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RESEARCH ARTICLE

Hibernation physiology, freezing adaptation and extreme freeze tolerance in a northern population of the wood frog

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SUMMARY

We investigated hibernation physiology and freeze tolerance in a population of the wood frog, *Rana sylvatica*, indigenous to Interior Alaska, USA, near the northernmost limit of the species' range. Winter acclimatization responses included a 233% increase in the hepatic glycogen depot that was subsidized by fat body and skeletal muscle catabolism, and a rise in plasma osmolality that reflected accrual of urea (to 106±10 µmol ml^{-1}) and an unidentified solute (to ~73 µmol ml^{-1}). In contrast, frogs from a cool-temperate population (southern Ohio, USA) amassed much less glycogen, had a lower uremia (28±5 µmol ml^{-1}) and apparently lacked the unidentified solute. Alaskan frogs survived freezing at temperatures as low as –16°C, some 10–13°C below those tolerated by southern conspecifics, and endured a 2-month bout of freezing at –4°C. The profound freeze tolerance is presumably due to their high levels of organic osmolytes and bound water, which limits ice formation. Adaptive responses to freezing (–2.5°C for 48 h) and subsequent thawing (4°C) included synthesis of the cryoprotectants urea and glucose, and dehydration of certain tissues. Alaskan frogs differed from Ohioan frogs in retaining a substantial reserve capacity for glucose synthesis, accumulating high levels of cryoprotectants in brain tissue, and remaining hyperglycemic long after thawing. The northern phenotype also incurred less stress during freezing/thawing, as indicated by limited cryohemolysis and lactate accumulation. Post-glacial colonization of high latitudes by *R. sylvatica* required a substantial increase in freeze tolerance that was at least partly achieved by enhancing their cryoprotectant system.

Key words: anuran, cryoprotectant, ecogeography, freeze tolerance, *Rana sylvatica.*

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INTRODUCTION

Several species of Holarctic amphibians and reptiles survive winter's cold by tolerating the freezing of their body tissues. Although some of the mechanisms underpinning freezing adaptation in vertebrate ectotherms are understood, relatively little is known about the factors driving evolution of this trait. Efforts directed thereto might profitably focus on anuran amphibians, which generally exhibit strong clinal variation in cold tolerance (Brattstrom, 1968; Snyder and Weathers, 1975). The wood frog, *Rana sylvatica* (LeConte 1825), is a singularly useful species in such studies owing to its broad geographic range, which extends to 69°N, within the Arctic Circle (Martof and Humphries, 1959).

Throughout its range, *R. sylvatica* overwinters in relatively exposed sites on the forest floor, where it potentially encounters subzero temperatures but nevertheless survives the freezing of up to two-thirds of its body water (Costanzo and Lee, 2013; Storey and Storey, 2004). Freezing adaptation in northern populations of this species has not been thoroughly examined, although preliminary findings indicate that frogs collected near Fairbanks, Alaska, USA, tolerate profound freezing, with many surviving exposure to temperatures below –18°C (Middle and Barnes, 2001). Within their hibernacula, indigenous frogs are commonly exposed to –10°C or below (Middle and Barnes, 2000), consistent with conditions within subnivean habitats of Interior Alaska (Barnes et al., 1996). In contrast, *R. sylvatica* endemic to the Great Lakes Region in North America encounter more modest hibernal temperatures and tolerate freezing only to –3 to –6°C (Costanzo and Lee, 2013). Discovering the physiological basis of this variation could provide important clues to the evolution of the freeze-tolerance adaptation.

One reasonable expectation is that frogs from colder environments possess enhanced mechanisms of cryoprotection, including a greater accumulation of colligatively active solutes. Two osmolytes paramount in freezing survival of *R. sylvatica* are urea, which accumulates during fall and early winter, and glucose, which is quickly and copiously mobilized from hepatic glycogen reserves in direct response to freezing. Both agents limit freezing injury by colligatively lowering the equilibrium freezing/melting point (FP_{eq}) of body fluids (and, hence, limiting ice formation), and by preserving the integrity of membranes and macromolecules (Costanzo and Lee, 2013; Storey and Storey, 2004). Cryoprotection is also conferred by a redistribution of water and its sequestration as ice within coelomic and lymphatic compartments, a process that limits intra-organ ice formation that could physically damage cells and tissues (Lee and Costanzo, 1998).

In this project, we investigated physiological preparations for hibernation, capacity for freeze tolerance, and freezing adaptation in a northern population of *R. sylvatica* that must endure extreme cold during an extended period of overwintering. For perspective, we also examined responses of conspecifics from a more temperate part of the species' geographic range.

MATERIALS AND METHODS

Experimental animals and acclimatization

We collected *R. sylvatica* from Fairbanks North Star Borough, near Fairbanks, Alaska, USA (64.8°N, 147.7°W; Fig. 1), during early

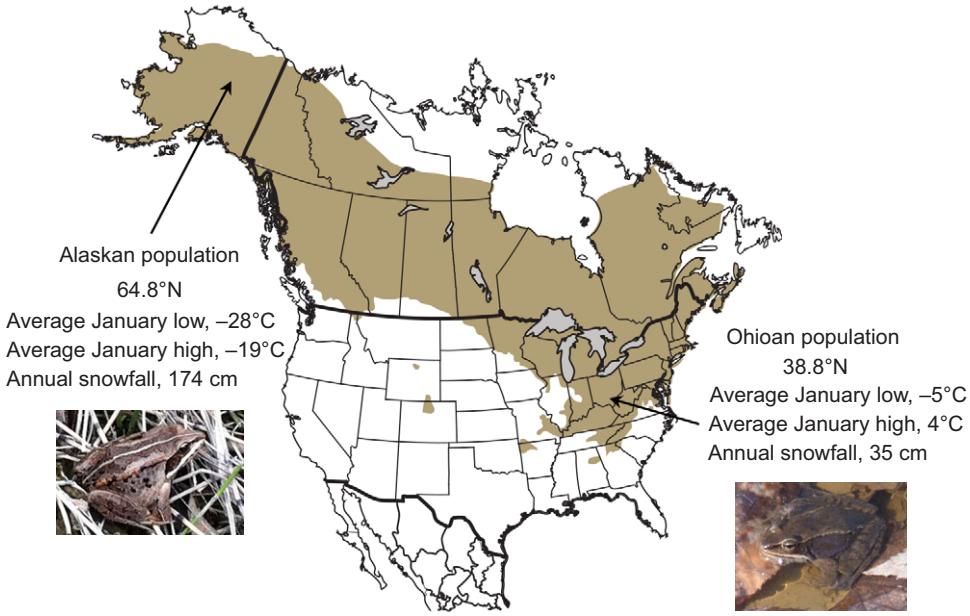

Fig. 1. Geographic range of *Rana sylvatica* (shading) and sources of animals used in the study, with key climatic variables of the collecting locales. Average daily air temperature and snowfall data were obtained from local meteorological stations.

August 2011. Frogs were topically treated with tetracycline HCl, placed in plastic cups containing a moist paper towel, and shipped under refrigeration to Miami University, where they were transferred individually to a clean plastic cup and held on damp paper. Several of these frogs were sampled promptly (hereafter 'late-summer frogs'), but most were inducted into a dormant state (winter acclimatized) by housing them in a programmable environmental chamber (model I-35X, Percival, Boone, IA, USA) and exposing them to dynamic, diel cycles of temperature and full-spectrum lighting, which, based on institutional records of weather, were seasonally appropriate to their origin. At the start of this 5-week regimen, temperature varied daily from 17.0 to 8.0°C and the photophase was 16.5 h; at its end, in mid-September, temperature varied daily from 13.0 to 2.5°C and the photophase was 13.3 h. Frogs were fed *ad libitum* with crickets dusted with a vitamin supplement (ReptoCal, Tetrafauna, Blacksburg, VA, USA), although most refused food after the first week in September. Following acclimatization, several frogs were sampled immediately (hereafter 'fall frogs'), whereas most were kept at 4°C, in darkness, for ~8 weeks in simulated hibernation until used in experiments in mid-November (hereafter 'winter frogs').

Wood frogs from a cool-temperate population were collected in late winter (21 February 2011) from vernal breeding pools in Adams County, south-central Ohio, USA (38.8°N, 83.3°W; Fig. 1). They were brought to laboratory facilities and kept, unfed, on damp moss within darkened plastic boxes (4°C) for the next 3 weeks. Thereafter they were kept in a 48 m^2 pen at the Ecology Research Center (39.5°N, 84.7°W), Miami University, until autumn. The pen was situated in a mature, deciduous woodlot and provided herbaceous cover and cool, moist conditions. Frogs had access to a pool of water and were fed vitamin-fortified crickets two to three times weekly, although this diet was supplemented by a host of arthropods that was attracted to a UV-A-emitting light. Feeding was suspended in late October. In November, frogs, on the verge of dormancy, were recaptured and placed inside covered, opaque boxes that were kept in a cold room (4°C). They remained quiescent and buried under sheets of damp moss until used in experiments 8 weeks later.

Frogs were collected using approved methods under appropriate permits issued by cognizant governmental agencies. Rearing and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Miami University.

Acclimatization experiment

We investigated the physiological changes in Alaskan frogs during preparation for hibernation by comparing morphometrics and select blood and tissue metabolite concentrations among late-summer, fall and winter frogs. Winter-acclimatized Ohioan frogs were also sampled for comparative purposes. Winter samples also served as reference (unfrozen) groups in the freeze/thaw time-course experiment.

Freeze/thaw time-course experiment

Winter frogs from both populations were experimentally frozen and thawed following a protocol (Costanzo et al., 1992) that facilitates cryoprotective responses, promotes survival and presumably mimics natural freezing and thawing episodes (i.e. slow freezing followed by gradual warming). They were purged of any bladder fluid *via* cloacal canulation, weighed (to 0.1 g) and individually placed inside a 50 ml polypropylene tube. A thermocouple placed against the abdomen allowed us to chart body temperature (T_b) at 30 s intervals on a multichannel data logger (model RD3752, Omega, Stamford, CT, USA). Tubes containing these frogs were plugged with flexible plastic foam and suspended in a refrigerated bath (model RTE 140, Neslab, Portsmouth, NH, USA) containing chilled ethanol. After each frog became supercooled (T_b approximately –1°C), we seeded the freezing of its tissues by applying aerosol coolant to the tube's exterior. Freezing continued for as long as 48 h as the frogs gradually cooled (0.05°C h^{-1}) to the ultimate T_b, –2.5°C, which was reached ~30 h after freezing commenced. Groups of frogs (N=4–6) were removed from the bath at intervals (2, 6, 30 or 48 h) and immediately euthanized to provide tissues for analysis. Additional frogs were frozen for 48 h, gently removed from their tubes and held on damp paper at 4°C, in darkness, for either 6 h or 5 days before being euthanized and sampled. A reference (unfrozen) group comprised frogs sampled directly from their containers in the cold room.

Morphometrics and physiological assays

Working inside a refrigerated (4°C) room, each frog was purged of any urine *via* cloacal cannulation, weighed (to 0.1 g), euthanized by

double-pithing, measured to determine snout–ischium length (to 0.1 mm) and dissected. Blood was immediately drawn into heparinized microcapillary tubes from an incision in the aortic trunk (or ventricle of still-frozen frogs) and centrifuged (2000 ***g***, ~5 min) in order to determine hematocrit and isolate the plasma, which was immediately frozen in liquid N_2.

We quickly excised the liver, heart, brain and two muscles, the gastrocnemius and the gracilis, from the right hindlimb. Mass of the intact liver was determined (to 0.01 g) after lightly blotting the organ on laboratory tissue. Destined for metabolite assays, portions of the liver and gracilis, and also the entire heart and brain, were immediately frozen in liquid N_2. Other portions of the liver and gracilis, plus the intact gastrocnemius, were blotted to remove excess surface moisture, weighed (to 0.01 mg), placed in a 65°C oven and reweighed after thoroughly dried. Initial water concentration in these samples was estimated by dividing the mass lost upon drying by the mass of the dried residue; an insufficient amount of tissue prohibited us from determining the hydration state of the heart and brain. Water concentration of the liver sample was used to estimate mass of the entire, dry liver; in turn, this value was used to compute the hepatosomatic index (HSI; g dry liver g^{-1} dry body×100) and hepatic glycogen content (μmol g^{-1} dry liver×g dry liver). We removed and weighed (to 0.01 mg) any coelomic fat body. The carcass was then weighed (to 0.01 g) and thoroughly dried in the same oven so that its water concentration could be determined. Extrapolating this result permitted us to estimate the mass of the dry, intact body, which was then used to compute glycogen richness by dividing the hepatic glycogen content (μmol) by the mass of the dry body (g). Using dial calipers we measured the length of the tibiofibula (to 0.1 mm) of the right hindlimb; this value was used to normalize mass of the dried gastrocnemius to body size.

Plasma and organ samples were stored at –80°C before metabolite analyses were carried out. Deproteinized organ extracts were prepared by homogenizing samples in cold 7% (w/v) perchloric acid and then neutralizing the aqueous portion of the homogenate with KOH. These extracts, plus an aliquot of plasma, were assayed for urea, glucose and lactate using urease, glucose oxidase and lactate oxidase procedures (Pointe Scientific, Canton, MI, USA), respectively; concentrations of these metabolites were expressed as μmol ml^{-1} plasma or μmol g^{-1} fresh tissue. Extracts of liver and muscle (gracilis) were also assayed for glycogen using an enzymatic procedure. A portion of whole-tissue homogenate (100 μl) was neutralized with KOH and incubated at 40°C for 2 h in a 0.2 mol l^{-1} sodium acetate buffer, pH 4.8, with amyloglucosidase (1 mg ml^{-1}). Following incubation, the reaction was stopped by adding cold 7% (w/v) perchloric acid and the free glucose was determined as described above; glycogen concentration was expressed as glucosyl units (μmol g^{-1} dry tissue or μmol mg^{-1} protein) after subtraction of the initial free glucose. Plasma osmolality was measured by vapor-pressure osmometry (model 5520, Wescor, Logan, UT, USA) or freezing point-depression osmometry (model 3320, Advanced Instruments, Norwood, MA, USA) using appropriate NaCl standards.

In the acclimatization experiment, we used a colorimetric assay to measure glutamate dehydrogenase (GDH) activity in the gracilis. Frozen muscle samples (~25 mg) were weighed (to 0.01 mg) and homogenized in 0.5 ml imidazole buffer (50 mmol l^{-1} imidazole at pH 7.5, 100 mmol l^{-1} NaF, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} EGTA, 0.1 mmol l^{-1} PMSF, 15 mmol l^{-1} β-mercaptoethanol) with a mincing-blade homogenizer. Homogenates were centrifuged at 13,000 ***g*** for 10 min. GDH activity was determined using an assay kit (BioVision, Mountain View, CA, USA) that can detect activity as low as 0.01 mU. The assay was run per kit instructions using 50 μl of sample; optical density was read at 450 nm with a SPECTRAmax PLUS^{384} microplate reader (Molecular Devices, Sunnyvale, CA, USA) before and after incubation at 37°C for 60 s. GDH activity was expressed as mU mg^{-1} protein, with 1 U representing the amount of enzyme generating 1.0 μmol NADH min^{-1} at 37°C, pH 7.6. Total protein in muscle homogenates was determined using the Bradford method (BioRad, Hercules, CA, USA) with bovine serum albumin as the standard.

For frogs used in the freeze/thaw time-course experiment, we measured free hemoglobin (Hb) in plasma using a modification of the Drabkin's reagent protocol (Sigma-Aldrich, St Louis, MO, USA). The assay was performed in a 96-well plate containing 10 μl plasma and 190 μl Drabkin's solution, with human Hb (H7379, Sigma-Aldrich) as the standard. The reaction was incubated at room temperature for 20 min before the absorbance at 540 nm was read with the aforementioned microplate reader. Hb concentration (mg ml^{-1}) was determined from a standard curve and then adjusted to match the sample volume/dilutant volume ratio from the original protocol.

Freeze tolerance trials

We examined freeze tolerance in a single group (N=10) of winter Alaskan frogs. These subjects were removed from their holding cups and placed, in separate groups of five frogs, amongst several layers of floral sheet moss inside each of two opaque plastic boxes (0.0162 m^3). The moss had been previously chilled (4°C) and hydrated with dechlorinated water that prevented the frogs from dehydrating and, once frozen, provided ice crystals to reliably initiate tissue freezing through contact inoculation. We placed crushed ice on the moss, closed the boxes with a lid and transferred them to a programmable incubator (Percival, model I-35X) set at 0°C. Temperature was recorded using several microprocessor-based loggers (Tidbit, Onset Computer Corporation, Pocasset, MA, USA) placed inside the incubator and within each box, both on top of and within the sheets of moss.

In the first trial, all 10 frogs were cooled (0.05°C h^{-1}) to –8°C, a process requiring 160 h, and held at that temperature an additional 6 h before being warmed to 4°C over the ensuing 24 h and, finally, examined for viability. In a second trial, which began following an ~30 h hiatus, nine of the original frogs were randomly assigned to either of two groups and cooled (0.05°C h^{-1}) to minima of –12°C (N=5) or –16°C (N=4). As before, frogs were held at either target temperature for 6 h; thus, the total time they spent frozen before rewarming to 4°C was 246 and 326 h, respectively.

We next used these frogs to test survival of prolonged freezing at a relatively mild temperature. This experiment was carried out as described above, except that the eight available frogs, which were evenly distributed between two boxes, were cooled (0.05°C h^{-1}) to –4°C over 80 h and then held at that temperature for either 8 or 12 weeks. Both boxes contained a central partition that separated two frogs previously exposed to –12°C from two frogs previously exposed to –16°C, thus enabling us to associate each individual's survival status with its thermal history. In all trials, we allowed frogs up to 7 days to demonstrate the righting reflex, our ultimate survival criterion, before determining survival status.

Statistical inferences

For the acclimatization experiment, seasonal variation in somatic and physiological variables in Alaskan frogs was tested using ANOVA, followed by the Student–Newman–Keuls multiple comparisons test. Means for winter Alaskan and Ohioan frogs were

compared using Student's *t*-tests. For the freeze/thaw time-course experiment, group means were compared within each population using ANOVA; samples of frozen or thawed frogs were distinguished from unfrozen frogs using Dunnett's *post hoc* test. Two-factor ANOVA was used to compare populations, with pairs of means distinguished using Bonferroni tests. Data sets were tested to ensure they met parametric assumptions of normality and homoscedasticity; those that did not were transformed or, if necessary, analyzed using nonparametric Kruskal–Wallis/Dunn's tests. Significance was judged at $P<0.05$.

RESULTS

We aimed to collect only adult males in an effort to eliminate any potential gender- and age-based differences in physiology. This objective was largely achieved for the sample Ohioan frogs, which, collected during the breeding season, contained only one female. However, because secondary sex characteristics are not evident in summer, and because northern *R. sylvatica* lack sexual dimorphism in body size (Martof and Humphries, 1959), we inadvertently collected both sexes of Alaskan frogs. This sample comprised approximately 32% females, which occurred randomly and were represented by at least one individual within the various treatment groups. Qualitative inspection of our data suggested that there was no gender-based variation that would materially influence the statistical results or overall conclusions of the study.

Winter acclimatization

Frogs collected in Interior Alaska during August were near the end of their activity season, as they typically enter hibernacula by mid-September (Kirton, 1974). Many had recently fed, as their guts usually contained invertebrate prey in various stages of digestion. In contrast, little or no alimentary matter was found in fall or winter frogs. Externally, frogs of all three groups appeared morphologically similar. They did not vary in body mass, snout–ischium length or body mass index, nor in body water content (range=3.81–3.91 g water g^{-1} dry tissue; Table 1). However, winter acclimatization induced marked changes in certain body components. The coelomic fat body present in late-summer frogs was virtually depleted during acclimatization. Concomitantly, relative mass of the liver, as represented by the HSI, increased ~1.5-fold. This change coincided with a 1.4-fold increase in the tissue's glycogen concentration, which rose by more than 1000 μmol g^{-1}; consequently, the liver glycogen depot in fall and winter frogs was nearly twice that in late-summer frogs. Muscle tissue also exhibited profound changes during winter acclimatization. Size of the gastrocnemius, as represented by its normalized dry mass, was reduced by 27%. The gracilis exhibited a marked decrease in protein concentration (reduced by 40% in winter frogs) and concomitant increase in GDH activity, which was 1.7-fold higher in winter frogs than in late-summer frogs. Glycogen concentration in this tissue rose during this period, reaching a maximal value in winter frogs.

Plasma levels of the two major cryoprotectants increased in Alaskan frogs during winter acclimatization (Table 1). However, whereas glucose increased by only several μmol ml^{-1}, urea concentration rose 10-fold, exceeding 100 μmol ml^{-1} in fall and winter frogs. Plasma osmolality in winter frogs, 419±9 mOsmol kg^{-1}, increased by 173 units over that in late-summer frogs. This rise exceeded the combined increment in glucose and urea (100 units), suggesting that an unidentified solute(s) was also accumulated during winter acclimatization.

Winter-acclimatized frogs representing the two populations differed in most of the measured variables (Table 1). Variation in morphological traits reflected the larger body size of frogs from the more temperate locale, as Ohioan frogs were 2.3-fold heavier and 1.2-fold longer than Alaskan frogs. The body mass index (length-adjusted body mass) of Ohioan frogs was nearly twice that of Alaskan frogs, despite the fact that their tissues contained relatively less water. Such differences probably reflect allometric scaling

Table 1. Somatic and physiological variables of Alaskan and Ohioan wood frogs before and after acclimatization to winter conditions

	Alaskan				Ohioan	
	Late summer	Fall	Winter	*P*	Winter	*P*
N	6	5	8		7	
Body mass (g)	6.0±0.8	5.9±0.5	7.2±0.5	0.245	16.7±1.1	<0.0001
Snout–ischium length (cm)	3.9±1.2	4.0±0.1	4.3±0.1	0.142	5.2±0.1	<0.0001
Body mass index (g cm^{-1})	1.53±0.13	1.45±0.09	1.68±0.10	0.335	3.25±0.23	<0.0001
Body water content (g g^{-1})	3.81±0.09	3.85±0.08	3.91±0.05	0.608	3.28±0.05	<0.0001
Coelomic fat body (mg)	23.6±7.5[a]	0.4±0.4[b]	1.5±0.8[b]	0.004	16.9±4.9	0.006
Liver						
Hepatosomatic index	13.8±1.4[a]	21.3±1.0[b]	22.4±0.9[b]	<0.0001	7.9±0.6	<0.0001
Glycogen (μmol g^{-1})	2705±122[a]	3719±529[b]	3549±88[b]	0.032	2884±79	<0.0001
Glycogen content (μmol)	502±102[a]	934±112[b]	1170±97[b]	0.0009	916±120	0.119
Glycogen richness (μmol g^{-1} frog)	379±47[a]	795±124[b]	794±33[b]	0.0005	229±21	<0.0001
Muscle						
Mass (mg mm^{-1} tibiofibula)	0.93±0.09[a]	0.68±0.06[b]	0.70±0.05[b]	0.049	2.15±0.12	<0.0001
Total protein (mg g^{-1})	549±65[a]	399±47[a,b]	327±30[b]	0.009	517±29	0.0006
GDH (mU mg^{-1} protein)	2.2±0.4[a]	3.1±0.7[a,b]	3.8±0.4[b]	0.014	1.9±0.1	0.0005
Glycogen (μmol g^{-1})	262±17[a]	358±34[a]	533±42[b]	0.0001	238±16	0.0003
Glycogen (μmol mg^{-1} protein)	0.5±0.1[a]	1.0±0.2[a,b]	1.8±0.04[b]	0.002	0.5±0.04	0.0003
Plasma						
Glucose (μmol ml^{-1})	2.9±1.4[a]	4.7±0.5[b]	7.2±1.3[b]	0.005	2.1±0.1	0.0001
Urea (μmol ml^{-1})	9.9±1.3[a]	106.4±6.0[b]	105.8±9.7[b]	<0.0001	28.8±4.8	<0.0001
Osmolality (mOsmol kg^{-1})	246±10[a]	397±17[b]	419±9[b]	<0.0001	245±7	<0.0001

Values are means ± s.e.m. Muscle mass values pertain to the gastrocnemius; other muscle variables pertain to the gracilis. Comparisons among three groups of Alaskan frogs were made using ANOVA/Student–Newman–Keuls or Kruskal–Wallis/Dunn's tests. Where statistical significance was achieved ($P<0.05$), groups are distinguished by dissimilar letters. Comparison of Alaskan winter frogs with Ohioan winter frogs was made using Student's *t*-test or the Mann–Whitney *U*-test.

relationships, although genetic variation may also be involved, as *R. sylvatica* from northern locales have considerably smaller and stockier bodies, with proportionately shorter hind limbs (Loomis and Jones, 1953). Tibiofibula–body length ratios of our Alaskan (0.41) and Ohioan (0.55) frogs were comparable to those previously reported for northern and southern frogs (Martof and Humphries, 1959).

Whereas fat body was virtually absent from winter Alaskan frogs, winter Ohioan frogs had a modest quantity of this tissue (Table 1). The HSI in Alaskan frogs was a remarkable 22.4%. The corresponding value for Ohioan frogs was only one-third as much, the difference chiefly reflecting the liver's 20% inferior glycogen concentration. Although the hepatic glycogen content was similar between populations, when considered relative to body mass (glycogen richness) this depot was actually 3.5-fold greater in Alaskan frogs. The gastrocnemius was comparatively lighter in Alaskan frogs. The gracilis from these frogs had a higher GDH activity and lower protein concentration, as well as more glycogen, relative to Ohioan frogs (Table 1).

Populations varied markedly in hematological variables (Table 1). Whereas the difference in glycemia was relatively minor, the plasma urea concentration in Ohioan frogs, 28.8±4.8 μmol ml^{-1}, was only 27% of that in Alaskan frogs and, consequently, their osmolality was comparatively low (245±7 *versus* 419±9 mOsmol kg^{-1}). However, some of the disparity in osmotic activity was due to the 73 μmol ml^{-1} of additional, unidentified solute in Alaskan frogs, as previously mentioned; Ohioan frogs contained no significant quantity of any unexpected solute.

Metabolite responses to freezing and thawing of winter frogs

Freezing commenced at a T_b near –1°C and was marked by an exotherm in the T_b record for each individual. Dissections revealed that ice progressively accumulated in frogs as time elapsed. Ohioan frogs sampled at 30 or 48 h contained substantial amounts of ice in the coelom, beneath the skin and within the muscles. Compared with Ohioan frogs, Alaskan frogs contained much less ice, which was primarily confined to subcutaneous spaces, and were relatively supple even after 48 h of freezing. Frogs sampled 6 h after thawing began contained no visible ice but were still inanimate. Excepting one Alaskan frog, which was omitted from analyses, all frogs sampled 5 days after thawing began exhibited normal neurobehavioral functions, including the righting reflex.

In Alaskan frogs, experimental freezing was accompanied by a progressive decrease ($F_{6,35}$=14.4, P<0.0001) in liver glycogen concentration, which fell from 3549±88 μmol g^{-1} in unfrozen frogs to 2164±245 μmol g^{-1} after 48 h of freezing (Fig. 2). This value remained low shortly after thawing but within 5 days rebounded to 2991±127 μmol g^{-1}, a level indistinguishable from that of unfrozen frogs. Ohioan frogs also lost liver glycogen during freezing and replenished it upon thawing ($F_{6,29}$=25.7, P<0.0001). However, this dynamic differed from that seen in Alaskan frogs ($F_{13,64}$=3.1, P=0.0095) primarily in that, after 48 h of freezing, glycogen concentration had fallen 83% in Ohioan frogs, but only 39% in Alaskan frogs. Moreover, liver glycogen concentration in unfrozen Ohioan frogs, 2883±79 μmol g^{-1}, was 19% less than that found in unfrozen Alaskan frogs (t=2.9, P<0.01).

Plasma glucose concentration in Alaskan frogs exhibited changes ($F_{6,35}$=128.2, P<0.0001) that mirrored the freezing/thawing dynamic in liver glycogen (Fig. 2). This also occurred with Ohioan frogs ($F_{6,29}$=155.8, P<0.0001); however, although glycemic levels achieved after 48 h of freezing were indistinguishable between Alaskan (217±9 μmol ml^{-1}) and Ohioan (231±24.4 μmol ml^{-1}) frogs,

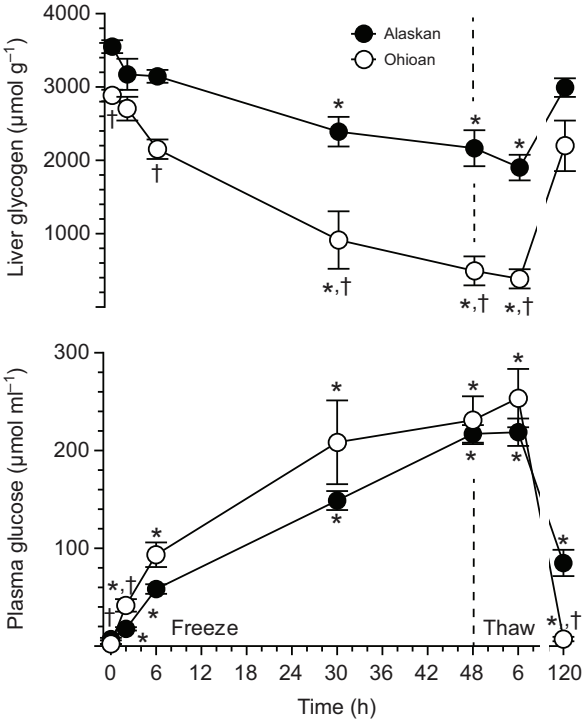

Fig. 2. Time course for changes in liver glycogen (μmol glucosyl units g^{-1} dry tissue) and plasma glucose associated with experimental freezing for up to 48 h and thawing for up to 5 days of Alaskan and Ohioan wood frogs. Means (±s.e.m.; N=4–8) identified by asterisks differed (P<0.05) from the corresponding mean for unfrozen frogs, which is given at the zero on the abscissa. Dagger indicates that the mean for Ohioan frogs differed (P<0.05) from the corresponding mean for Alaskan frogs.

the nature of the mobilization response differed between populations ($F_{13,64}$=30.7, P<0.0001). Notably, the glycemic increase in the northern phenotype appeared essentially linear, whereas the response in Ohioan frogs was asymptotic, with little additional increase occurring after 30 h. Populational differences in post-thaw glucose clearance were also evident. Glycemic levels in frogs sampled 5 days after thawing began were higher than those in unfrozen frogs, although the disparity was relatively small in Ohioan frogs; at this time Alaskan frogs remained strongly hyperglycemic (85.0±13.4 μmol ml^{-1}).

Alaskan frogs exhibited a strong increase with freezing, and subsequent reduction upon thawing, in glucose levels of liver ($F_{2,16}$=433.2, P<0.0001), heart ($F_{2,16}$=430.7, P<0.0001), brain ($F_{2,16}$=251.5, P<0.0001) and skeletal muscle ($F_{2,16}$=677.3, P<0.0001; Fig. 3). Concentrations in frogs frozen for 48 h were 54- to 80-fold higher than those in unfrozen frogs, the actual levels varying by organ, being highest in liver (194±16 μmol g^{-1} fresh tissue) and lowest in skeletal muscle (62±3 μmol g^{-1} fresh tissue). Although Ohioan frogs incurred grossly comparable changes (liver: $F_{2,13}$=81.6, P<0.0001; heart: $F_{2,13}$=291.5, P<0.0001; brain: $F_{2,13}$=235.3, P<0.0001; skeletal muscle: $F_{2,13}$=414.6, P<0.0001), populations differed in freeze/thaw dynamics with respect to liver ($F_{5,29}$=4.8, P=0.015), heart ($F_{5,29}$=49.9, P<0.0001) and brain ($F_{5,29}$=29.3, P<0.0001). Glucose levels in these organs were similar between Alaskan and Ohioan frogs frozen for 48 h, but were comparably higher in Alaskan frogs sampled 5 days after thawing began. Skeletal muscle differed from other organs in that the overall pattern of glucose accumulation with freezing and depletion upon thawing was similar between populations ($F_{5,29}$=2.3, P=0.124) and,

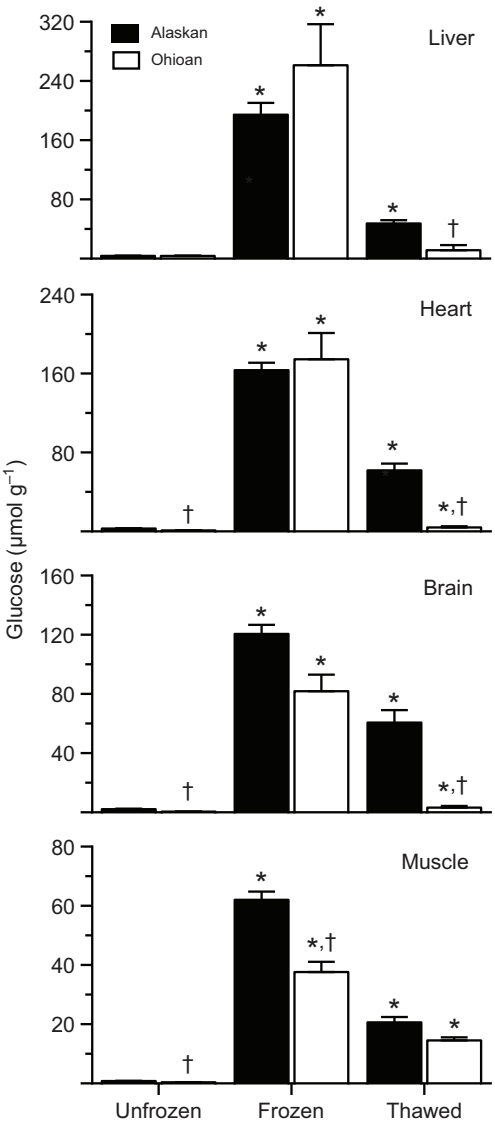

Fig. 3. Variation in glucose concentration (μmol g^{-1} fresh tissue) in several organs associated with experimental freezing (48 h) and thawing (5 days) of Alaskan and Ohioan wood frogs. Sample sizes and symbology as in Fig. 2.

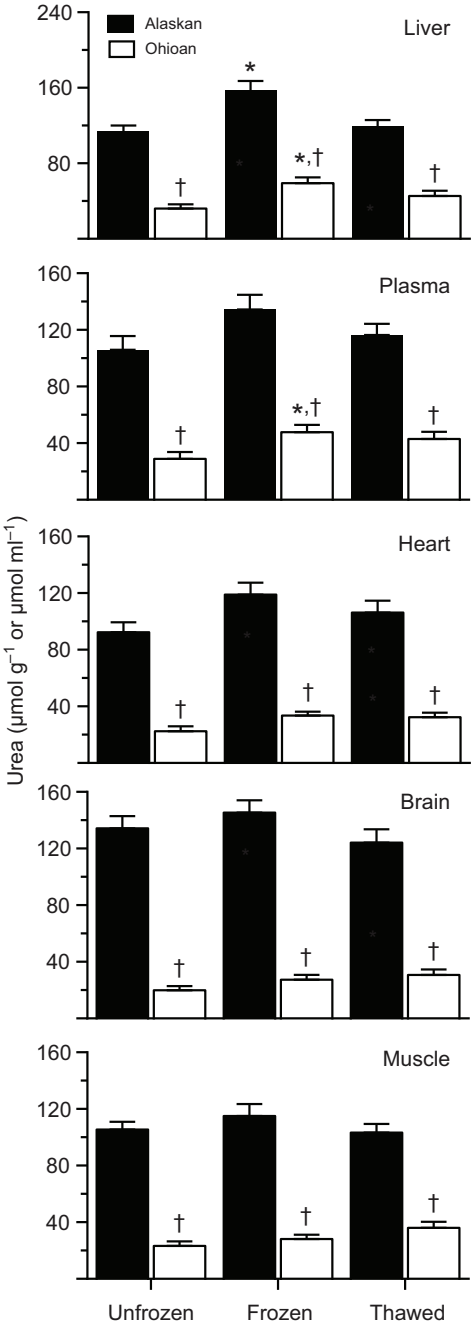

Fig. 4. Variation in concentration of urea in several organs (μmol g^{-1} fresh tissue) and plasma associated with experimental freezing (48 h) and thawing (5 days) of Alaskan and Ohioan wood frogs. Sample sizes and symbology as in Fig. 2.

in the case of 48-h frozen frogs, glucose levels were markedly higher (t=3.2, P<0.05) in the northern phenotype (Alaskan: 62±3 μmol g^{-1} fresh tissue; Ohioan 38±3 μmol g^{-1} fresh tissue).

Urea accumulated in the liver during freezing, the concentration in Alaskan frogs frozen for 48 h reaching 157.2±9.9 μmol g^{-1} fresh tissue, a value substantially higher ($F_{2,16}$=9.8, P=0.002) than that (114.1±5.8 μmol g^{-1} fresh tissue) found in unfrozen frogs (Fig. 4). Following a similar pattern of response ($F_{5,29}$=1.5, P=0.24), hepatic urea levels in Ohioan frogs also varied ($F_{2,13}$=7.0, P=0.009) with freezing/thawing, yet were 62–72% lower than those in Alaskan frogs. Generally, urea concentrations in other organs and blood of 48-h frozen frogs were nominally higher than corresponding levels in unfrozen frogs, but the difference was significant ($F_{2,13}$=4.1, P=0.042) only for the plasma of Ohioan frogs. There was good concordance between urea concentrations in blood and non-hepatic tissues, except that the level in brain of Alaskan frogs exceeded that in plasma. Tissue urea levels returned to respective basal levels within 5 days after thawing began.

Hydric responses to freezing and thawing of winter frogs

Experimental freezing and thawing of Alaskan frogs was accompanied by a measured rise ($F_{6,35}$=17.4, P<0.0001) in the water concentration of liver tissue, which, after 48 h of freezing, had increased 36% over that in unfrozen frogs (2.0±0.04 g water g^{-1} dry tissue; Fig. 5). This state persisted throughout thawing, but was rectified within 5 days after thawing began. In Ohioan frogs, hydration of liver also changed with freezing/thawing ($F_{6,29}$=25.7, P<0.0001), although the variation principally reflected a 1.7-fold spike in recently thawed frogs; consequently, populations differed in their pattern of response ($F_{13,64}$=6.2, P<0.0001).

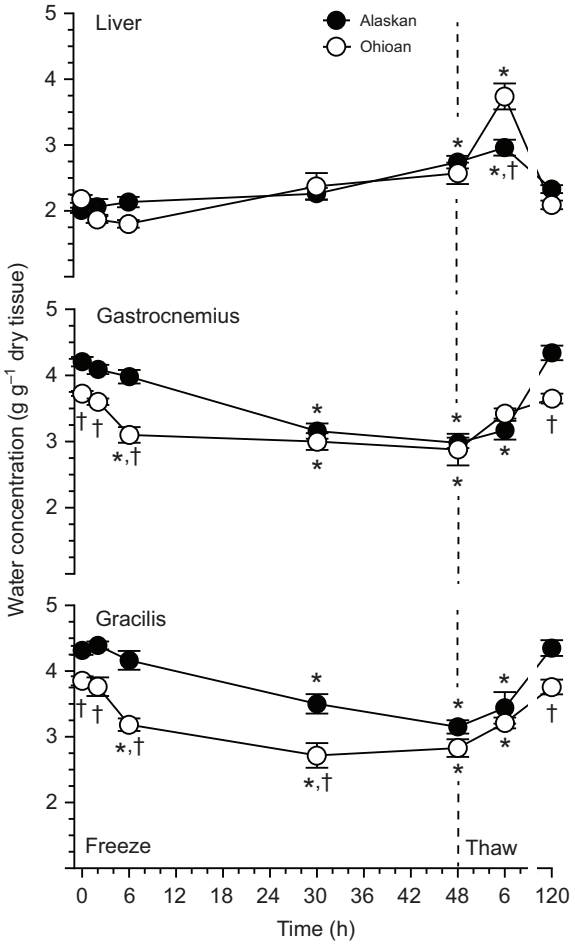

Fig. 5. Time course for changes in water concentration in several organs associated with experimental freezing (48 h) and thawing (5 days) of Alaskan and Ohioan wood frogs. Sample sizes and symbology as in Fig. 2.

Contrary to the case with liver, skeletal muscles dehydrated during freezing and rehydrated upon thawing (Fig. 5). In Alaskan frogs, changes in water concentration of both the gastrocnemius ($F_{6,35}$=32.3, P<0.0001) and the gracilis ($F_{6,35}$=14.4, P<0.0001) reflected a reduction from basal values by 30 h of freezing, followed by repletion within 5 days after thawing began. Similar changes occurred in Ohioan frogs (gastrocnemius: $F_{6,29}$=8.9, P<0.0001; gracilis: $F_{6,29}$=15.6, P<0.0001), although their tissues lost water more rapidly during freezing. The water concentration in muscles of 48-h frozen frogs was 23–29% less than that in unfrozen frogs, the differential being greater in Alaskan frogs. Hydric responses of muscles apparently differed between Alaskan and Ohioan frogs (Fig. 5), although the difference was significant for gastrocnemius ($F_{13,64}$=6.2, P<0.0001), but not quite so for gracilis ($F_{13,64}$=2.0, P=0.080).

Hematocrit, the percentage by volume of the blood occupied by formed elements, is a useful index of the hydration status of the blood. Hematocrit of Alaskan frogs changed slightly, albeit significantly ($F_{6,34}$=2.8, P=0.027), with freezing and thawing, although only the value for 48-h frozen frogs, 28.1±2.1%, differed from that of unfrozen frogs, 18.2±1.4% (Fig. 6). By contrast, Ohioan frogs exhibited a rapid and robust rise ($F_{6,27}$=13.7, P<0.0001) in hematocrit with freezing, the value in 48-h frozen frogs, 49.6±1.2%, being 2.4-fold higher than that of unfrozen frogs, 21.0±3.2%.

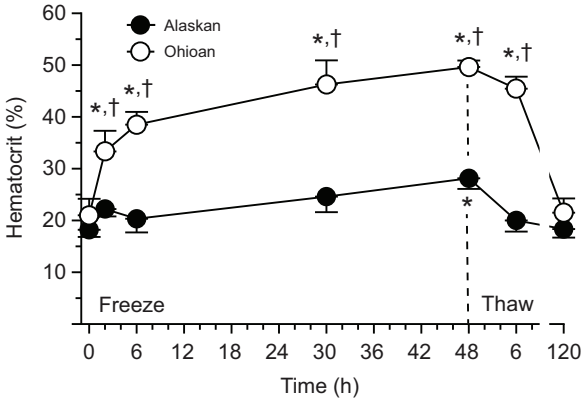

Fig. 6. Variation in hematocrit associated with experimental freezing (48 h) and thawing (5 days) of Alaskan and Ohioan wood frogs. Sample sizes and symbology as in Fig. 2.

Hematocrit returned to basal levels within 5 days after thawing began.

Freezing/thawing stress in winter frogs

Experimental freezing coincided with a rise in the lactate concentration of all tissues examined. By 48 h of freezing, lactate in plasma of Alaskan frogs had increased ($F_{2,16}$=82.7, P<0.0001) 5.4-fold over that measured in unfrozen frogs (Fig. 7). Ohioan frogs also accumulated lactate in plasma ($F_{2,13}$=97.9, P<0.0001), although the increment was nearly 15-fold; consequently, lactemia in these frogs was greater (t=3.7, P<0.01) than that in Alaskan frogs. For both populations, lactate values returned to basal levels within 5 days after thawing began. This pattern of accumulation during freezing followed by clearance after thawing was observed for all other tissues (data not shown), although the levels achieved with freezing were consistently higher (P<0.01, all cases) in Ohioan frogs. For example, after 48 h of freezing, lactate concentrations in Ohioan frogs ranged from 10.8±0.8 μmol g^{-1} fresh tissue in muscle to 27.2±2.3 μmol g^{-1} fresh tissue in brain, whereas corresponding values for Alaskan frogs were only 3.2±0.3 and 12.8±1.4 μmol g^{-1} fresh tissue, respectively.

Plasma Hb concentration served to index the magnitude of cellular freezing stress (Fig. 8). Hb concentration in Alaskan frogs frozen for 48 h was slightly higher than that in their unfrozen counterparts, but the difference was not quite significant ($F_{2,17}$=3.1, P=0.072). By contrast, the concentration in frozen Ohioan frogs was 5.5-fold greater ($F_{2,13}$=5.8, P=0.016) than the basal level, indicating that substantial cryohemolysis had occurred in these frogs. For both populations, Hb levels in thawed frogs were comparable to those measured in unfrozen frogs.

Freeze tolerance trials with winter frogs

Our method of experimentally freezing winter Alaskan frogs exploited the tenet that frogs physically contacting ambient ice cannot resist freezing (i.e. remain supercooled) and that inoculative freezing will occur at a T_b close to the equilibrium freezing point of body fluids, approximately –0.7°C (Lee and Costanzo, 1998). Although we could not monitor the freezing status of frogs during cooling, we quickly examined the contents of one box after it had reached –8°C, confirming that the moss within was solidly frozen and the frogs themselves were icy and rigid.

Frogs frozen to –8°C were alert and responded to tactile stimulation when first examined, 2 days after thawing began. At this

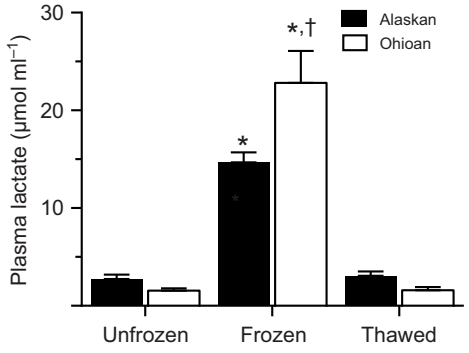

Fig. 7. Variation in plasma concentration of lactate associated with experimental freezing (48 h) and thawing (5 days) of Alaskan and Ohioan wood frogs. Sample sizes and symbology as in Fig. 2.

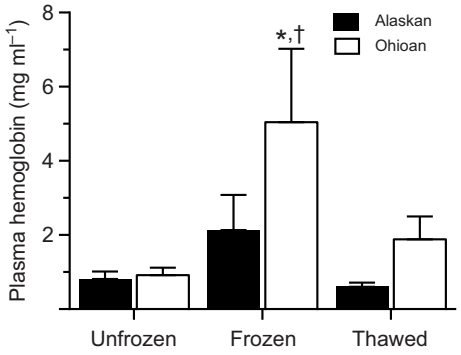

Fig. 8. Variation in plasma concentration of hemoglobin (Hb) associated with freezing (48 h) and thawing (6 h) of Alaskan and Ohioan wood frogs. Sample sizes and symbology as in Fig. 2.

time, nine of these 10 frogs also exhibited the righting response and thus were used in subsequent, more challenging trials; the remaining subject was omitted from further testing but ultimately regained its righting response within the 7 day recovery period and thus was deemed to have survived. All five of the frogs frozen to –12°C and all four of the frogs frozen to –16°C were alert and exhibited normal posture and the righting response by 2 days post-thawing. However, one member of the –12°C group, which gradually declined in condition and ultimately died, was not used in further testing.

In freeze-endurance trials, we tested the ability of winter Alaskan frogs to recover from extended periods of freezing at –4°C. Of the four frogs held frozen for 8 weeks, three initially showed signs of viability, two of which ultimately met the survival criterion. The two mortalities included one frog that was previously frozen to –12°C and another that had earlier survived freezing at –16°C. The four frogs kept frozen at –4°C for 12 weeks were inanimate following thawing and were scored as mortalities.

DISCUSSION

Cold tolerance in ectotherms can vary substantially among populations of conspecifics inhabiting diverse climatological regimes (Bennett et al., 2005; Hilbish, 1981; Klok and Chown, 2005; Rasmussen and Holmstrup, 2002; Slotsbo et al., 2008; Williams and Lee, 2008). Molecular, biochemical and physiological mechanisms underpinning phenotypic variability in this trait are incompletely understood, but are perhaps most profitably studied in species, such as *R. sylvatica*, for which fundamental adaptations are reasonably well known. In the present study, we investigated hibernation physiology, extreme freeze tolerance and freezing adaptation in a northern population of *R. sylvatica*, finding that this phenotype is well suited to the harsh and prolonged winter characteristic of its native habitat.

Overwintering preparations

Freeze-tolerant organisms commonly accumulate cryoprotective osmolytes during seasonal cold hardening (Storey and Storey, 1988). Among amphibians, this response is best exemplified by members of the gray treefrog complex (genus *Hyla*), which potentially can accumulate glycerol to concentrations of 50–80 μmol ml^{-1} blood during cold acclimation (Layne and Stapleton, 2009; Zimmerman et al., 2007). *Rana sylvatica* (and perhaps other species) can accrue urea to comparable levels during autumn and early winter, coincident with seasonal declines in ambient temperature and water potential (Costanzo and Lee, 2005). In the present study this species amassed urea even in the absence of osmotic stress, a potent driver of urea accumulation in amphibians (Jørgensen, 1997). Indeed, kept in moist conditions, these frogs remained fully hydrated but nevertheless achieved a high uremia (>100 μmol ml^{-1} in Alaskan frogs) during winter acclimatization. This unexpected finding incites questions about the seasonal regulation of nitrogen metabolism in *R. sylvatica* and, particularly, the northern phenotype, which accumulated more than three times as much urea as Ohioan frogs.

Mechanisms to facilitate the distribution and cellular uptake of copious amounts of urea synthesized during winter acclimatization could potentially include increased expression of urea transporters in the liver and other organs. Whether this occurs is an open question, although, in *R. sylvatica*, elevated urea in the absence of tissue dehydration reduces the abundance of these proteins in the kidney and urinary bladder (Rosendale et al., 2012). Urea permeability could be enhanced through other mechanisms. For example, expression of the gene for HC-3, one of several amphibian orthologs of the mammalian aquaporin, AQP3, which transports glycerol and urea in addition to water, increases during cold acclimation in various tissues of a freeze-tolerant tree frog (Zimmerman et al., 2007).

Urea accrual in Alaskan frogs likely stemmed from cold-induced oliguria and high activity of the regulatory enzyme carbamoyl phosphate synthetase I (Schiller et al., 2008), as well as a targeted reorganization of certain metabolic processes. In addition, reductions in dry mass and protein content of skeletal muscle during winter acclimatization coincided with increased activity of GDH, a key mitochondrial enzyme mediating protein turnover and, ultimately, urea synthesis in liver. By contrast, levels of muscle protein and GDH activity in winter Ohioan frogs were unremarkable; thus, heightened proteolysis may underlie the exceptional uremia achieved in the northern phenotype. Given that acclimatizing frogs consumed prey and exhibited certain anabolic processes (e.g. glycogenesis in liver and muscle), muscle atrophy must be a regulated process and not simply a starvation response. Skeletal muscle is a highly plastic tissue that can change to meet physiological demands (James, 2010) and, indeed, the muscle catabolism seen in some anurans under osmotic stress is key to providing additional nitrogen for urea synthesis (Hoffman et al., 1990; Jones, 1980; McClanahan, 1972). The extent to which the observed atrophy compromises mechanical performance remains to be determined, but, given that *R. sylvatica* engages in intense spawning behavior immediately upon arousal, the cost–benefit implications of this scenario are interesting to contemplate.

Our Alaskan frogs attained levels of urea that, albeit impressive, were perhaps more modest than could be achieved under osmotic challenge, such as occurs in nature when the surrounding soil freezes (Costanzo and Lee, 2005). Nevertheless, their capacity to accumulate this osmolyte to high levels enhances winter survival because, in addition to its cryoprotective properties, urea can promote hypometabolism in dormant ectotherms (Muir et al., 2010). In this role, transfer between arousal and dormancy is achieved through shifts between the active/inactive states of key regulatory enzymes in response to changing urea concentration, pH and temperature. Elevated urea is associated with reduced metabolism in both intact frogs and isolated tissues, and this response would particularly benefit frogs in Interior Alaska, which remain dormant for nearly 8 months of the year (Kirton, 1974).

Temperate amphibians undergo distinct, seasonal patterns of nutrient cycling exemplified by the hepatic glycogen depot peaking in autumn or early winter and regressing following vernal spawning (Pinder et al., 1992). In some species, pre-hibernal glycogenesis is subsidized by increased lipolysis in fat bodies and other tissues (Farrar and Dupre, 1983; Koskela and Pasanen, 1975; Krawczyk, 1971; Pasanen and Koskela, 1974), and this seems to be the case with our Alaskan *R. sylvatica*, which substantially reduced their fat bodies during the period of glycogen deposition. Whereas winter Ohioan frogs retained a modicum of this adipose, Alaskan frogs expended their fat bodies whilst preparing for hibernation. Enhanced lipolysis in high-latitude populations of *R. sylvatica*, and also freeze-tolerant tree frogs (Irwin and Lee, 2003), likely improves survival by magnifying the glycogen reserve. Additionally, more of the energy in lipid, which cannot be oxidized in frozen (hypoxic) tissues, is converted to a more useful substrate; indeed, *R. sylvatica* undergo a seasonal downregulation of certain enzymes that would curtail lipid use in hibernation (Kiss et al., 2011).

Glycogen deposition in liver (and perhaps muscle) during the pre-hibernal period seemingly was supported by proteolysis in skeletal muscle. Albeit energetically expensive, this process would not only help synthesize glycogen *via* gluconeogenesis, but, as mentioned earlier, would also promote urea accumulation. Proclivity for muscle degeneration in Alaskan frogs, but apparently not in Ohioan frogs, may underlie the substantially larger glycogen reserves amassed by the former.

Anuran species, and populations within species, inhabiting especially cold climates tend to stockpile more glycogen, and thus have heavier livers, than counterparts from milder locales (Chen et al., 2011; Jönsson et al., 2009; Pasanen and Koskela, 1974). Accordingly, our Alaskan frogs had extremely rich glycogen deposits and an extraordinary HSI; indeed, the percentage of body mass represented by the liver was 22%, as compared with 8% in our Ohioan frogs. Fall-collected *R. sylvatica* native to Ontario, near the 45th north parallel, have an intermediate value, 12% (Sinclair et al., 2013). This clinal pattern might suggest that frogs need greater energy reserves in colder climates to survive aphagia during prolonged dormancy. However, *R. sylvatica* (and likely other freeze-tolerant species) preferentially conserves this substrate – an important source of cryoprotectant – throughout hibernation, ultimately expending it to help fuel post-emergent reproductive activities (Costanzo et al., 2012). Maximizing the size of the glycogen depot, and thus capacity for cryoprotectant generation, is likely a key trait contributing to winter survival at high latitudes. Disparity in glycogen provisioning between northern and southern phenotypes is particularly evident when considering the size of the glycogen reserve relative to the mass of tissue requiring cryoprotection: the supply was more than 3.5 times as great in Alaskan frogs (Table 1).

Between late summer and winter, Alaskan frogs accumulated much glycogen in skeletal muscle, a response also seen in other freeze-tolerant anurans (Higgins and Swanson, 2013). Abundant glycogen may be advantageous in cold climates, as levels in Alaskan frogs were 2.2-fold greater than those in Ohioan frogs. Furthermore, the pre-hibernal supply of muscle glycogen in a Minnesotan population (~47.6°N) of the gray tree frog was nearly twice that of conspecifics from Missouri (~37.9°N) (Irwin and Lee, 2003). Muscle stores of this substrate probably contribute little to whole-animal metabolism in winter (Pasanen and Koskela, 1974; Tattersall and Ultsch, 2008), but may help fuel occasional movements to new hibernacula, which have been observed for *R. sylvatica* wintering in an outdoor enclosure (J.P.C. and J. T. Irwin, unpublished). Such relocations, which are made by other ranids that hibernate beneath forest duff, even in mid-winter and at near-freezing temperatures (Holenweg and Reyer, 2000), may help diminish the stimulus for disuse atrophy of skeletal muscle (James, 2010). However, the most crucial role of the muscle glycogen depot is likely to support the reproductive activities that commence upon spring arousal (Wells and Bevier, 1997).

During winter acclimatization of Alaskan frogs, the observed increment in plasma osmolality, 173 mOsmol kg^{-1}, was only partly explained by a rise in glucose and urea, which jointly contributed ~100 mOsmol kg^{-1} to this increase. We thus deduced that the blood of winter frogs contained ~73 μmol ml^{-1} of some other osmolyte(s), which, like glucose and urea, serves a cryoprotective role by colligatively reducing FP_{eq}. We further deduced that this agent was absent from Ohioan frogs because, omitting glucose and urea, their plasma osmotic pressure would be ~214 mOsmol kg^{-1}, which can be wholly attributed to the principal ionic electrolytes (Shoemaker and Nagy, 1977). Additional study is needed to identify this solute and determine the extent to which it contributes to exceptional freeze tolerance in the northern phenotype. Our preliminary results indicate that it is not glycerol, as this compound was absent (or at least present at concentrations below 0.25 μmol ml^{-1}) from the plasma of late-summer, fall and winter frogs.

Extreme freeze tolerance

Freeze-tolerant frogs of cool-temperate regions commonly experience thermal minima of –5 or –7°C within their shallow, terrestrial hibernacula (MacArthur and Dandy, 1982; Schmid, 1982), although lower temperatures and longer chilling excursions probably affect populations at higher latitudes. Temperatures in the winter microenvironment occupied by *R. sylvatica* in Interior Alaska are indeed more severe (Middle and Barnes, 2000; Barnes et al., 1996) and presumably demand a commensurately greater tolerance to freezing. Whereas frogs indigenous to the Great Lakes Region tolerate freezing only to –4 to –6°C, our results confirm and extend a preliminary report (Middle and Barnes, 2001) that this northern phenotype can survive at temperatures below –16°C. Freeze tolerance is also considered in terms of survivable duration, which reflects the ability to cope with additional stresses, such as prolonged hypoxia and energy depletion. Whereas *R. sylvatica* from a cool-temperate population survive continuous freezing for perhaps several weeks (Layne, 1995; Layne et al., 1998), some Alaskan frogs revived after being frozen at –4°C for 2 months. None tolerated a 3-month exposure, although the subjects used in these trials, which had recently been frozen at –12 or –16°C, may have been unduly stressed. Additional study is needed to delineate freeze endurance in this population.

Freeze-tolerant animals can survive the freezing of as much as two-thirds of their body water, but may reach this limit at markedly

different T_b values (Storey and Storey, 1988). Such variation partly reflects differences in osmotic potential, which can be increased by accruing organic osmolytes before and/or during freezing. Ice formation in our Alaskan frogs would be curbed by their high plasma osmolality, which, at 419 mOsmol kg^{-1} before freezing commenced, is exceptional for a fully hydrated amphibian (Shoemaker and Nagy, 1977). Tissue FP_{eq} would be further reduced by the glucose and urea synthesized during freezing, although such agents may also limit ice formation by increasing the proportion of body water that is bound or 'unfreezable' by virtue of its close association with macromolecules and cellular structures (Storey and Storey, 1988). Indeed, disparate capacities for freeze tolerance in Alaskan and Ohioan frogs may owe considerably more to variation in bound water than to the difference in osmotic potential, which in frozen frogs was only ~150 mOsmol kg^{-1}. Using a mathematical model of the colligative properties of solutions, FP_{eq} and T_b (Claussen and Costanzo, 1990), we estimate that the bound water component in our Alaskan frogs, having an approximate FP_{eq} of –1.22°C (equivalent to 657 mOsmol kg^{-1} in frozen animals), must exceed 26% in order that total body ice content remains below the lethal threshold, 67%, when frozen at –16°C (Table 2). It would be instructive to empirically determine ice contents of frogs frozen to such temperatures; however, it is telling that bound water proportions in *R. sylvatica* from lower latitudes are only 12–20% (Costanzo et al., 1993; Layne, 1995). Accordingly, the model's estimate for our Ohioan frogs, which presumably attain the lethal threshold ice content at –5°C, is 15%.

Osmolyte responses to freezing and thawing

The well-described glycemic response to freezing in *R. sylvatica* derives from a sympathetic stimulation of hepatocytes that initiates glycogenolysis and copiously produces glucose, which is circulated to tissues throughout the body (Storey and Storey, 2004). The process commences immediately and continues until the glycogen reserve is exhausted or freezing reaches an advanced stage. Glucose levels in blood and most tissues quickly rise but usually reach an asymptote within a day or two. In the present study, frogs expressed a strong glycogenolytic response to freezing, the glycemic level increasing rapidly, reaching ~220 µmol ml^{-1} in both phenotypes. This value is within the range reported for winter *R. sylvatica* from various locales [albeit ~2.9 times higher than that earlier reported for this Ohioan population; see discussion and table 4 in Costanzo and Lee (Costanzo and Lee, 2005)]. Although the glycemic level achieved by Alaskan frogs was not extraordinary, some evidence suggests that their cryoprotectant system actually is superior. Because Ohioan frogs lost considerably more vascular water during freezing (as evidenced by higher hematocrits; Fig. 6), much of the glycemic increase seen in these frogs reflects the freeze concentration of glucose, rather than its *de novo* synthesis, which was comparatively greater in Alaskan frogs. Furthermore, whereas Ohioan frogs nearly depleted their hepatic glycogen reserve by 48 h of freezing, Alaskan frogs retained a substantial reserve capacity (Fig. 2). Indeed, extrapolating from the glycemic yield suggests that, if all remaining glycogen were mobilized (e.g. during an extended freezing bout), blood glucose concentration would approach 0.55 mol l^{-1}. Whether Alaskan frogs actually attain such glycemic levels with extended or more extreme freezing, such as occurs in other freeze-tolerant species (Slotsbo et al., 2008), remains to be determined.

Tissues of frogs frozen for 48 h showed the familiar variation in glucose concentration, with the highest level occurring in liver, core organs (heart and brain) having intermediate levels, and skeletal muscle having the lowest (Storey and Storey, 2004). The meager amount in the latter likely reflects the rapidity with which peripheral structures freeze and become isolated from circulating glucose (Costanzo et al., 1997b; Rubinsky et al., 1994; Storey, 1984). Our finding that more glucose accumulated in muscle of the northern phenotype than in Ohioan frogs suggests that perfusion persisted longer in the former, perhaps because their tissues formed less ice.

Glucose mobilized with freezing is cleared from most tissues, returned to the liver and reconverted to glycogen usually within 24–48 h of thawing (Costanzo and Lee, 2013; Storey and Storey, 2004). Expectedly, glucose levels in blood and organs of Ohioan frogs returned to near-basal values within 5 days after thawing began. However, Alaskan frogs remained strongly hyperglycemic, their mean glucose level, 85 µmol ml^{-1}, being some 12-fold higher than that of unfrozen frogs. Uncharacteristically high levels of glucose also remained in the liver, the heart and especially the brain of these frogs. We speculate that clearance could be delayed by high urea levels, as this phenomenon is also seen in frogs experimentally rendered hyperuremic before freezing (Costanzo and Lee, 2008). Tests using a *Xenopus* oocyte expression system (A.J.R., R.E.L. and J.P.C., unpublished) showed that high urea (150 µmol ml^{-1} medium) strongly inhibits glucose flux through GLUT2, the facilitated transporter that mediates glucose transfer between the liver and the blood, and reabsorption in the kidney and, possibly, the bladder (Costanzo et al., 1997a). Implications of delayed glucose clearance are not entirely clear, although sustained hyperglycemia could potentially facilitate recovery (e.g. fueling tissue repair) or enhance cryoprotectant levels achieved during subsequent freezing excursions (Costanzo et al., 1992).

Alaskan frogs accumulated substantial amounts of urea even before freezing began. In contrast to the case with Ohioan frogs, whose heart, brain and skeletal muscle had uniform levels of this cryoprotectant (20–23 µmol g^{-1}), urea concentrations in Alaskan

Table 2. Parameters associated with ice content in Alaskan and Ohioan wood frogs frozen to various temperatures

				Body ice content (% of total water)		
	Osmolality (mOsmol kg^{-1})	FP_{eq} (°C)	Bound water (%)	–2.5°C	–5°C	–16°C
Alaskan	657	–1.22	25.7	25.4	49.9	**66.7**
Ohioan	493	–0.92	14.9	48.4	**66.7**	79.4

Osmolality was estimated by adding the osmotic activity of urea and glucose (1 µmol ml^{-1}=1 mOsmol kg^{-1}) in plasma of frogs frozen for 48 h to the plasma osmolality (less contributions from urea and glucose) of unfrozen frogs.

The equilibrium freezing/melting point (FP_{eq}) was determined from the relationship between a solution's osmolality and FP_{eq} (–1.86°C/Osmol kg^{-1}).

Bound water was determined using a mathematical model of the colligative properties of solutions, FP_{eq} and body temperature (Claussen and Costanzo, 1990) and stipulating that the threshold lethal ice content, 67%, is attained at –16°C in Alaskan frogs and –5°C in Ohioan frogs (boldface values). The stipulation for Ohioan frogs has some empirical support (Costanzo et al., 1993; Layne, 1995), but for Alaskan frogs is false because they survived freezing to –16°C; therefore, the bound water and ice content values predicted for these frogs are necessarily underestimates and overestimates, respectively, of the true values.

frogs varied markedly among organs, ranging from 92±7 μmol g^{-1} in the heart to 134±9 μmol g^{-1} in the brain. The exceptional level in the brain, which even exceeded that in the liver, the organ of its genesis, is noteworthy in light of its extreme sensitivity to osmotic and freezing/thawing stress. Assuming that urea was uniformly distributed across the plasma membrane, and that each gram of brain tissue contained 830 mg water (Costanzo et al., 1992), we estimate that urea concentration in cytosol was 163 μmol ml^{-1}, considerably higher than that in blood plasma, 106±10 μmol ml^{-1}. Sequestration of urea in the brain, *via* its uptake against the blood–tissue concentration gradient, implies the work of an active urea transporter. Confirmation of this notion awaits further study, although experimental evidence suggests that amphibian tissues can express such transporters (Ehrenfeld, 1998; Garcia-Romeu et al., 1981). In this organ, elevated urea likely contributes to antioxidation (Wang et al., 1999) and limits damage, such as myelinolysis, resulting from osmotic upheaval during freezing and thawing (Soupart et al., 2007).

The urea concentration in livers of Ohioan and Alaskan frogs frozen for 48 h exceeded that in unfrozen frogs by 40 and 80%, respectively, attesting that somatic freezing stimulates urea synthesis in this organ. However, ureagenesis apparently occurs relatively late in freezing because it barely augmented the solute already present in other tissues of winter-acclimatized frogs. This interpretation corroborates an earlier finding (Costanzo and Lee, 2005) and underscores the importance of prehibernal accumulation of this agent, which contributes substantively to total cryoprotectant load, particularly within peripheral tissues, which accrue relatively little glucose during freezing (Fig. 9).

Dynamics of water and ice in freezing and thawing

Dissection of the frogs used in our freeze/thaw time-course experiment revealed that the northern phenotype had accumulated considerably less ice, a distinction attributable to their higher osmolality, lower FP_{eq}, and, as argued above, greater abundance of bound water. Using the same parameters in the aforementioned model (Claussen and Costanzo, 1990), we calculated that the ice content of specimens frozen to –2.5°C was <25% for Alaskan frogs, but ~48% for Ohioan frogs (Table 2). The latter estimate is in excellent accord with calorimetric data reported for *R. sylvatica* from similar climates (Layne and Lee, 1991; Storey, 1984).

Changes in hydro-osmotic status during freezing and thawing are driven by transmembrane water fluxes associated with the formation and subsequent melting of ice, as well as innate cryoprotective responses, including accrual of osmotically active solutes and extra-organ sequestration of ice (Lee and Costanzo, 1998). Freezing may result in hypovolemia, which in still-frozen frogs is evidenced by a marked increase in hematocrit, a proxy for vascular water balance. In Ohioan frogs, for example, freezing at a moderate T_b typically induces a twofold to threefold increase in hematocrit (Costanzo et al., 1997b) (Fig. 6). The comparatively small response seen in our Alaskan frogs is further testament to their limited ice content.

In *R. sylvatica*, reversible organ dehydration during freezing limits the amount of ice forming within tissues through both the physical removal of water and the concentration of solutes in a reduced solvent volume; much of this water freezes innocuously within coelomic, subdermal and lymphatic spaces (Lee and Costanzo, 1998). With progressive freezing, organs usually dehydrate to variable degrees, with some, such as liver and intestine, losing over one-half of their initial water (Lee et al., 1992). However, in the present study, liver showed a net increase in water concentration, followed by a return to basal levels after thawing. Hyperhydration of this tissue probably derived from water liberated in glycogenolysis; it has not been observed in spring frogs, which have a smaller glycogen reserve (Storey and Storey, 2004). Skeletal muscles showed the expected dehydration during freezing, the quicker response in Ohioan frogs bolstering the supposition that their tissues froze more rapidly. However, water redistribution is also influenced by factors other than ice formation, because muscle of frogs in both populations ultimately reached a similar level of dehydration (23–29%).

Freezing/thawing stress

Myriad stresses of freezing and thawing include metabolic and homeostatic perturbations, hypoxic and oxidative damage, and osmotic/mechanical injury to delicate structures, such as organelles and cell membranes (Storey and Storey, 1988). Severity of freezing injury is conveniently indexed by leakage of intracellular proteins, such as Hb, from damaged cells into the bloodstream. Elevated levels of urea and glucose limit intravascular cryohemolysis, providing direct evidence of their cryoprotective efficacy *in vivo* (Costanzo and Lee, 2008; Costanzo et al., 1993). That our Alaskan frogs incurred no significant Hb leakage is consistent with their low ice content, but may also reflect their high levels of urea, a known stabilizer of cell membranes (Chakraborty et al., 2005; Costa-Balogh et al., 2006).

Freezing also depletes high-energy phosphates and promotes accumulation of anaerobic metabolites in ischemic tissues; thus, lactate is another useful marker of freezing stress, its abundance in tissues indicating propensity for oxidative damage (Storey and Storey, 1988). Alaskan frogs accumulated less lactate than did Ohioan frogs, perhaps because they formed less ice (and better sustained tissue perfusion) and/or used oxygen reserves more efficiently. Similarly, gray tree frogs in a northern population accumulated less lactate with freezing than did more southerly conspecifics (Irwin and Lee, 2003). Mitigating hypoxic stress, perhaps by enhancing metabolic downregulation, or forming ice more slowly, may contribute to survival in extremely cold habitats.

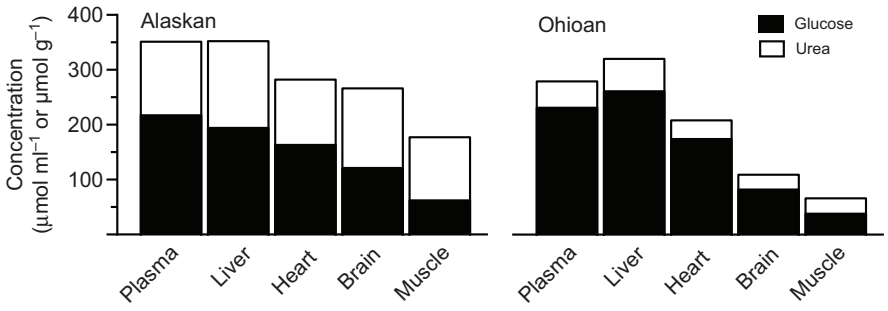

Fig. 9. Relative contribution of urea and glucose to total cryoprotectant load in plasma and several organs in Alaskan and Ohioan wood frogs experimentally frozen for 48 h. Derived from values for group means as depicted in Figs 2–4.

Implications

Wood frogs indigenous to Interior Alaska, near the northern limit of their geographical range, exhibit distinct, prehibernal changes in physiology and a substantially greater capacity for freeze tolerance compared with conspecifics from more temperate regions. Such variation may have genetic underpinnings, as a phylogeographic analysis (Lee-Yaw et al., 2008) suggested that frogs from our Alaskan and Ohioan populations belong to separate mtDNA clades. The northwestern portion of their extant range was populated following glacial retreat by colonizers emanating from a high-latitude, ice-free refugium located in present-day Wisconsin. Because populations in the Great Lakes Region have a similar origin but clearly lack such extreme cold hardiness, the unique traits of Alaskan frogs may reflect local adaptation driven by demands imposed by an extremely harsh winter environment. It would be instructive to determine winter preparatory and freezing responses expressed by other populations along the same northwest expansion route (Lee-Yaw et al., 2008).

LIST OF SYMBOLS AND ABBREVIATIONS

FP_{eq}	equilibrium freezing/melting point (°C)
GDH	glutamate dehydrogenase
Hb	hemoglobin
HSI	hepatosomatic index (g dry liver g^{-1} dry body × 100)
T_b	body temperature (°C)

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AUTHOR CONTRIBUTIONS

J.P.C. and M.C.F.A conceived the study; J.P.C., M.C.F.A and A.J.R. designed the experiments and collected the data; J.P.C. analyzed the data and wrote the paper; J.P.C., M.C.F.A, A.J.R. and R.E.L. contributed substantially to interpreting the data and developing the manuscript and take full responsibility for the content of the paper.

COMPETING INTERESTS

No competing interests declared.

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