Journal of Zoology

ZSL LIVING CONSERVATION

Journal of Zoology. Print ISSN 0952-8369

Seasonal variation and freezing response of glucose transporter 2 in liver of the wood frog: implications for geographic variation in freeze tolerance

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Keywords
Rana sylvatica; wood frog; population; freeze tolerance; glucose transporter.

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Editor: Mark-Oliver Rödel

Received 15 December 2014; revised 24 March 2015; accepted 10 April 2015

doi:10.1111/jzo.12255

Abstract

Subarctic populations of the wood frog *Rana sylvatica* survive freezing to temperatures at least 10–13°C below those of more southerly conspecifics. This profound freeze tolerance is due in part to an enhanced glucosic cryoprotectant system that requires rapid mobilization of glucose from hepatocytes during the early hours of freezing. To determine if glucose transporter 2 (GLUT2) in the liver contributes to geographic variation in freeze tolerance, we examined changes in the protein's abundance seasonally and in response to experimental freezing in frogs from Interior Alaska and southern Ohio, USA. Using immunoblotting techniques, we found that GLUT2 abundance increased in preparation for winter in both populations, but tests with Ohioan frogs showed that that altered temperature alone does not cause these seasonal changes. In Ohioan frogs, transporter expression apparently was regulated transcriptionally, as mRNA levels, assessed using quantitative real-time polymerase chain reaction, changed in accordance with protein abundance. However, this pattern was not observed in Alaskan frogs, suggesting that other mechanisms of regulation are important in this phenotype. Overall, GLUT2 abundance was constitutively greater and more responsive to freezing in Alaskan *R. sylvatica*, suggesting that GLUT2 contributes substantively to the extreme freeze tolerance of subarctic wood frogs.

Introduction

The wood frog *Rana sylvatica* is one of a small group of amphibians that tolerate the freezing of their body fluids as an adaptation to survive the thermal challenges of winter (Costanzo & Lee, 2013). In its relatively exposed hibernacula on the forest floor, the wood frog routinely encounters low temperatures, particularly toward the northern limit of its range, which extends north of the Arctic Circle (Martof & Humphries, 1959). Populational variation in the lower lethal temperature of *R. sylvatica* reflects the severity of the frigorific conditions experienced in different climes. *Rana sylvatica* from Interior Alaska, near the northern limit of its range, can survive freezing at temperatures below −16°C (Costanzo *et al.*, 2013; Larson *et al.*, 2014), whereas frogs from the more temperate Great Lakes Region of North America can only survive freezing from −3 to −6°C (Costanzo & Lee, 2013).

The use of cryoprotective solutes is an important physiological adaptation in animal freeze tolerance (see recent review by Costanzo & Lee, 2013). Because their protective benefits derive at least partly from colligative properties, an individual's capacity to accumulate cryoprotectants to high levels strongly influences its degree of freeze tolerance (Costanzo, Lee & Lortz, 1993; Williams & Lee, 2008). In *R. sylvatica*, glucose contributes to freezing survival in a concentration-dependent manner by reducing ice content and cell shrinkage, and by fueling postfreeze repair processes (Costanzo *et al.*, 1993; Costanzo & Lee, 2013). Freezing of peripheral tissues triggers catabolism of hepatic glycogen stores, and glucose is rapidly exported from the liver to target tissues before circulation ceases (Storey & Storey, 2004); thus, the liver is the primary source of the cryoprotectant mobilized during freezing.

The rate of glucose export from the liver is an important factor determining the efficacy of the glycemic response in *R. sylvatica*. Transmembrane movement of glucose is accomplished by specialized carrier proteins, facilitative glucose transporters (GLUTs) (Manolescu *et al.*, 2007). GLUT2 is the isoform primarily responsible for glucose transport in the liver, and its particular kinetic properties (low affinity, high capacity; Leturque, Brot-Laroche & Le Gall, 2009) allow for the efficient efflux of high concentrations of glucose that is necessary when *R. sylvatica* freezes. Uptake of the circulating glucose by cells of nonhepatic tissues is also critical to freezing survival, but in some organs, such as skeletal muscle, is a much more complex process that is influenced by various factors, including blood flow, metabolic activity and hormonal stimulation (Wasserman *et al.*, 2011).

The purpose of this study was to explore the role of hepatic GLUT2 in the wood frog's cryoprotectant system, with a particular focus on the importance of this protein in the extreme freeze tolerance of northern frogs. Work by King, Rosholt & Storey (1993) suggests that the greater glucose transport capacity in the liver of *R. sylvatica*, as compared with that in the closely related but freeze-intolerant leopard frog *R. pipiens*, contributes to the difference in freeze tolerance between these species. However, there is no variation in GLUT2 kinetics (Rosendale *et al*., 2014b) that could explain putative disparities in glucose transport capacity between subarctic and temperate *R. sylvatica*. Rather, we hypothesize that the exceptional freeze tolerance of the northern phenotype is due at least in part to a cryoprotectant system that is enhanced by maintaining a larger population of glucose transporters in hepatocytes. To test this hypothesis, we examined seasonal and freezing-induced changes in GLUT2 abundance in livers of frogs indigenous to Alaska and Ohio, USA. Additionally, we studied the effect of acclimation temperature on hepatic GLUT2 abundance in Ohioan frogs.

Materials and methods

Acquisition and acclimation of Ohioan *R. sylvatica*

Adult male *R. sylvatica* was collected from a vernal pool in southern Ohio, USA (Adams County; 38.8°N, 83.3°W) during February 2011 and February 2012. Frogs were transported to our laboratory, placed in darkened boxes containing damp moss, and kept at 4°C for several weeks. Thereafter, they were transferred to a 48 m^2 outdoor enclosure in a wooded area of the Miami University Ecology Research Center (39.5°N, 84.7°W). Frogs were provided with a pool of water and offered crickets dusted with a vitamin supplement (ReptoCal, Tetrafauna, Blacksburg, VA, USA) three times weekly. Feeding was supplemented with arthropods attracted to an ultraviolet-A light that hung in the enclosure until late October. Frogs were sampled in April, late July, October and November (hereafter 'spring', 'summer', 'fall' and 'late-fall' frogs, respectively; $n = 7$, each group). In November, additional frogs were collected from the enclosure, brought to the laboratory, and kept on damp moss in darkened boxes at 4°C until used in experiments in January (hereafter 'winter' frogs; $n = 6$).

Acquisition and acclimation of Alaskan *R. sylvatica*

Male and female frogs representing the northern phenotype of *R. sylvatica* were collected from wetlands in Southeast Fairbanks Census Area, Alaska, USA (63.8°N, 143.6°W) in late May 2011 after emerging from hibernacula and occupying breeding sites. Frogs were shipped on moist substratum inside coolers containing ice packs to our laboratory, where they were communally housed on damp moss in darkened boxes at 4°C. These frogs (hereafter 'spring' frogs; $n = 7$) were sampled 3 days later.

Additional Alaskan frogs were collected in and around Fairbanks, Alaska (64.8°N, 147.7°W) during early August 2011. Frogs were topically treated with tetracycline HCl and shipped on moist substratum in refrigerated containers to our laboratory. Upon arrival, they were transferred individually to a clear plastic cup and kept on damp paper in a programmable environmental chamber (Percival, model I-35X; Boone, IA, USA). 'Late-summer' frogs ($n = 6$) were sampled promptly, whereas most were acclimatized over 5 weeks using dynamic diel cycles of temperature and ambient light, which, based on institutional long-term records of weather (obtained from the National Oceanic and Atmospheric Administration's National Climatic Data Center), were seasonal and appropriate to the frogs' origin. Initially, temperature varied daily from 17 to 8°C and the photophase was 16.5 h; by the end of acclimatization, in mid-September, temperature varied daily from 13 to 3°C and the photophase was 13.3 h. Throughout, frogs were fed three times weekly with vitamin-fortified crickets. Following acclimatization, 'fall' frogs ($n = 6$) were sampled immediately, whereas the remaining frogs were kept on moist substratum in the dark at 4°C for ~8 weeks in simulated hibernation until used in experiments in mid-November (hereafter 'winter' frogs; $n = 8$). By utilizing Ohioan and Alaskan frogs kept in simulated hibernation conditions in the laboratory, frogs were available for experimentation at a time of year when collection from outdoor enclosure is generally impractical. Additionally, we were able to control if and/or when frogs froze, a factor that can be highly variable among natural microhabitats.

All protocols were approved by Miami University's Institutional Animal Care and Use Committee.

Thermal acclimation experiment

In November, when Ohioan frogs were brought into the laboratory for winter acclimatization, a separate set of 15 frogs was divided into three treatment groups ($n = 5$ per group) and placed individually in deli cups on a substratum of damp paper towel. They were kept unfed in darkness at 0, 4 or 10°C inside environmental chambers and then sampled (as described below) after 21 days. Because of the limited number of Alaskan frogs available, the thermal acclimation experiment was restricted to the Ohioan population.

Freezing and thawing experiment

Freeze/thaw experiments were performed following Costanzo *et al*. (2013) on winter frogs in November (Alaskan) or January (Ohioan), *c*. 2 months after they began hibernating in their respective native locales. This acclimatory period is necessary for the frogs to accumulate cryoprotectants and become fully cold hardened (Costanzo *et al*., 2015). To begin, we removed any bladder fluid via cloacal cannulation. Each frog was individually placed inside a 50-mL polypropylene tube with a thermocouple probe placed against its abdomen. Thermocouples were used to monitor body temperature (T_b) at 30 s intervals on a multichannel data logger (RD3752, Omega, Stamford, CT, USA) throughout the experiment.

After the frogs were cooled to −1°C in a refrigerated bath (RTE 140, Neslab, Portsmouth, NH, USA) containing chilled ethanol, aerosol coolant was applied to the tube's exterior so that ice crystals formed inside, causing the frog to freeze through ice inoculation. Initiation of freezing was confirmed for each frog by the occurrence of a freezing exotherm. Freezing continued for up to 48 h as the temperature of the bath was gradually decreased (−0.05°C h^{-1}) over 30 h to an ultimate T_b of −2.5°C. Groups of frogs (n = 5–6) were removed from the bath and sampled at 2, 6, 30 or 48 h after freezing commenced. Additional frogs were removed from the bath 48 h after ice inoculation, gently removed from their tubes, and kept on damp paper in darkness at 4°C for 6 h or 5 days before being sampled. Unfrozen controls (n = 7) were sampled directly from their holding containers kept at 4°C.

Tissue sampling and preparation

Frogs were euthanized by double pithing and dissected in a cold room. Their livers were quickly excised, frozen in liquid N_2, and stored at −80°C until analyzed. For protein analysis, liver samples were homogenized in STE buffer (250 mM NaCl, 10 mM Tris-HCl, pH 8.3, 5 mM EDTA) containing a protease-inhibitor cocktail (Sigma, St. Louis, MO, USA) using a shearing-type homogenizer. The homogenate was centrifuged at 500×***g*** for 10 min at 4°C to remove cellular debris, and the resulting supernatant was centrifuged at 16 000×***g*** for 20 min at 4°C. The pellet, containing the crude membrane fraction, was resuspended in STE buffer, aliquoted, and frozen at −80°C. Final protein concentration was determined using a Bradford protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

For mRNA analysis, liver samples (~100 mg) were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted following the manufacturer's protocol. RNA samples were treated with DNase I and the RNA was concentrated using the GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific, Pittsburgh, PA, USA). RNA quantity was determined at 260 nm; RNA quality was confirmed at 260:280 nm and 260:230 nm.

Immunoblotting analysis

Samples of protein (10 μg) were mixed with Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol, incubated for 10 min at room temperature (RT; ~21°C), and analyzed by SDS-PAGE on a 4–15% gradient gel (Bio-Rad). Precision Plus protein standard (Bio-Rad) was used as a mass reference. Protein was then transferred to a nitrocellulose membrane (Bio-Rad) and membranes were stained with 0.2% (w/v) Ponceau S (Sigma) containing 5% acetic acid. Membranes were digitally scanned and then destained using 0.1 M NaOH. Total protein was analyzed by densitometry (described below) to verify equal protein loading in all lanes and that transfer was equivalent across the gel. Membranes were incubated at RT for 2 h in Tris-buffered saline and Tween 20 (TBS-T; 10 mM Tris, 100 mM NaCl and 0.1% Tween 20 at pH 7.5) containing 10% nonfat milk. Membranes were then incubated overnight at 4°C in TBS-T containing 5% nonfat milk and an anti-GLUT2 primary antibody (0.2 μg mL^{-1}). The anti-GLUT2 antibody, designed against an oligopeptide corresponding to a C-terminus region of the *R. sylvatica* GLUT2, was highly specific, detecting a single band at 54 kDa (described in Rosendale *et al.*, 2014b). Membranes were washed with TBS-T and then incubated for 2 h at RT with goat anti-rabbit IgG-HRP conjugates secondary antibody (GE Healthcare, Piscataway, NJ, USA), which was diluted 1:2000 in TBS-T containing 5% nonfat milk. Membranes were then washed with TBS-T, incubated 2 min in enhanced chemiluminescence detection reagents (GE Healthcare), and imaged using a digital imaging system (FluorChem FC2; Alpha Innotech, San Leandro, CA, USA). GLUT2 protein bands and the total protein for each sample were semiquantified using AlphaViewspot densitometry (Alpha Innotech). Densitometric values determined for GLUT2 were standardized to total protein densitometry values. All samples were run in duplicate; the average of the two densitometric values was then used in statistical analyses. To facilitate comparison of protein abundance among experimental groups, each gel contained at least one sample from each season, acclimation temperature or time point for both populations.

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR reactions (10 μL) consisted of iTaq™ Universal SYBR® Green reaction mix (Bio-Rad), 1 ng RNA, iTaq reverse transcriptase, 300 nM forward and reverse primers, and nuclease-free water. Primers for the reference gene β-actin and *R. sylvatica* GLUT2 were designed as previously described (Rosendale, Lee & Costanzo, 2014a). Primers for 18s (Forward: 5′-CAAGACGAACCAAAGCGAAAG-3′, Reverse: 5′-TCGGAACTACGACGGTATCT-3′) were designed from areas of conserved nucleic acid sequence of various ranid species. Reference genes were chosen for their stable expression in other organisms during thermal acclimation and freezing (Zimmerman *et al.*, 2007; Wu, Storey & Storey, 2009). Primers were validated as described in Rosendale *et al.* (2014a). qRT-PCR reactions, which consisted of a reverse transcription step at 50°C for 10 min, polymerase activation at 95°C for 1 min, 45 cycles of denaturation at 95°C for 15 s, annealing/extension at 60°C for 30 s and denaturation at 95°C for 1 min, were run on a Rotor-Gene thermal cycler (Qiagen, Germantown, MD, USA). Following amplification, a melt-curve analysis was performed from 65 to 95°C, with 0.5°C increments every 10 s. Each sample was run in triplicate, and the average quantification cycle (Cq) value was determined. A no-template control was run with each primer pair to check for primer-dimers and reagent contamination, and a negative control (no reverse transcriptase) was run for each sample to check for genomic DNA contamination. The suitability of β-actin and 18s as reference genes was assessed using the qBase$^+$ model (Hellemans *et al.*, 2007), and GLUT2 mRNA levels were normalized to β-actin and 18s using the qBase$^+$ model (Biogazelle, Zwijnaarde, Belgium).

Statistical analysis

Summary statistics are reported as mean ± SE. Comparisons of protein levels and also mRNA levels in the seasonal experiment were made within each population using one-way analysis of variance (ANOVA). Post hoc comparisons among groups within the seasonal and thermal acclimation experiments were made using Tukey's tests, whereas in the freeze/thaw time course experiment, a Dunnett's test was used to distinguish each group of experimentally treated frogs from that of a corresponding group of unfrozen (control) frogs. In the latter experiment, two-factor ANOVA was used to compare protein abundance between populations, with matched pairs of means distinguished using Bonferroni tests. Two-factor ANOVA was also used to test for overall populational effects in the seasonal study, but only values from the two (equitable) winter groups were compared using the Student's *t*-test.

Results

Seasonal variation and effect of thermal acclimation on GLUT2 expression

All frogs appeared in good health and showed no signs of distress at the time they were sampled. A detailed description of the changes in somatic and physiological variables in these frogs seasonally and in response to freezing was previously reported (Costanzo *et al.*, 2013, 2014). GLUT2 protein abundance varied seasonally in both Alaskan ($F_{3,23} = 8.3$, $P = 0.0006$) and Ohioan ($F_{4,29} = 6.1$, $P = 0.001$) frogs (Fig. 1a). In Alaskan frogs, GLUT2 levels were lowest in the summer, with GLUT2 abundance being 2.8-fold greater in fall and winter. In spring, GLUT2 protein levels were also high, being 3.2-fold greater than in summer, but did not differ from winter values. In Ohioan frogs, GLUT2 abundance was lowest in the summer, increased in the fall, and was ~2.3-fold greater in late fall and winter than in summer. In spring, GLUT2 protein levels did not differ from any of the other seasons. Overall, GLUT2 protein was more abundant in Alaskan than Ohioan frogs ($F_{8,52} = 6.3$, $P = 0.015$), specifically 1.8-fold greater ($t_{12} = 3.6$, $P < 0.01$) when winter frogs were compared.

Levels of GLUT2 mRNA varied seasonally in both Alaskan ($F_{3,23} = 46.1$, $P < 0.0001$) and Ohioan ($F_{2,29} = 14.3$, $P < 0.0001$) frogs (Fig. 1b). In Alaskan frogs, levels were 2.9- and 12-fold lower in winter and spring, respectively, than in late summer. In Ohioan frogs, levels were maximal in late fall, when they were ~10-fold greater than those determined in summer and spring.

Ohioan frogs acclimated in late fall to 0, 4 or 10°C showed no difference ($F_{2,12} = 0.8$, $P = 0.481$) in GLUT2 protein abundance, as represented by arbitrary densitometric units. Values for these groups were 1.3 ± 0.1, 1.4 ± 0.2 and 1.5 ± 0.1, respectively.

Effect of experimental freeze/thaw cycle on GLUT2 abundance

Frogs reached an equilibrium temperature of –2.5°C after 30 h during the 48-h experimental freezing treatment. Frogs

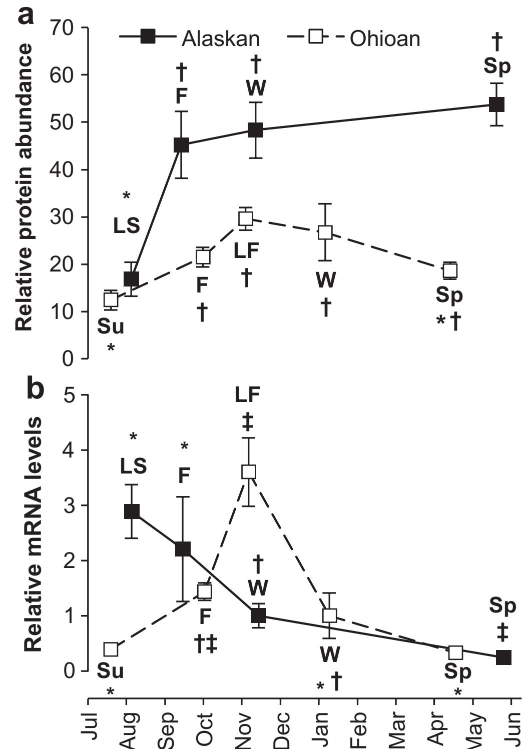

Figure 1 Seasonal variation in abundance of GLUT2 (a) protein and (b) mRNA in liver of Alaskan and Ohioan *Rana sylvatica*. Su = summer; LS = late summer; F = fall; LF = late fall; W = winter; Sp = spring. Values are expressed as mean ± SE (*n* = 6–8 frogs per group). Within a population, mean values identified by different symbols were statistically distinguishable (one-way ANOVA/Tukey; *P* < 0.05).

sampled after 30 or 48 h of freezing were visually examined for ice content upon dissection; qualitatively, Ohioan frogs contained substantially more internal ice within and among organs than Alaskan frogs. All frogs permitted to thaw and monitored for 5 days exhibited regular cardiac rhythm, tissue perfusion, normal posture and locomotor ability, indicating that they had survived the freezing exposure.

Freezing significantly increased ($F_{6,35} = 2.8$, $P = 0.022$) GLUT2 protein abundance in Alaskan frogs, but had no effect ($F_{6,29} = 1.0$, $P = 0.419$) in Ohioan frogs (Fig. 2). Alaskan frogs frozen for 48 h, and those frozen for 48 h and then thawed for 6 h, had about twice as much of the protein as unfrozen controls. Overall, levels of GLUT2 protein in Alaskan frogs were greater ($F_{1,64} = 47.3$, $P < 0.001$) than those in Ohioan frogs, with the differences ranging from 1.9- (unfrozen control) to 2.6-fold (48 h frozen).

Discussion

Among temperate ectotherms, the degree of freeze tolerance in terms of minimum tolerable temperature and maximal duration can vary substantially among geographically distinct populations of conspecifics (Williams & Lee, 2008).

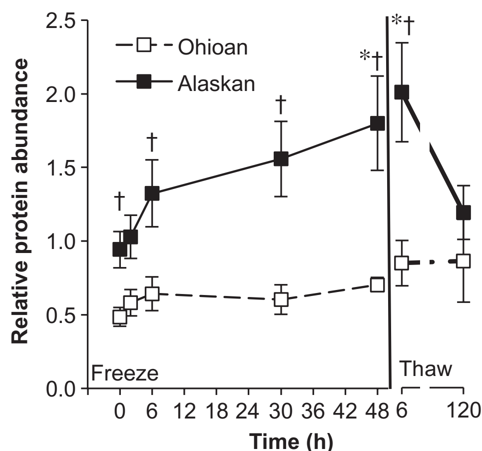

Figure 2 Changes in abundance of GLUT2 protein in liver of Alaskan and Ohioan *Rana sylvatica* associated with experimental freezing for up to 48 h and thawing for up to 120 h. Freezing experiments were performed 2 months after frogs began hibernating in their respective native locales, November and January for the Alaskan and Ohioan frogs, respectively. Mean values (±SE, n = 4–8 frogs per group) indicated by asterisks differed from the corresponding value for unfrozen (control) frogs (one-way ANOVA/Dunnett's test, $P < 0.05$); daggers denote corresponding values that differed between populations (two-way ANOVA/Bonferroni, $P < 0.05$).

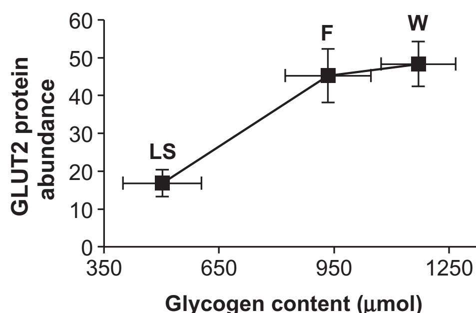

Figure 3 Relationship between abundance of GLUT2 protein (arbitrary units; data from Fig. 1) and glycogen content in liver of Alaskan *Rana sylvatica* sampled in late summer (LS), fall (F) and winter (W). Values are expressed as mean ± SEM (n = 5–8 frogs per group). Glycogen data are from Costanzo *et al.* (2013).

Cryoprotectant accumulation is an important factor underpinning this variability, with northern populations typically achieving higher cryoprotectant levels during freezing than more southerly counterparts (Irwin & Lee, 2003; Holmstrup *et al.*, 2007; Costanzo *et al.*, 2013). In *R. sylvatica*, the glycemic response is critical in freeze tolerance, and whereas freezing-induced glycogenolysis is well described (Storey & Storey, 2004), little is known about the role of transport proteins in glucose distribution to nonhepatic tissues. GLUT2, which abounds in liver and manages high concentrations of glucose (Leturque *et al.*, 2009), is ideally suited to facilitate this process. In fact, King *et al.* found that glucose transport is enhanced in the liver of *R. sylvatica* as compared with a freeze-intolerant congener (1993), and that GLUT2 abundance increases from summer to fall (1995). Our study provides novel evidence of this protein's importance in amphibian freeze tolerance, including its role in the evolution of extreme freeze tolerance in subarctic *R. sylvatica*.

Seasonal changes in GLUT2

Freeze-tolerant organisms commonly accrue large quantities of cryoprotectants during seasonal cold hardening (Storey & Storey, 1988). Although circulating levels of glucose are fairly static, temperate anurans undergo seasonal cycling of hepatic glycogen reserves, with glycogen deposits peaking in autumn or early winter and falling after hibernal emergence (Pinder, Storey & Ultsch, 1992). In *R. sylvatica*, this ensures that a source of glucose is available when the risk of freezing is greatest. In both Ohioan and Alaskan *R. sylvatica*, GLUT2 abundance in liver increased from summer and reached heightened levels in late fall and winter. These changes in transporter abundance closely corresponded with the pattern of glycogen cycling (Fig. 3; Costanzo *et al.*, 2013), suggesting that capacities for glucose synthesis and transport are synchronized. Because the kinetics of glucose transport (presumably via GLUT2) in the liver of *R. sylvatica* does not change from summer to autumn (King *et al.*, 1995), the increase in GLUT2 abundance we observed is likely the sole mechanism enhancing glucose transport capacity in preparation for winter. Seasonal changes in GLUT2 abundance in Alaskan frogs generally followed the pattern in Ohioan frogs, except that GLUT2 abundance remained high during spring. This difference likely reflects their continued need to mobilize glucose as a cryoprotectant, as spring frogs remain freeze tolerant, albeit to a lesser degree than winter-acclimatized frogs (Costanzo *et al.*, 2014).

Glycogen depots of anurans indigenous to cold climates tend to be more substantial than those of conspecifics from more temperate locales (Jönsson *et al.*, 2009; Chen, Zhang & Lu, 2011). Among freeze-tolerant species, larger glycogen reserves enhance glucose mobilization during freezing (Costanzo & Lee, 1993). In *R. sylvatica*, Alaskan frogs develop especially rich glycogen deposits which are much larger than those of Ohioan frogs, particularly when normalized to body mass (Costanzo *et al.*, 2013). Correspondingly, Alaskan frogs generally maintained a greater abundance of GLUT2, which, in winter, was ~2-fold greater than in Ohioan conspecifics (Fig. 1a). Their greater abundance of GLUT2 presumably affords this phenotype an enhanced capacity for glucose transport, which contributes to its exceptional freeze tolerance.

We examined seasonal dynamics in mRNA levels to determine if the observed changes in GLUT2 protein abundance could have resulted from transcriptional regulation. Although levels of protein and mRNA are often congruent, a disconnect can occur when organisms undergo large-scale physiological changes, such as preparing for dormancy (Storey & Storey,

2013). Seasonal changes in mRNA levels corresponded reasonably well to changes in protein abundance in Ohioan frogs, but this was not the case with Alaskan frogs. Thus, the role of transcriptional regulation in the seasonal mediation of GLUT2 abundance apparently differs between these populations. Future work should explore the possibility that regulation of transporter number is achieved not only transcriptionally, but also by mechanisms such as varying mRNA and/or protein stability, adjusting translation rates, or altering cellular compartmentalization of existing proteins.

Thermal acclimation

Among ectotherms, cold acclimation is commonly associated with changes in gene expression, which can enhance freeze tolerance (Storey & Storey, 2013). Although cold acclimation does not elicit a glycemic response in *R. sylvatica* (Storey & Storey, 1984), upregulation of hepatic GLUT2 presumably prepares frogs for impending bouts of freezing. However, in our study, acclimation temperature did not affect this protein's abundance in Ohioan frogs, suggesting that thermal change alone is an insufficient stimulus for the observed seasonal dynamics in GLUT2 expression. However, varying environmental temperature may work in concert with other factors, such as photoperiod, to regulate transporter abundance.

Freezing response of GLUT2

During freezing, ectotherms employ a suite of physiological, biochemical and molecular responses that permit them to survive the freezing of their body fluids (Storey & Storey, 2004; Costanzo & Lee, 2013). In *R. sylvatica*, the cryoprotective efficacy of the glycemic response is well documented, with high levels of glucose directly improving freeze tolerance (Costanzo *et al*., 1993). Initiation of somatic freezing causes a sympathetic stimulation of glycogenolysis in hepatocytes, resulting in the copious production of glucose, which, to serve its protective role, must be transported across the membrane, distributed throughout the body, and internalized within cells before circulation ceases (Storey & Storey, 2004). Expeditious glucose export is especially critical in subarctic *R. sylvatica*, which, to survive extreme cold, must mobilize massive amounts of cryoprotectant but can quickly freeze by virtue of their small size and low thermal capacitance (Costanzo *et al*., 2013). Our findings that Alaskan frogs maintain constitutively high levels of GLUT2 in winter, and these winter frogs double the transporter population upon freezing (achieving levels up to 2.6-fold greater than those in freezing Ohioan frogs), suggest that this northern phenotype has evolved a highly efficient system for mobilizing cryoprotectant. This enhanced transport system complements the ability of Alaskan frogs to produce and mobilize extraordinarily high levels of glucose, as compared with more southerly populations (Costanzo *et al*., 2013, 2015; Larson *et al*., 2014).

An upregulation of GLUT2 abundance with freezing would seemingly benefit *R. sylvatica* from any population, although we did not observe this response in our Ohioan frogs. However, other work has shown that, when frozen more slowly, Ohioan frogs do exhibit an ~2-fold upregulation in GLUT2 abundance (Rosendale *et al*., 2014a). We conjecture that GLUT2 upregulation occurs universally in *R. sylvatica* in response to freezing, but the response in subarctic frogs is heightened as compared with their temperate counterparts.

Conclusions

Wood frogs are the most northerly distributed of North American amphibians, and phylogeographic analysis suggests that, following glacial retreat, northern populations, including our Alaskan frogs, were established from emigrants from ice-free refugia in the American Midwest (Lee-Yaw, Irwin & Green, 2008). The considerable variation in freeze tolerance among populations likely developed as wood frogs evolved unique traits to adapt to local conditions. Among the unique adaptations associated with the extreme freeze tolerance of subarctic frogs is an augmented glucosic cryoprotectant system that is bolstered by inordinately large glycogen reserves (Costanzo *et al*., 2013), an enhanced glucose mobilization capacity (Costanzo *et al*., 2015) and, as suggested by our present results, a high abundance of hepatic GLUT2.

Acknowledgments

We thank B. Barnes for sharing his knowledge on the biology of Alaskan wood frogs. We thank D. Larson and D. Russell for assisting in the logistical aspects of collecting frogs in Alaska. A. Reynolds and C. do Amaral assisted with the frog and tissue collections. C. do Amaral and two anonymous reviewers contributed constructive comments on the paper. This work was supported by the National Science Foundation (IOS1022788 to J.P.C.).

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