RESEARCH ARTICLE

Hepatocyte Responses to *In Vitro* Freezing and β-Adrenergic Stimulation: Insights into the Extreme Freeze Tolerance of Subarctic *Rana sylvatica*

JOURNAL OF EXPERIMENTAL ZOOLOGY
PART A
ECOLOGICAL GENETICS AND PHYSIOLOGY
A Journal of Integrative Biology

M. CLARA F. DO AMARAL*,
RICHARD E. LEE JR., AND JON P. COSTANZO
Department of Zoology, Miami University, Oxford, Ohio

ABSTRACT

The wood frog, *Rana sylvatica* LeConte 1825, is a freeze-tolerant amphibian widely distributed in North America. Subarctic populations of this species can survive experimental freezing to temperatures below −16 °C, whereas temperate populations tolerate freezing only at temperatures above −6 °C. We investigated whether hepatocytes isolated from frogs indigenous to Interior Alaska (subarctic) or southern Ohio (temperate) had distinct characteristics that could contribute to this variation in freeze tolerance capacity. Following *in vitro* freezing, cell damage, as assessed from lactate dehydrogenase leakage, was similar between samples from Alaskan and Ohioan frogs. Preincubation of cells in media containing glucose or urea, the two primary cryoprotectants used by *R. sylvatica*, markedly reduced freezing damage to hepatocytes; however, results suggested that cells of the northern phenotype were comparatively more amenable to cryoprotection by urea. Stimulation of isolated hepatocytes with β-adrenergic agonists, which simulates the freezing-induced cryoprotectant mobilization response, gave rates of glucose production from endogenous glycogen reserves that were similar between the populations. Our findings suggest that extreme freeze tolerance in subarctic *R. sylvatica* does not require an enhanced ability of the liver to resist freezing stress or rapidly mobilize cryoprotectant. *J. Exp. Zool. 323A:89–96, 2015.* © 2015 Wiley Periodicals, Inc.

How to cite this article: do Amaral MCF, Lee Jr. RE, Costanzo JP. 2015. Hepatocyte responses to *in vitro* freezing and β-adrenergic stimulation: insights into the extreme freeze tolerance of subarctic *Rana sylvatica*. J. Exp. Zool. 323A:89–96.

The wood frog, *Rana sylvatica* LeConte 1825, is one of several amphibians known to tolerate corporal freezing as an adaptation to survive winter's cold (Costanzo and Lee, 2013). This species is widely distributed in North America, ranging from the Piedmont of western Georgia, USA, to north of the Arctic Circle (Martof and Humphries, '59). Throughout its range, *R. sylvatica* hibernates in shallow depressions in the soil, beneath leaf litter, where it is exposed to the harsh temperatures of winter, but nevertheless survives the freezing of up to two-thirds of its body water (Storey and Storey, 2004; Costanzo and Lee, 2013).

Freeze tolerance in *R. sylvatica* is based, in part, on its uremic and glycemic cryoprotectant systems. This frog accumulates organic osmolytes that colligatively act to reduce ice content and limit cellular dehydration, and also exert specific protective effects (i.e., independent of solute concentration) on membranes and proteins, enabling cellular components to withstand freezing

Grant sponsor: National Science Foundation; grant number: IOS 1022788; grant sponsor: Portuguese Science and Technology Foundation; grant number: SFRH/BD/63151/2009.
Conflict of interest: None.
*Correspondence to: M. Clara F. do Amaral, Department of Biology, Wittenberg University, 200 W. Ward St Springfield, OH 45505.
E-mail:doamaralm@wittenberg.edu
Received 14 August 2014; Revised 17 October 2014; Accepted 22 October 2014
DOI: 10.1002/jez.1905
Published online 7 January 2015 in Wiley Online Library (wileyonlinelibrary.com).

(Costanzo and Lee, 2013). Studies with *R. sylvatica* have shown that supplementing cryoprotectant levels in frogs (e.g., Costanzo and Lee, 2008) and adding cryoprotectants to isolated cell suspensions (e.g., Storey and Mommsen, '94) increases freezing survival. Whereas urea is accumulated in the fall and early winter, glucose is quickly generated from liver glycogen with the onset of freezing and transported to other organs before circulation ceases (Storey and Storey, 2004; Costanzo and Lee, 2013). Freezing hastens hepatic glycogenolysis and the consequent production of glucose, a response likely mediated through hepatic β-adrenergic receptors (Mommsen and Storey, '92; Storey and Storey, '96). Rapid production and distribution of glucose throughout the blood and tissues early in the freezing process is essential, as cryoprotectant distribution is curtailed as freezing progresses and ice accumulates (Costanzo et al., '92).

Subarctic populations of *R. sylvatica* are substantially more freeze tolerant than their temperate counterparts, as they can survive freezing to temperatures at least as low as −16 °C in the laboratory (Costanzo et al., 2013), and to even lower temperatures in natural hibernacula (Larson et al., 2014). By contrast, frogs indigenous to the Midwestern United States and southern Canada tolerate experimental freezing only to temperatures as low as −4 to −6 °C (Storey and Storey, 2004; Costanzo and Lee, 2013). Extreme freeze tolerance in subarctic frogs derives, in part, from an ability to accrue extremely high levels of urea before hibernating and to rapidly mobilize large amounts of glucose during the early stages of freezing (Costanzo et al., 2013; do Amaral et al., 2013). Rapid production and distribution of cryoprotectant seems especially important in subarctic frogs, which presumably freeze quickly due to their diminutive size (Martof and Humphries, '59) and the severe cold and high cooling rates that characterize their hibernal microhabitat (Larson et al., 2014). Accordingly, we hypothesized that the hepatocytes of these frogs are specially adapted to withstand cryoinjury and to hasten glucose production during freezing.

In the present study, we conducted two experiments that further elucidate the nature of the extreme freeze tolerance in northern *R. sylvatica*. We compared the viability of hepatocytes isolated from frogs representing temperate and subarctic populations following *in vitro* freezing with and without added cryoprotectants. To gain insights into the rapidity of glucose mobilization during freezing, we also compared maximal rates of glucose production in isolated hepatocytes under β-adrenergic stimulation.

MATERIALS AND METHODS

Experimental Animals and Acclimatization

For the purposes of this study we collected seventeen *R. sylvatica* of both sexes on public lands near Fairbanks, Alaska, USA (64.8 ° N and 147.7 °W), during early August, in 2011 and 2012, under a permit issued by the Alaskan Department of Fish and Game. Frogs were shipped under refrigeration to our laboratory and housed in clean plastic cups (floor area, 56.7 cm^2) with either damp paper or moss as a substratum. They were kept in a programmable environmental chamber (Percival, model I-35X, Boone, IA, USA) and exposed over 5 weeks to dynamic, diel cycles of temperature and ambient light, which, based on long-term records of weather (obtained from the National Oceanic and Atmospheric Administration's National Climatic Data Center, NOAA NCDC), were seasonal and appropriate to their origin. Initially, temperature varied daily from 17–8 °C and the photophase was 16.5 hr, but by the end of acclimatization, in mid-September, temperature varied daily from 13–3 °C and the photophase was 13.3 hr. Throughout, frogs were fed three times weekly with crickets that were dusted with a vitamin supplement (ReptoCal, Tetrafauna, Blacksburg, VA, USA). Following acclimatization, frogs were kept at 4 °C, in darkness, in simulated hibernation until used in mid-November (2012 cohort) or mid-December (2011 cohort).

An additional nine frogs (all male) were collected in late winter (February, 2012) in Adams County, south-central Ohio, USA (38.8 °N and 83.3 °W), on private land with the permission of the landowner, under a permit issued by the Ohio Division of Wildlife. They were kept, unfed, on damp moss within darkened plastic boxes (4 °C) for 3 weeks after collection from the field. Thereafter they were kept outside in a 48-m^2 pen at the Ecology Research Center (39.5 °N and 84.7 °W), Miami University, until autumn. Frogs had continuous access to a pool of water and were fed vitamin-fortified crickets three times weekly, and this diet was supplemented by a host of arthropods that was attracted to an UVA-emitting "black light" hung in the pen. Feeding was suspended in late October, and in November, the frogs, on the verge of hibernation, were recaptured, returned to our laboratory, and kept on damp moss in dark plastic boxes (4 °C) to simulate hibernation, until used in January. All rearing and experimental protocols were approved by the Institutional Animal Care and Use Committee of Miami University (research protocol number 812).

Preparation of Hepatocyte Suspensions

Hepatocytes were harvested from winter-acclimatized frogs (N = 26) by *in situ* perfusion of the liver with Liberase TM (Roche, Nutley, NJ, USA), a preparation containing a mixture of collagenase I and II, followed by differential centrifugation, as previously described (Mommsen and Storey, '92; Mommsen et al., '94). The entire procedure was conducted at room temperature. Frogs were double-pithed and cannulated through the ventral abdominal vein using a 27- or 30-gauge needle fitted with silicone tubing (0.05 cm internal diameter × 0.09 cm outer diameter). Livers were flushed of blood with "frog saline," a solution containing 114 mM NaCl, 1 mM $MgSO_4$, 2.25 mM KCl, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 13 mM $NaHCO_3$, 2% defatted bovine serum albumin (BSA), and 10 mM Hepes (pH 7.63 at 22 °C). The livers were then perfused with Liberase TM

in frog saline (0.096 U mL^{-1}) for ca. 30 min at 2 mL $min^{-1}g^{-1}$ liver.

Following perfusion, the liver was excised, placed on a Petri dish, and doused with ice-cold saline. It was then thoroughly minced, and the resulting mixture was passed sequentially through 253-μm and 73-μm mesh nylon filters. Harvested cells were suspended in frog saline containing 2 mM $CaCl_2$, collected by centrifugation (50 × *g*, 2 min at 4 °C), and then washed and centrifuged thrice using the same medium. Following the final wash, hepatocytes destined for use in freezing experiments were resuspended at a density of 30 mg cells mL^{-1} in frog saline containing 2 mM $CaCl_2$, whereas cells used in glucose production experiments were resuspended at a density of 25 mg cells mL^{-1} in frog saline containing 2 mM $CaCl_2$ and 4.5% BSA. Suspensions were reserved on ice until experiments were conducted. A subsample of each suspension was checked for erythrocyte contamination and assayed for viability by trypan blue exclusion (Mommsen and Storey, '92).

In vitro Freezing

Several 100-μL aliquots of each cell suspension, prepared individually from Ohioan (N = 9) and Alaskan frogs (N = 9), were placed in separate 0.5-mL microcentrifuge tube and mixed with 20 μL frog saline containing 2 μg mL^{-1} of Snomax® Snow Inducer (York Snow, Inc., Centennial, CO, USA), a preparation containing ice-nucleating proteins from *Pseudomonas syringae*; preliminary testing showed this additive reliably initiated freezing of the samples at temperatures between −3 and −3.5 °C. Following a 90-min incubation on ice, samples representing each suspension were kept on ice (unfrozen) or chilled to −4 or −8 °C by placing them in 25-mL glass tubes immersed in a refrigerated ethanol bath (model RTE 140, Neslab Instruments, Portsmouth, NH, USA) whose temperature was reduced from 0 °C at ca. 0.6 °C min^{-1}. We monitored the temperature of the samples during cooling via a thermocouple attached to the tubes and a multichannel data logger (model RD3752, Omega, Stamford, CT, USA). After the samples reached the target temperature, they were removed from the bath, thawed at 4 °C, and assessed for cryoinjury (see below).

We tested the efficacy of cryoprotectants in reducing cryoinjury using additional 100-μL samples of the same cell suspensions. The samples were prepared as described above, except that they also received glucose (final concentration, 120 or 240 mM) or urea (final concentration, 80 or 240 mM). Glucose and urea concentrations were chosen to match plasma levels previously reported for *R. sylvatica* (Costanzo et al., 2013), with the exception of the highest concentration of urea (240 mM), which was chosen to allow us to directly compare the efficacies of the two cryoprotectants. Samples were incubated on ice for 90 min before being frozen to −8 °C as described above. After the target temperature was reached, the samples were removed from the bath, thawed at 4 °C, and assessed for cryoinjury.

Cryoinjury Assessment

Membrane damage in the various 100-μL samples of hepatocyte suspension was inferred from the cells' leakage of lactate dehydrogenase (LDH), a cytoplasmic enzyme, following Storey and Mommsen ('94). Samples were gently centrifuged (50 × *g*, 2 min at 4 °C) and a 55-μL subsample of the supernatant was transferred to a clean tube in which we determined LDH activity using a reagent kit (Pointe Scientific, Canton, MI, USA). The cell pellet (along with remaining supernatant) was twice sonicated with a ultrasonic processor (model CP 130PB-1, Cole-Parmer, Vernon Hills, IL, USA) for 15 sec to release intracellular elements, and the LDH activity in the processed material was then determined using the same assay; subtracting the activity associated with the supernatant from this value gave an estimate of the activity derived from the pellet. We used the ratio of extracellular (supernatant) activity to total (extracellular plus intracellular) activity to estimate LDH leakage in the sample. Duplicate samples were tested in each experimental condition; thus, the ratio used in the statistical analyses was an average of values for the replicates.

Maximal Glucose Production Rate

We investigated maximal rates of glucose production in hepatocytes isolated from the same Ohioan frogs used in the freezing experiments, and from a separate group (N = 8) of Alaskan frogs. Rates were determined from the difference in glucose contents in the samples at the beginning and end of a 90-min incubation on ice; preliminary experiments showed that glucose production rate was constant during this period, as has been previously found (Storey and Mommsen, '94). Glycogen concentration was also measured in the samples before and after the 90-min incubation period (see below).

To conduct the experiment, several 120-μL aliquots of each cell suspension were placed in a separate 0.5-mL microcentrifuge tube to which we added 80 μL of frog saline alone, or frog saline containing one of two adrenergic agonists: epinephrine (Sigma Aldrich, St. Louis, MO, USA), which acts via β-adrenergic receptors (Hemmings and Storey, 94), or 8-Bromo-cAMP (8-Br-cAMP) (Sigma–Aldrich), a relatively stable cAMP analogue that readily traverses the plasma membrane (Scheurich et al., '89). Dose-dependence trials with agonist concentrations ranging from 10^{-9} to 10^{-3} showed that maximal glucose production rates were achieved with 10^{-5} M epinephrine or 10^{-3} M 8-Br-cAMP (data not shown); thus, we selected these concentrations for use in the experiments. Duplicate samples were collected at the beginning (time zero) and end of incubation, deproteinized by adding 70% $HClO_4$, immediately frozen in liquid N_2, and stored at −80 °C until glucose and glycogen assays were conducted.

Glucose and Glycogen Assays

Acidified cell samples were thawed on ice, sonicated twice for 15 sec, and centrifuged (14,000 × *g*, 30 sec at 4 °C). The supernatant was neutralized with KOH and a subsample (100 μL) was

assayed for glucose using a colorimetric assay kit (Pointe Scientific). An additional subsample (100 μL) was incubated with amyloglucosidase (1 mg mL^{-1}) at 40 °C for 2 hr in a 0.2 M sodium acetate buffer, pH 4.8 (Edwards et al., 2000), following which the reaction was stopped by adding cold 70% (w/v) $HClO_4$ and the liberated glucose was assayed; this glucose (less the amount present in the undigested sample) equated to glycogen content. Glycogen concentration was expressed as μmol glucosyl units g^{-1} cells.

Rates of glucose production were determined for each experimental condition (no agonist, epinephrine, or 8-Br-cAMP) from the change in glucose concentration before (time zero) and after incubation. Rates were normalized to the mass of cells present and expressed as μmol g^{-1} h^{-1}. The average of rates determined for two samples per experimental condition was used to represent the response for each frog.

Statistical Analysis

Mean and standard error of the mean (SEM) are the descriptive summaries used for the variables measured. A two-way, repeated-measures analysis of variance (ANOVA) was used to examine the response of experimental variables as a function of population or treatment, or their interaction (population × treatment). Within each population, mean values were compared using a one-way, repeated-measures ANOVA, followed by Bonferroni *post hoc* test. In some cases, a Student's t-test was used to compare means between two select treatment groups. The relationship between basal glucose production rate and initial (time zero) glycogen concentration was tested separately for each population using linear regression. As necessary, data were transformed to fulfill the parametric tests' assumptions. Analyses were performed using JMP (SAS, Cary, NC, USA); significance was accepted at $P < 0.05$.

RESULTS

Hepatocytes isolated from both Alaskan and Ohioan frogs excluded trypan blue (average viability >90%) and contained few red blood cells (<2% of total cells). Cells were responsive to hormonal stimulation for at least 2 hr following isolation, as indicated by the increase in glucose production in our experiments.

In vitro Freezing and Cryoinjury

In hepatocyte samples incubated for 90 min at 0 °C (unfrozen), less than 15% of the total LDH was extracellular (Fig. 1). Freezing increased ($F_{[2,10]} = 362.8$, $P < 0.0001$) this amount to a similar degree in Alaskan and Ohioan frogs (population × treatment, $F_{[2,10]} = 2.9$, $P = 0.1$; Fig. 1), with the value for cells frozen at −8 °C being approximately double that of cells frozen at −4 °C.

Adding glucose to the samples reduced LDH leakage with freezing ($F_{[2,10]} = 93.0$, $P < 0.0001$; Fig. 2), the effect being similar between populations (population × treatment, $F_{[2,10]} = 2.0$, $P = 0.18$). For Ohioan frogs, incubation of hepatocytes with 240 mM glucose reduced the LDH leakage occurring at −8 °C to half of that observed in samples without added cryoprotectant; for Alaskan frogs, the reduction was ca. 40% with 120 mM glucose and 60% with 240 mM glucose. Urea also had a cryoprotective effect in cells from both Alaskan ($F_{[2,4]} = 69.8$, $P = 0.0008$) and Ohioan frogs ($F_{[2,5]} = 6.1$, $P = 0.04$), although the former better tolerated freezing (population × treatment, $F_{[2,10]} = 9.8$, $P = 0.004$), albeit only at the higher concentration tested (Fig. 3). Incubation of cells in urea or glucose at a

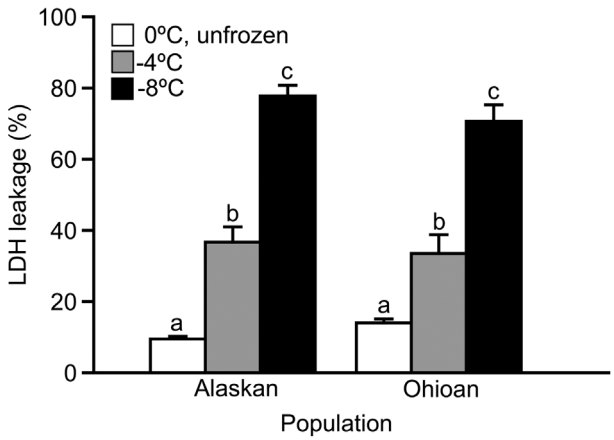

Figure 1. Cryoinjury in hepatocytes isolated from Alaskan and Ohioan wood frogs (*Rana sylvatica*) as assessed by LDH leakage. Within each population, means (± SEM; N = 6–7) identified by different letters were statistically distinguishable (Bonferroni *post hoc*, $P < 0.05$).

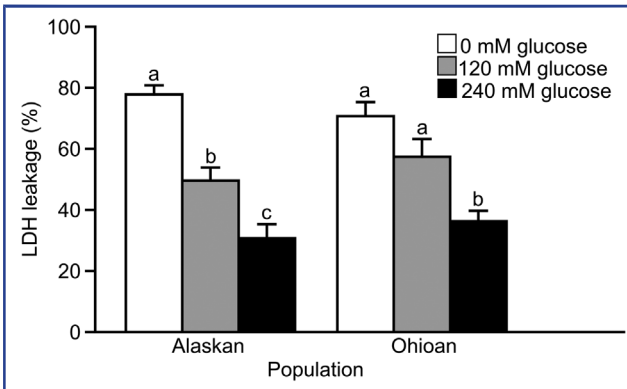

Figure 2. Cryoinjury in hepatocytes isolated from Alaskan and Ohioan wood frogs (*Rana sylvatica*) and frozen at −8 °C in frog saline with 0, 120 or 240 mM glucose. Within each population, means (± SEM; N = 6–7) identified by different letters were statistically distinguishable (Bonferroni *post hoc*, $P < 0.05$).

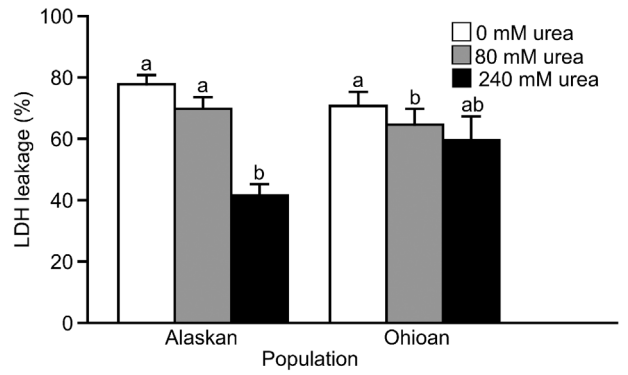

Figure 3. Cryoinjury in hepatocytes isolated from Alaskan and Ohioan wood frogs (*Rana sylvatica*) and frozen at −8 °C in frog saline with 0, 80 or 240 mM urea. Within each population, means (± SEM; N = 6–7) identified by different letters were statistically distinguishable (Bonferroni *post hoc*, $P < 0.05$).

concentration of 240 mM was equally effective ($t = 2.5$, d.f. = 5, $P = 0.05$) in cryopreserving cells of Alaskan frogs. However, in cells from Ohioan frogs, LDH leakage with urea was markedly higher than with glucose (60 ± 8% vs. 36 ± 3%; $t = 3.2$, d.f. = 6, $P = 0.019$).

Maximal Glucose Production Rate

Freshly-isolated cells produced glucose even without added agonist, as observed by Mommsen and Storey ('92). Hepatocytes from Alaskan frogs contained nearly twice as much glycogen as those from Ohioan frogs ($t = 2.4$, d.f. = 13, $P = 0.034$), but similar amounts of glucose ($t = 0.3$, d.f. = 13, $P = 0.75$; Table 1). The basal rate of glucose production, which did not differ between populations ($t = 1.0$, d.f. = 13, $P = 0.34$), was positively correlated with glycogen concentration in Ohioan frogs ($R^2 = 0.7$, $F_{[1,6]} = 12.1$, $P = 0.013$), but not in Alaskan frogs ($R^2 = 0.03$, $F_{[1,5]} = 0.2$, $P = 0.69$).

Incubation of hepatocytes with either agonist increased the rate of glucose production ($F_{[2,12]} = 63.4$, $P < 0.0001$; Fig. 4), with the cells of Alaskan and Ohioan frogs responding similarly (population × treatment, $F_{[2,12]} = 0.3$, $P = 0.74$). The increase over basal rates with epinephrine stimulation was similar ($t = 0.4$, d.f. = 11, $P = 0.39$) between Alaskan and Ohioan frogs, being 6.0- and 7.3-fold, respectively. Similarly, with stimulation by 8-Br-cAMP there was no difference ($t = 0.5$, d.f. = 11, $P = 0.61$) in the magnitude of increase of glucose production in cells of Alaskan (4.6-fold) and Ohioan (5.3-fold) frogs. We found no significant effect of either agonist on glycogen levels in hepatocytes from Alaskan ($F_{[3,11]} = 1.0$, $P = 0.34$) or Ohioan ($F_{[3,11]} = 2.2$, $P = 0.14$) frogs, as the glycogen concentrations determined for these samples were similar to those measured before incubation.

DISCUSSION

Extreme freeze tolerance in subarctic *R. sylvatica* derives from myriad adaptations including an enhanced cryoprotectant system (Costanzo et al., 2013; do Amaral et al., 2013; Costanzo et al., 2014; Rosendale et al., 2014). Our primary objective in the present study was to determine whether this enhanced tolerance is supported by a superior ability of hepatocytes to withstand freezing and rapidly produce glucose, such as occurs during the early hours of freezing. Cells isolated from the liver of *R. sylvatica* maintain a high degree of structural and metabolic integrity long after their harvest, making them a suitable system to study freezing survival and glucose production *in vitro* (Mommsen and Storey, '92; Storey and Mommsen, '94).

Effect of Freezing on Isolated Hepatocytes

We determined hepatocyte cryoinjury from LDH leakage, rather than dye exclusion, as it is a more expedient, sensitive, and quantitative metric for assessing the effect of freezing and thawing stress on cells (Storey and Mommsen, '94). In the absence of added cryoprotectants, freezing injury to hepatocytes of both Alaskan and Ohioan frogs was considerable, as, for example, LDH leakage from cells frozen at −4 °C increased over that in cells held on ice (0 °C) by an average of 3.1 fold. An identical response was seen in hepatocytes isolated from *R. sylvatica* indigenous to eastern Ontario, Canada (Storey and Mommsen, '94). In cells from our frogs, freezing at −8 °C caused even more cryoinjury, consistent with the greater stress the cells would encounter at this lower temperature. Our values for LDH leakage at −8 °C (ca. 75%) are substantially higher than those determined by Storey and

Table 1. Metabolite contents and glucose production rates of hepatocytes isolated from Ohioan and Alaskan wood frogs, *Rana sylvatica*.

	Ohioan	Alaskan
Glycogen (μmol glucosyl units g^{-1} cells)	216 ± 67	426 ± 35*
Glucose (μmol g^{-1} cells)	1.4 ± 0.3	1.6 ± 0.4
Glucose production (μmol g^{-1} cells h^{-1})	1.5 ± 0.5	2.9 ± 0.8
N	8	7

Variables were compared between populations using Student's t-test; asterisk indicates that the value differed between populations ($P = 0.034$).
*Values are mean ± SEM.

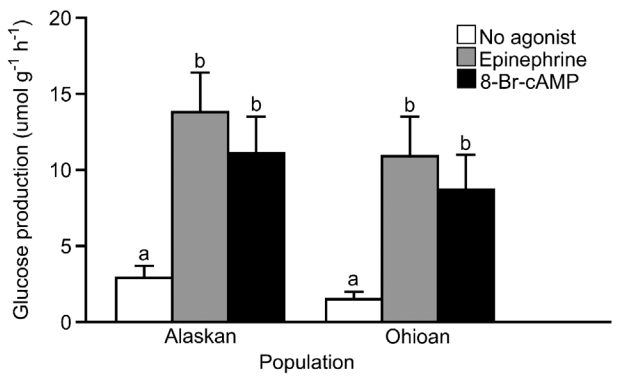

Figure 4. Glucose production in hepatocytes isolated from Alaskan and Ohioan wood frogs (*Rana sylvatica*) and incubated in frog saline without agonist, or in saline containing 10^{-5} M epinephrine or 10^{-3} M 8-Br-cAMP. Within each population, means (± SEM; N = 7–8) identified by different letters were statistically distinguishable (Bonferroni *post hoc*, $P < 0.05$).

Mommsen ('94) for hepatocytes from Canadian *R. sylvatica*, although this disparity probably reflects differences in experimental protocols rather than phenotypic variation. In particular, the cell suspensions prepared from the Canadian frogs contained BSA, an effective cryoprotectant (Matsuoka et al., 2006), which was intentionally omitted from our preparations.

Resistance to freezing damage depends on the cells' concentration of cryoprotectants, membrane fluidity, and tolerance to osmotic stress, among other things (Storey and Storey, 2004; Overgaard et al., 2009; Costanzo and Lee, 2013). Our Alaskan frogs presumably had exceptionally high concentrations of urea at the time they were sampled (see Costanzo et al., 2013), although probably much of this cryoprotectant diffused out of hepatocytes during the saline/Liberase perfusion and multiple washes performed throughout the isolation procedure. Furthermore, because glycogenolysis is not enhanced in isolated hepatocytes during freezing (Storey and Mommsen, '94), cells from both Alaskan and Ohioan frogs probably had similar, low levels of glucose, as well. Our finding that cryoinjury was comparable between these groups suggests that the extreme freeze tolerance of Alaskan frogs does not require a superior intrinsic capacity of hepatocytes to withstand cryoinjury, even though these cells, which are critical to mounting a cryoprotective response, play a central role in the freezing survival of the whole animal. Storey and Mommsen ('94) reported that hepatocytes isolated from *R. sylvatica* and the leopard frog (*Rana pipiens*) tolerate freezing at −4 °C equally well, despite the fact that *R. pipiens* is decidedly intolerant of somatic freezing; thus, the results of *in vitro* experiments with hepatocytes cannot necessarily be extrapolated to the *in vivo* condition. However, it is possible that other tissues, such as nervous tissue, when frozen *in vitro*, may better reflect organismal freeze tolerance. Notwithstanding, variation in freeze tolerance among species, or populations within species, likely involves interplay among various factors, such as tissue-specific responses to freezing and thawing stress, as well as efficiency of cryoprotectant production and distribution (Storey and Storey, 2004; Costanzo and Lee, 2013).

Glucose and urea are the two major cryoprotectants in *R. sylvatica*, with mean hepatic concentrations in Alaskan frogs experimentally frozen to −2.5 °C reaching 194 and 160 μmol g^{-1} fresh tissue, respectively (Costanzo et al., 2013). Both agents act colligatively to reduce extracellular ice formation, but each also provides certain other benefits, such as membrane stabilization, glycolytic fuel supply, antioxidation, metabolic depression, and protection of macromolecules from iono-osmotic perturbation (Storey and Storey, '88; Costanzo and Lee, 2008; Costanzo and Lee, 2013). Hepatocytes isolated from this species are metabolically active and continue to synthesize both osmolytes; however, given the modest *in vitro* rates of production of glucose (Table 1) and urea (Storey and Mommsen, '94), the prefreeze accrual of each solute in our experiments would be minimal (i.e., <5 mM). These cells readily take up glucose from an extracellular medium (Storey and Storey, 2004), and apparently import urea as well (Costanzo and Lee, 2005), so it is reasonable to assume that the intracellular concentration of cryoprotectant they ultimately attained was proportional to its concentration in the incubation medium. However, a limited availability of cells precluded us from determining the actual intracellular concentrations of these osmolytes.

Glucose is a relatively uncommon cryoprotectant among freeze-tolerant animals, although its efficacy in reducing cryoinjury in *R. sylvatica* has been amply demonstrated at cellular, tissue, and organismal levels of organization (Storey, '88; Costanzo and Lee, 2013). Our present results provide additional evidence of its protective action on cells of this species. Raising the glucose concentration of the incubation medium from 120 to 240 mM further diminished LDH leakage from hepatocytes, consistent with the tenet that glucose acts to colligatively reduce ice formation and limit cellular dehydration. A previous study documented the efficacy and concentration dependence of this agent in cryopreserving isolated hepatocytes from Canadian *R. sylvatica* (Storey and Mommsen, '94). Our finding that glucose provided equal protection to cells from Alaskan and Ohioan frogs implies that its mechanism(s) of action is similar between these populations.

Like glucose, urea was effective in reducing LDH leakage from hepatocytes frozen *in vitro*, although at the higher concentration (240 mM) the benefit was substantially greater in cells from Alaskan frogs. Conceivably, this could reflect a greater permeation of exogenous urea into the cells of Alaskan frogs. On the other hand, cryoprotectants can vary in efficacy among species (e.g., Costanzo et al., '93) and even among tissues of the same individual (e.g., Costanzo and Lee, 2005), so urea's superior effect in cells from

the northern phenotype could be an adaptive phenomenon. Composition of the hepatocyte membrane differs between Alaskan and Ohioan frogs (Reynolds et al., 2014), that of the former ostensibly being more fluid and potentially more amenable to cryoprotection by urea. Urea may be an especially effective cryoprotectant in northern populations of *R. sylvatica*, which naturally accrue exceptionally high levels of this compound during the pre-hibernal period (Costanzo et al., 2013; Costanzo et al., 2014). Future studies should evaluate this notion by determining whether urea's superior efficacy in northern frogs extends to other tissues and the whole-organism level.

Maximal Glucose Production Rate

At the onset of freezing, glycogenolysis in hepatocytes is heightened, rapidly generating copious amounts of glucose in both Alaskan and Ohioan frogs (do Amaral et al., 2013). Although the triggering mechanism of freezing-induced glycogenolysis has not been elucidated, it likely involves β-adrenergic stimulation of the liver (Storey and Storey, '96), which accelerates glycogenolysis through an increased activation of specific enzymes involved in the process (Storey and Storey, 2004). Glycogenolysis rate is influenced by the amount and activity of glycogen phosphorylase, a key enzyme in the pathway, as well as the abundance of its substrate. In some systems, such as fish liver, the rate of glycogenolysis is dependent on glycogen content, presumably because cells with low glycogen concentrations have reduced amounts of phosphorylase (Mommsen, '86; Pereira et al., '95). We found no correlation between glycogen concentration and glycogenolytic rate in hepatocytes from Alaskan *R. sylvatica*, and neither did Mommsen and Storey ('92) for Canadian *R. sylvatica*. However, in our Ohioan frogs the two variables were positively correlated. The reason for this difference and its significance are unclear, although earlier work indicates that the livers of unfrozen Ohioan and Alaskan frogs have similar amounts of phosphorylase activity (do Amaral et al., 2013). Ultimately, given their exceptionally large hepatic glycogen reserves, northern *R. sylvatica* have the greater potential to mobilize glucose during freezing (Costanzo et al., 2014).

Stimulation of isolated hepatocytes with epinephrine or 8-Br-cAMP induced a similar, marked increase in glucose production in Alaskan and Ohioan frogs. We did not detect a significant reduction in glycogen content, perhaps due to high variability among samples and the relatively brief incubation used in our experiments. Nevertheless, we presume that the *de novo* glucose resulted primarily from glycogenolysis, as the suspension medium lacked gluconeogenic substrates and rates of gluconeogenesis from endogenous lactate or alanine reportedly are very low in *R. sylvatica* (Mommsen and Storey, '92).

In a study similar to ours, both epinephrine and dibutyryl-cAMP doubled the rate of glucose production in hepatocytes isolated from Canadian *R. sylvatica* (Mommsen and Storey, '92). The rates determined in that study were nominally higher than those we observed, although this disparity probably is attributable to methodological inconsistencies, such as differences in acclimatization regimen and the dosage and specific agonist used. Because our purpose was to determine if hepatocytes of Alaskan and Ohioan frogs differed in their ability to rapidly mobilize glucose, we chose concentrations of agonists that gave maximal rates of glucose production in both populations. Overall, our results imply that subarctic *R. sylvatica* do not have a superior ability to rapidly mobilize glucose during freezing. However, because *in vitro* experiments may not accurately replicate conditions *in vivo*, these results should be interpreted with caution. Indeed, during the early stages of freezing, intact Alaskan frogs have a higher activity of glycogen phosphorylase in liver, and mobilize glucose faster, as compared to Ohioan frogs (do Amaral et al., 2013). Our present findings suggest that this advantage derives not from superior enzymatic function, but rather from a stronger adrenergic stimulus and/or a higher population of hepatic β-adrenergic receptors in the northern phenotype.

ACKNOWLEDGMENTS

We thank Andrew Rosendale and Alice Reynolds for aiding with frog and tissue collection, and David Russell for aiding with the logistical challenges of collecting frogs in Alaska. We thank Michael Hughes from the Statistical Consulting Center of Miami University for assistance with statistical analyses. We thank Andrew Rosendale for providing constructive comments on an earlier version of the manuscript.

LITERATURE CITED

Costanzo JP, Lee RE. 2005. Cryoprotection by urea in a terrestrially hibernating frog. J Exp Biol 208:4079–4089.

Costanzo JP, Lee RE. 2008. Urea loading enhances freezing survival and postfreeze recovery in a terrestrially hibernating frog. J Exp Biol 211:2969–2975.

Costanzo JP, Lee RE. 2013. Avoidance and tolerance of freezing in ectothermic vertebrates. J Exp Biol 216:1961–1967.

Costanzo JP, Lee RE, Wright MF. 1992. Cooling rate influences cryoprotectant distribution and organ dehydration in freezing wood frogs. J Exp Zool 261:373–378.

Costanzo JP, Lee RE, Lortz PH. 1993. Physiological responses of freeze-tolerant and intolerant frogs: clues to evolution of anuran freeze tolerance. Am J Physiol 265:R721–R725.

Costanzo JP, do Amaral MCF, Rosendale AJ, Lee RE. 2013. Hibernation physiology, freezing adaptation and extreme freeze tolerance in a northern population of the wood frog. J Exp Biol 216:3461–3473.

Costanzo JP, do Amaral, Rosendale MCF, Lee AJ. 2014. Seasonality of freeze tolerance in a subarctic population of the wood frog, *Rana sylvatica*. Int J Zool 1–13.

do Amaral MCF, Lee RE, Costanzo JP. 2013. Enzymatic regulation of glycogenolysis in a subarctic population of the wood frog: implications for extreme freeze tolerance. PLoS ONE 8:e79169.

Edwards JR, Koster KL, Swanson DL. 2000. Time course for cryoprotectant synthesis in the freeze-tolerant chorus frog, *Pseudacris triseriata*. Comp Biochem Physiol A Mol Integr Physiol 125:367–375.

Larson DJ, Middle L, Vu H, et al. 2014. Wood frog adaptations to overwintering in Alaska: new limits to freezing tolerance. J Exp Biol 217:2193–2200.

Martof BS, Humphries RL. 1959. Geographic variation in the wood frog *Rana sylvatica*. Am Mid Nat. 61:350–389.

Matsuoka T, Imai H, Kohno H, Fukui Y. 2006. Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. J Reprod Dev 52:675–683.

Mommsen TP. 1986. Comparative gluconeogenesis in hepatocytes from salmonid fishes. Can J Zool 64:1110–1115.

Mommsen TP, Storey KB. 1992. Hormonal effects on glycogen metabolism in isolated hepatocytes of a freeze-tolerant frog. Gen Comp Endocrinol 87:44–53.

Mommsen TP, Moon TW, Walsh PJ. 1994. Hepatocytes: isolation, maintenance and utilization. In: Hochachka PW, Mommsen TP, editors. Biochemistry and molecular biology of fishes. Amsterdam: Elsevier B.V. p 355–372.

Overgaard J, Tollarova M, Hedlund K, Petersen SO, Holmstrup M. 2009. Seasonal changes in lipid composition and glycogen storage associated with freeze-tolerance of the earthworm *Dendrobaena octaedra*. J Comp Physiol B 179:569–577.

Pereira C, Vijayan MM, Storey KB, Jones RA, Moon TW. 1995. Role of glucose and insulin in regulating glycogen synthase and phosphorylase activities in rainbow trout hepatocytes. J Comp Physiol B 165:62–70.

Reynolds AM, Lee RE, Costanzo JP. 2014. Membrane adaptation in phospholipids and cholesterol in the widely distributed, freeze-tolerant wood frog, *Rana sylvatica*. J Comp Physiol B 184: 371–383.

Rosendale AJ, Philip BN, Lee RE, Costanzo JP. 2014. Cloning, characterization, and expression of glucose transporter 2 in the freeze-tolerant wood frog, *Rana sylvatica*. Biochem Biophys Acta 1840:1701–1711.

Scheurich P, Kobrich G, Pfizenmaier K. 1989. Antagonistic control of tumor necrosis factor receptors by protein kinases A and C. Enhancement of TNF receptor synthesis by protein kinase A and transmodulation of receptors by protein kinase C. J Exp Med 170:947–958.

Storey KB, Mommsen TP. 1994. Effects of temperature and freezing on hepatocytes isolated from a freeze-tolerant frog. Am J Physiol 266: R1477–R1482.

Storey KB, Storey JM. 1988. Freeze tolerance in animals. Physiol Rev 68:27–84.

Storey JM, Storey KB. 1996. β-Adrenergic, hormonal, and nervous influences on cryoprotectant synthesis by liver of the freeze-tolerant wood frog *Rana sylvatica*. Cryobiology 33:186–195.

Storey KB, Storey JM. 2004. Physiology, biochemistry and molecular biology of vertebrate freeze tolerance: the wood frog. In: Benson E, Fuller B, Lane N, editors. Life in the frozen state. Boca Raton, FL: CRC Press. p 243–274.