of sperm were similar among samples ranging from 50 to 100% SB (110–220 mosmol/kg), but were reduced at lower osmolalities (Fig. 2). Osmolality also influenced sperm motility ($F = 5.1$, $P = 0.017$), which was invariably low at the higher osmolalities, but increased with decreasing osmotic concentration to a maximum rate of 41 ± 13% in 50% SB. Motility was reduced with further dilution of the suspension medium, ultimately to <5% for cells in 2.5% SB (Fig. 2). Our experiments used 50% SB as the primary suspension medium, because this preparation maximized cell motility without causing spermolysis.

Sperm counts in samples from *R. sylvatica* and *R. pipiens* generally decreased with increasing osmotic concentration ($F = 106.7$, $P < 0.0001$), although the pattern of attrition differed between species (species × temperature: $F = 3.5$, $P = 0.04$). Spermolysis in 2.0 and 3.0 osmol/kg solutions was 72.8 and 84.5%, respectively, for *R. sylvatica*, but comparatively higher (83.6 and 96.4%) for *R. pipiens* (Fig. 3). Sperm incubated in isotonic and hypertonic solutions were immotile (Fig. 2).

Freeze tolerance. Freezing of suspensions reduced sperm count in samples from *R. sylvatica* ($F = 13.6$, $P < 0.0001$) and from *R. pipiens* ($F = 24.8$, $P < 0.0001$). Generally, the effect of temperature on spermolysis was similar between *R. sylvatica* and *R. pipiens* (species × temperature: $F = 0.76$, $P > 0.55$). However, whereas *R. sylvatica* sperm readily tolerated mild subzero temperatures (i.e., samples incubated at −2 and −4°C contained as many sperm as the unfrozen control, 14,200 ± 1,320 cells/μl), spermolysis occurred in samples from *R. pipiens* at −4°C (Fig. 4*A*). Only ~30% of sperm from

Table 1. *Morphometric comparisons between wood frogs (Rana sylvatica) and leopard frogs (R. pipiens)*

	R. sylvatica	*R. pipiens*	*F*	*P*
Body mass, g	14.7 ± 0.4	27.5 ± 1.2	154.6	<0.0001
Testis mass, mg	11.6 ± 0.8	23.0 ± 1.3	58.9	<0.0001
Sperm count				
Cells × 10^5/mg testis	8.7 ± 0.5	8.5 ± 0.5	0.1	0.770
Cells × 10^7/testis	1.0 ± 0.1	1.9 ± 0.1	29.7	<0.0001
Sperm dimensions				
Length, μm	26.9 ± 0.5	17.6 ± 0.5	163.6	<0.0001
Width, μm	1.5 ± 0.04	2.1 ± 0.06	70.0	<0.0001

Values are means ± SE for $n = 17$ (*R. sylvatica*) or $n = 8$ (*R. pipiens*) animals except for dimensions, which are means ± SE for $n = 36$ cells combined from 3 animals.

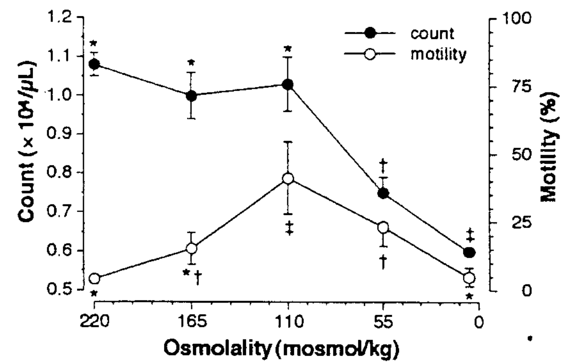

Fig. 2. Hyposmotic injury to *R. sylvatica* sperm as indicated by reduced cell count (spermolysis) and motility. Data are means ± SE for suspensions prepared from $n = 3$ animals. Within each data set, means identified by different superscripts were statistically distinguishable ($P < 0.05$).

both species remained intact in suspensions frozen to −8°C.

Vital dye assays performed on cells that resisted cryospermolysis revealed that viability, as judged by membrane integrity, was temperature dependent in sperm from *R. sylvatica* ($F = 7.4$, $P < 0.0001$) and *R. pipiens* ($F = 9.2$, $P = 0.0002$). However, viability of sperm in frozen/thawed samples differed from unfrozen control samples (78–79%) only in suspensions incubated at the lowest temperature, −8°C (Fig. 4*B*). Motility of *R. sylvatica* sperm was reduced ($F = 4.1$, $P = 0.014$) in suspensions frozen at −6 and −8°C relative to the control sample, 38.2 ± 6.0% (Fig. 4*C*). Sperm from *R. pipiens* also exhibited this general pattern, although mean values did not differ ($F = 1.11$, $P > 0.37$) among the treatment groups.

Comparisons of densities of viable cells and motile cells, computed as the products of cell count and viability, and cell count and motility rate, respectively, among treatment groups and between species, revealed that sperm from *R. sylvatica* generally were more

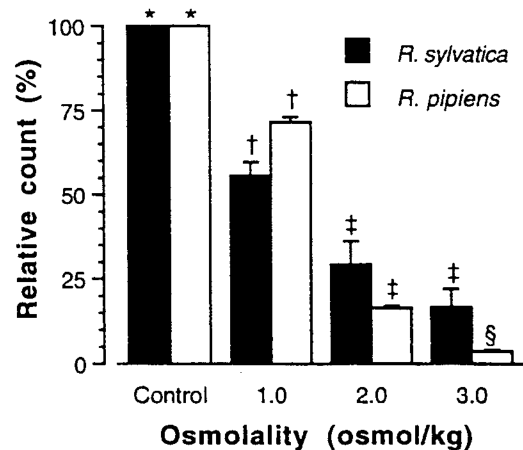

Fig. 3. Hyperosmotic injury to sperm of *R. sylvatica* and *R. pipiens* as indicated by reduction in cell count (spermolysis) relative to the control, which was isosmotic with blood plasma (220 mosmol/kg). Control samples contained 1.03 ± 0.15 × 10^4 sperm/μl (*R. sylvatica*) or 1.10 ± 0.01 × 10^4 sperm/μl (*R. pipiens*). Data are means ± SE for suspensions prepared from $n = 3$ animals. Means identified by different superscripts were statistically distinguishable ($P < 0.05$).

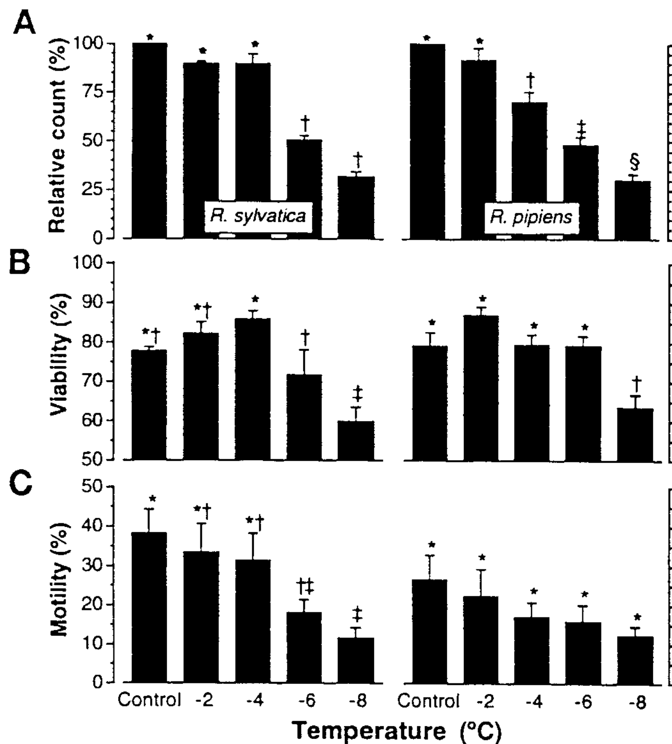

Fig. 4. Freezing/thawing injury to sperm of *R. sylvatica* and *R. pipiens* as indicated by reduction in *A*) cell count (spermolysis) relative to the unfrozen control; *B*) viability of sperm as judged by vital dye assay; and *C*) sperm motility. Control samples contained $1.42 \pm 0.13 \times 10^4$ cells/µl (*R. sylvatica*) or $1.38 \pm 0.11 \times 10^4$ cells/µl (*R. pipiens*). Data are means ± SE for suspensions prepared from n = 5 animals. Within each data set, means identified by different superscripts were statistically distinguishable ($P < 0.05$).

tolerant of freezing/thawing than *R. pipiens* sperm. Reduction in the number of intact sperm retaining functional plasma membranes occurred at temperatures less than −4°C for sperm from *R. sylvatica* and at temperatures less than −2°C for sperm from *R. pipiens*. Similarly, the threshold temperature for decrease in motility rate was lower for *R. sylvatica* (−4 to −6°C) than *R. pipiens* (−2 to −4°C).

Cryoprotection. Addition of glucose or glycerol to the suspension markedly improved freeze tolerance of *R. sylvatica* sperm incubated at −8°C with respect to cell count (F = 28.4, $P < 0.0001$), viability (F = 14.4, P = 0.001), and motility (F = 4.1, P = 0.03). Whereas 150 mM glucose or glycerol markedly reduced cryoinjury, low concentrations (1.5 and 15 mM) of glucose were ineffectual (Fig. 5). Equimolar concentrations of glucose and glycerol provided similar cryoprotection, although counts of sperm in these solutions were 45–48% below unfrozen control values.

Viability of sperm frozen within the testis. Exposure of *R. sylvatica* sperm to −8°C reduced cell count (F = 279.2, $P < 0.0001$) and motility (F = 19.1, P = 0.003) relative to the unfrozen control. Freezing diminished sperm count by ~68% and motility by ~60%, regardless of whether sperm were incubated in aqueous suspension or within the intact organ (Fig. 6).

DISCUSSION

Our finding that the larger testes of heavier frogs generally contain more spermatozoa is consistent with other evidence that fecundity is size dependent in anurans (15, 16, 29). No differences occurred between *R. sylvatica* and *R. pipiens* testes in mass-corrected sperm counts, although the former was tested during the peak of breeding season and the latter was used ~3 mo before its breeding season. Vital dye assay of sperm in an isotonic medium indicated that, in both species, 80–85% of the sperm population is viable (Fig. 4).

Sperm of amphibians and fishes that spawn in fresh water are sensitive to changes in extracellular osmotic concentration. Within the testes, sperm are largely quiescent, but motility is triggered by acute hyposmotic exposure, such as occurs during spawning (2, 22, 25). Sperm of fish and frogs incur hyposmotic injury, as manifested by decreased motility, within seconds to minutes of activation (2, 15, 25, 35). Accordingly, ~50%

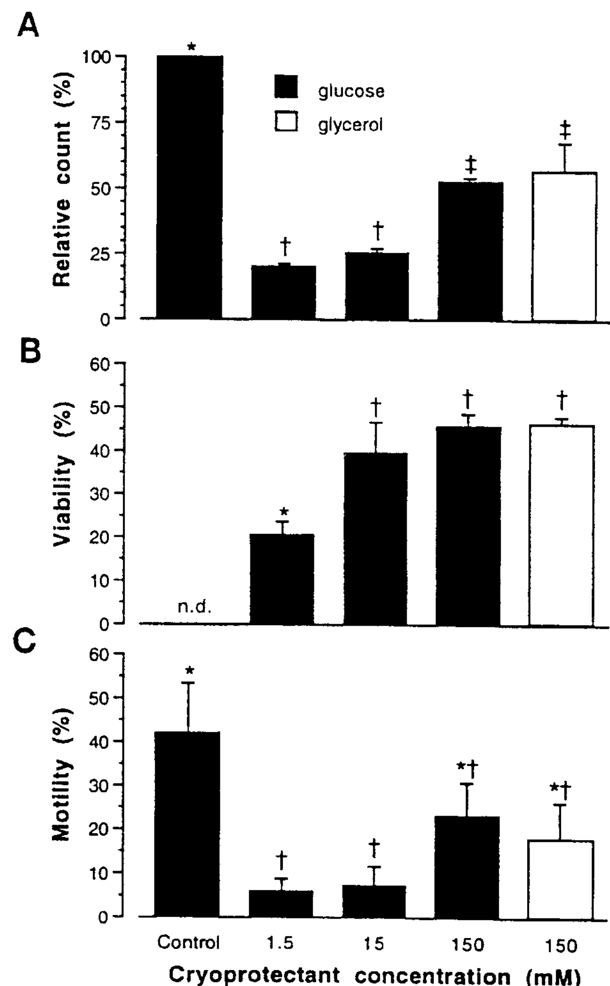

Fig. 5. Efficacy of glucose and glycerol in mitigating cryoinjury to sperm of *R. sylvatica* frozen at −8°C as indicated by reduction in *A*) cell count (spermolysis) relative to the unfrozen control; *B*) viability of sperm as judged by vital dye assay; and *C*) sperm motility. Control samples contained $1.41 \pm 0.14 \times 10^4$ cells/µl. Data are means ± SE for suspensions prepared from n = 3 animals. Within each data set, means identified by different superscripts were statistically distinguishable ($P < 0.05$). ND, not determined.

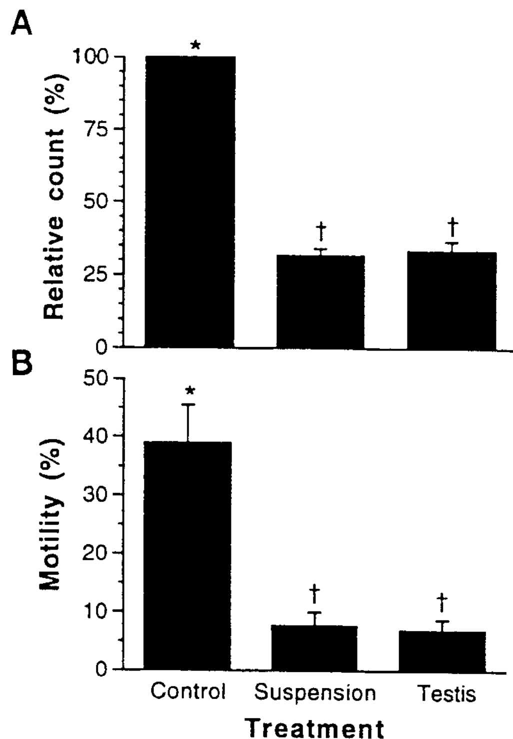

Fig. 6. Cryoinjury to sperm of *R. sylvatica* during freezing at −8°C within isolated unperfused testis or in suspension and frozen at −8°C, as indicated by reduction in *A*) cell count (spermolysis) relative to the unfrozen control and *B*) sperm motility. Control samples contained 1.52 ± 0.02 × 10^4 cells/μl. Data are means ± SE for suspensions and organs prepared from n = 3 animals. Within each data set, means identified by different superscripts were statistically distinguishable ($P < 0.05$).

of *R. sylvatica* sperm exposed to 5.5 mosmol/kg, an osmotic concentration equivalent to that of pond water, had lysed within 10 min and few of the surviving sperm were motile. The brief delay in making counts and motility measurements likely underrepresents the number of motile sperm actually available for fertilizing eggs, because fertilization presumably occurs within seconds or minutes of sperm release.

Under our sampling regimen the optimal osmolality for sustaining motility of *R. sylvatica* sperm was ~110 mosmol/kg, approximately one-half the concentration of blood plasma. Sperm motility is maximal at similar osmolalities in other frogs (15) and fish (24). Newt (*Notophthalmus viridescens*) sperm remain quiescent in a 115 mosmol/kg solution, but this is the typical osmolality of seminal fluids (14).

We performed our tests of freeze tolerance on motile sperm because preliminary experiments indicated that freezing predisposed cells to damage resulting from the mechanical stress (centrifugation) and hyposmotic exposure required to activate sperm after treatment. Consequently, our results may not accurately represent responses of (unactivated) sperm frozen in vivo (although good agreement was obtained for sperm frozen in suspension and sperm frozen within intact testes; Fig. 6).

Sperm frozen and thawed in aqueous suspensions are exposed to an environment that becomes progressively hypertonic with decreasing temperature and then hypotonic during thawing. The breadth of osmotic tolerance, spanning nearly three orders of magnitude, enables *R. sylvatica* sperm to survive these extremes. The range of osmotic tolerance for sperm, relative to that of somatic cells, is skewed toward lower osmolalities, consistent with the demands of the fertilization environment. Notably, the osmotic concentration of the hyposmotic medium producing 50% cytolysis is 10-fold lower for spermatozoa than for erythrocytes (Fig. 7).

The finding that *R. sylvatica* sperm better tolerated hyperosmotic exposure than *R. pipiens* sperm is consistent with previous studies of these species and likely reflects fundamental cellular adaptations to the more terrestrial (i.e., potentially desiccating) habits of the former (10). Moreover, unlike *R. pipiens*, *R. sylvatica* is well adapted to survive increases in extracellular osmolality induced during freezing of its body fluids. In addition to our present data for spermatozoa, differences in innate freezing tolerance between these species have been demonstrated for erythrocytes, hepatocytes, skeletal muscle, and cardiac muscle (10, 17, 30).

Even in the absence of exogenous cryoprotectant, sperm of *R. sylvatica* tolerate freezing without apparent injury to a minimum temperature between −4 and −6°C, within the range of thermal tolerance of intact animals (8, 19, 32). However, exposure to lower temperatures caused extensive spermolysis. Loss of unprotected cells incubated at −8°C (Figs. 4*A* and 6) was ~70%, lower than that (86%) of erythrocytes treated identically (7), but greater than that of hepatocytes (~50%) also frozen in vitro to −8°C (30). To promote their freeze tolerance, animals such as *R. sylvatica* accumulate cryoprotectants, which preserve cell function during freezing and thawing. Concentrations of glucose or glycerol within organs of fully frozen frogs vary with the degree and persistence of perfusion after freezing begins, the degree of tissue desiccation, and the efficacy of intracellular uptake, but typically are 0.15–0.3 M in deep visceral tissues (8, 32). Glucose, the cryoprotectant used by *R. sylvatica*, enhances in vitro freezing viability of erythrocytes (7), hepatocytes (30), and cardiocytes (4). Glycerol is known to reduce cryoinjury to frog sperm frozen at high subzero temperatures

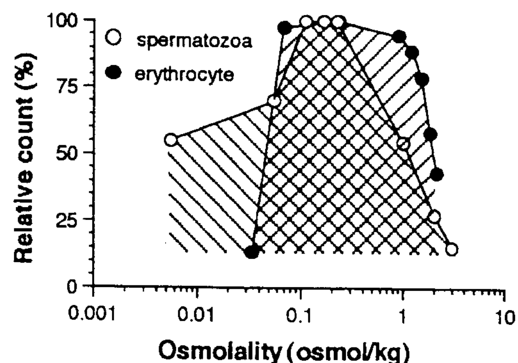

Fig. 7. Osmotic tolerance of *R. sylvatica* sperm and erythrocytes, depicting the fraction of cells resisting lysis. Data are means based on samples from n = 3 (spermatozoa) or n = 10 (erythrocyte) animals. Data for erythrocytes were derived from Costanzo and Lee (7).

(26). In 0.15 M concentrations, glucose and glycerol were equally effective in reducing cryospermolysis and preserving cell motility at −8°C. However, unlike the case with frog sperm (Fig. 5), glycerol is a more effectual cryoprotectant with erythrocytes, perhaps because it better permeates their membranes (7). Glycerol and glucose mitigate cryoinjury by reducing the transmembrane osmotic gradient, reducing interactions among subcellular elements by maintaining a greater cytoplasmic volume, and by stabilizing membranes through as yet undetermined mechanisms (1, 23).

Cryoinjury to cells in a frozen suspension may be partly due to mechanical stresses produced by the compression of cells in the unfrozen channels between growing ice crystals (23). Sperm frozen in situ within (unperfused) testes incurred the same degree of injury as those frozen in suspension, suggesting that the intimacy between sperm and testicular tissues confers no particular protection. However, during somatic freezing the testes may serve to sequester glucose and thus improve survival of spermatozoa frozen in vivo.

Perspectives

Because breeding occurs in late winter (e.g., February for populations in southern Ohio), *R. sylvatica* may endure freezing episodes shortly before spawning (6). Frogs are more susceptible to cryoinjury at this time owing to their diminished capacity to mobilize cryoprotectant (8, 19, 32). Our data suggest that mild freezing episodes would not depreciate sperm viability; however, deeper freezing (e.g., lower than −4°C) may reduce the number of viable cells available during spawning.

The development of protocols for cryopreserving sperm of fishes, birds, and mammals has been promulgated by economic and conservatory agendas (13), with current efforts aimed at limiting cryoinjury and extending storage life (34). With the lack of such initiatives, cryopreservation protocols are currently unavailable for use with amphibian gametes, although the recent concern for sustained population declines (3, 33) seems sufficiently compelling. Our data on cryoprotectant efficacy suggest the feasibility of developing gamete cryopreservation measures that may ultimately be used to safeguard genetic integrity and facilitate culture of amphibians of special concern.

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