

Effect of Physiological Stress on Expression of Glucose Transporter 2 in Liver of the Wood Frog, *Rana sylvatica*



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ABSTRACT

Glucose transporters (GLUTs) have been implicated in the survival of various physiological stresses in mammals; however, little is known about the role of these proteins in stress tolerance in lower vertebrates. The wood frog (*Rana sylvatica*), which survives multiple winter-related stresses by copiously mobilizing hepatic glycogen stores, is an interesting subject for the study of glucose transport in amphibians. We examined the effects of several physiological stresses on GLUT2 protein and mRNA levels in the liver of *R. sylvatica*. Using immunoblotting techniques to measure relative GLUT2 abundance, we found that GLUT2 numbers increased in response to organismal freezing, hypoxia exposure, and glucose loading; whereas, experimental dehydration and urea loading did not affect GLUT2 abundance. GLUT2 mRNA levels, assessed using quantitative real-time polymerase chain reaction, changed in accordance with protein abundance for most stresses, indicating that transcriptional regulation of GLUT2 occurs in response to stress. Overall, hepatic GLUT2 seems to be important in stress survival in *R. sylvatica* and is regulated to meet the physiological need to accumulate glucose. *J. Exp. Zool.* 321A:566–576, 2014.

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Facilitative glucose transporters (GLUTs) are specialized carrier proteins that function in the movement of glucose across cell membranes. These proteins were first identified in humans (Mueckler et al., '85), and 14 isoforms of the mammalian GLUT (SLC2A) protein family have since been described (Thorens and Mueckler, 2010). These isoforms are structurally similar but differ in their tissue distribution, subcellular localization, kinetic characteristics, and regulatory properties (Thorens and Mueckler, 2010). In addition to mammals, GLUTs have been characterized in various vertebrates including fish (Castillo et al., 2009), birds (Wang et al., '94), and, more recently, amphibians (Rosendale et al., 2014).

In all vertebrates, GLUTs play an important role in maintaining glucose homeostasis, particularly during periods of physiological stress. For example, in hypoxia, changes in GLUT abundance facilitate utilization of glucose, which serves as a source of metabolic energy and protects cells from hypoxic injury (Bunn

and Poyton, '96; Lin et al., 2000). GLUT expression is also modified by dehydration (Vannucci et al., '94), glucose deprivation (Ismail-beigi, '93), hyperosmolality (Ramasamy et al., 2001), and high pH (Ismail-beigi, '93). Although GLUTs have been extensively studied in stress tolerance in mammals, little is known about their role in lower vertebrates, particularly amphibians.

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The wood frog, *Rana sylvatica*, is an excellent subject for the study of GLUTs during physiological stress, particularly freezing. This species is the most northerly distributed of North American amphibians, ranging north of the Arctic Circle (Martof and Humphries, '59), and can successfully exploit terrestrial hibernacula due to its tolerance of multiple physiological stresses. *R. sylvatica* survives the freezing of ~65% of its body water at temperatures at least as low as -16°C (Costanzo et al., 2013), tolerates the loss of over 50% of its body water during dehydration (Churchill and Storey, '93), and endures anoxia for at least several days (Holden and Storey, '97).

R. sylvatica has evolved various physiological adaptations to survive winter-related stresses, and primary among them is the use of cryoprotective solutes. Cryoprotectants accumulate gradually with the seasonal accrual of urea or rapidly with the freezing-induced mobilization of glucose. Both glucose and urea greatly improve freeze tolerance by limiting cellular water loss and reducing ice formation, and purportedly by stabilizing cellular components (Costanzo and Lee, 2013). Additionally, in many species of frogs, hyperglycemia also occurs during dehydration and anoxia (Churchill and Storey, '93; Ultsch et al., 2004). Glucose likely acts as an osmoprotectant during dehydration and as a metabolic fuel during both dehydration and anoxia. During stress, glycemic increase occurs primarily through glycogenolysis in the liver (Storey and Storey, 2004). The rapidity of glucose mobilization depends on various factors including the rate of glucose export from the liver, a process that requires moving glucose across cell membranes.

Due to the limited permeability of membranes to glucose and the need to rapidly mobilize glucose during freezing, it is likely that transporters play an important role in freezing survival of *R. sylvatica*. Because glucose transporter 2 (GLUT2) is a low-affinity, high-capacity carrier that is primarily responsible for glucose transport in mammalian liver (Thorens and Mueckler, 2010), it is conceivable that this isoform is also involved in the freezing-induced export of cryoprotectant in *R. sylvatica*. To address this question, and to elucidate the role of GLUT2 in stress tolerance in general, we examined a GLUT2 isoform present in high abundance in liver of *R. sylvatica* (Rosendale et al., 2014). Changes in protein abundance and mRNA expression of GLUT2 in the liver were determined for *R. sylvatica* exposed to organismal freezing, dehydration, or hypoxia exposure. The effect of hyperglycemia, which in the absence of other stresses can cause changes in GLUT expression (Asano et al., '92), was also studied through glucose-loading experiments. Additionally, we examined changes in the protein's expression in response to high uremia, which has the potential to inhibit GLUT2 function (Rosendale et al., 2014). To verify that our experimental treatments successfully elicited the desired stress and to clarify the physiological environments that may influence transporter regulation, we also examined appropriate physiological variables. Finally, we separately determined GLUT2 abundance

in the plasma membrane and entire cell in both unfrozen and frozen frogs to elucidate the importance of newly synthesized transporters in meeting the demand for increased glucose transport under stress.

MATERIALS AND METHODS

Animals

Male *R. sylvatica* were collected from a vernal pool in southern Ohio (Adams County) during March, 2009 and February, 2012. They were transported to our laboratory, placed inside boxes containing damp moss, and kept in darkness at 4°C . In early April, they were transferred to a 48 m^2 outdoor enclosure in a wooded area of the Miami University Ecology Research Center. Frogs were provided a pool of water and offered crickets dusted with a vitamin supplement (ReptoCal, Tetrafauna, Blacksburg, VA, USA) three times per week. Feeding, which was supplemented with arthropods attracted to an ultraviolet-A light hung in the enclosure, continued until late October. In November, frogs, which were on the verge of entering dormancy, were recaptured, brought to the laboratory, and kept on damp moss in darkened boxes at 4°C until used in experiments in January. Immediately prior to receiving experimental treatment, standard body mass was determined by removing any fluid present in the bladder (by inserting a polished glass cannula into the cloaca and applying gentle pressure to the lower abdomen) and then weighing the frog on an electronic balance. Control frogs ($N=5-7$) were sampled directly from their holding containers kept at 4°C , some frogs were used as controls in more than one experiment. All experiments were conducted in compliance with the Institutional Animal Care and Use Committee at Miami University.

Effect of Experimental Freeze/Thaw Cycle on GLUT2 Expression

Freezing experiments were performed using a protocol (modified from Costanzo et al., '93) that promotes cryoprotective responses and presumably mimics natural freezing and thawing episodes. Frogs were individually placed inside a 50-mL polypropylene tube, and the opening of the tube was blocked with foam. Body temperature (T_b), monitored by a thermocouple probe that was placed against the frog's abdomen, was continuously recorded on a multichannel data logger (Omega, model RD3752, Stamford, CT, USA). Tubes containing these frogs were subsequently cooled to -0.7°C inside a refrigerated bath (Neslab, model RTE 140, Portsmouth, NH, USA) containing chilled ethanol. Once the T_b reached -0.7°C , aerosol coolant was applied to the tube's exterior; ice crystals forming inside the tube initiated the freezing of the frog through ice inoculation. After freezing commenced, frogs were gradually cooled ($-0.0375^{\circ}\text{C hr}^{-1}$) over 48 hr. Frogs were held at -2.5°C for 6 hr, following which some were sampled in the fully frozen state ($N=6$) and others, destined to be sampled after thawing, were held at 4°C for 24 hr ($N=5$).

Effect of Experimental Dehydration on GLUT2 Expression

Body water content was reduced by experimentally dehydrating frogs following an established procedure (Muir et al., 2007; Rosendale et al., 2012). Briefly, frogs ($N=6$) were transferred individually from their holding boxes to a plastic container with a perforated lid and substratum of dry paper towels. Frogs were kept in darkness at 4°C and allowed to gradually lose water through evaporation. They were weighed daily to track any change in their body mass, which was assumed to represent only moisture, until they had lost on average ~40% of their initial body water, a process that took 14 days.

Effect of Experimental Hypoxia on GLUT2 Expression

Frogs were exposed to hypoxia following a protocol modified from Holden and Storey ('97). Frogs ($N=7$) were individually transferred to an acrylic metabolic chamber (Qubit Systems no. G115, Kingston, ON) with valved incurrent and excurrent ports and placed on moist paper towels. Chambers were flushed with N₂ gas for 15–20 min before the excurrent ports were closed and the chambers were filled with N₂ gas. Frogs were kept in their chambers, in darkness, at 4°C for 34 hr. Following hypoxia exposure, but prior to removing them from the chambers, a gas sample was drawn from the excurrent port into a 60-mL Hamilton syringe fitted with a Teflon stopcock and analyzed for partial pressure of oxygen using an oxygen analyzer (model S-3A/I; AEI Technologies, Pittsburgh, PA).

Effect of Experimental Glucose Loading on GLUT2 Expression

Glycemia was manipulated using a protocol modified from Costanzo et al. ('93). Frogs ($N=7$) were transferred individually from their holding boxes to a plastic container and placed on a substratum of moist paper towels. They were administered isotonic phosphate-buffered saline (PBS; 100 mM NaCl, 2 mM KCl, 6 mM Na₂HPO₄, 1 mM KH₂PO₄; 230 mosmol kg⁻¹) containing 1.5 M glucose. The volume (0.3–0.5 mL) of the solution, injected into the dorsal lymph sac using a 27.5-gauge needle, approximated 3% of standard body mass. Following the initial injection, frogs were kept in darkness at 4°C for 2 days after which they received a second injection of the same dosage of PBS/glucose solution. They were held at 4°C for an additional 2 days prior to being sampled.

Effect of Experimental Urea Loading on GLUT2 Expression

Uremia was manipulated using a protocol that simultaneously introduces exogenous urea and inhibits urea excretion by subjecting frogs to conditions that stimulate antidiuretic responses (Muir et al., 2007; Rosendale et al., 2012). Frogs ($N=7$) were kept on a substratum of dry paper towels, and over 3 days they lost ~0.5 g of body mass, presumably through evaporative water loss. Next, frogs were administered a single dosage of isotonic PBS containing 1.5 M urea (as described for the glucose loading in the preceding section) and kept in darkness at 4°C for 2 days prior to being sampled.

Tissue Sampling and Metabolite Assays

Working inside a cold room (4°C), frogs were euthanized by double pithing and dissected. Aortic blood was collected into heparinized capillary tubes, which were centrifuged at 2,000g for 5 min to isolate plasma. The excised liver and plasma were frozen in liquid N₂ and stored at –80°C until analyzed. Carcasses were dried in a 65°C oven and the mass of evaporated water determined to estimate body water content (expressed as g water per g dry tissue). Plasma concentrations of glucose, urea, and lactate were determined colorimetrically using glucose oxidase, urease, and lactate oxidase assays (Pointe Scientific, Canton, MI, USA), respectively. All physiological variables were measured for every frog; however, only variables that differed significantly between groups and/or were particularly relevant to a given treatment were reported.

GLUT2 Abundance by Immunoblot Analysis

Liver samples collected from frogs in control and treatment groups 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 (model 985370, BioSpec Products, Bartlesville, OK, USA). The homogenate was centrifuged at 500g for 10 min at 4°C to remove cellular debris, and the resulting supernatant was centrifuged at 16,000g for 20 min at 4°C. The pellet, representing a crude plasma 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 (BSA) as the standard. Protein (10 µg) samples were mixed with Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol, incubated for 10 min at room temperature (~21°C), and analyzed by immunoblotting and densitometry techniques as previously described (Rosendale et al., 2012). An oligopeptide corresponding to a C-terminus region of the *R. sylvatica* GLUT2 was used to produce an anti-GLUT2 antibody (described in Rosendale et al., 2014). The anti-GLUT2 antibody was used in immunoreactions at a final concentration of 0.2 µg mL⁻¹ in TBS-T (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20 at pH 7.5) containing 5% non-fat milk. All samples were run in duplicate; the average of the two densitometric values was used in statistical analyses.

To check specificity, the anti-GLUT2 antibody was preincubated for 2 hr with a 400-fold molar excess of its antigenic peptide (0.53 µM) in a competition assay (Fig. 1). Additionally, the antibody was used to probe *Xenopus* oocytes expressing mRNA for GLUT2 from *R. sylvatica* (described in Rosendale et al., 2014). When the antibody was pre-incubated with the antigenic peptide, there was complete ablation of the band at 54 kDa; additionally, this band was present in oocytes injected with GLUT2 mRNA but not in sham-injected oocytes, demonstrating that this antibody is specific for GLUT2. The ability of the antibody to discern differences in GLUT2 abundance among samples was verified by probing liver samples of varying concentrations of total protein (Fig. 1).

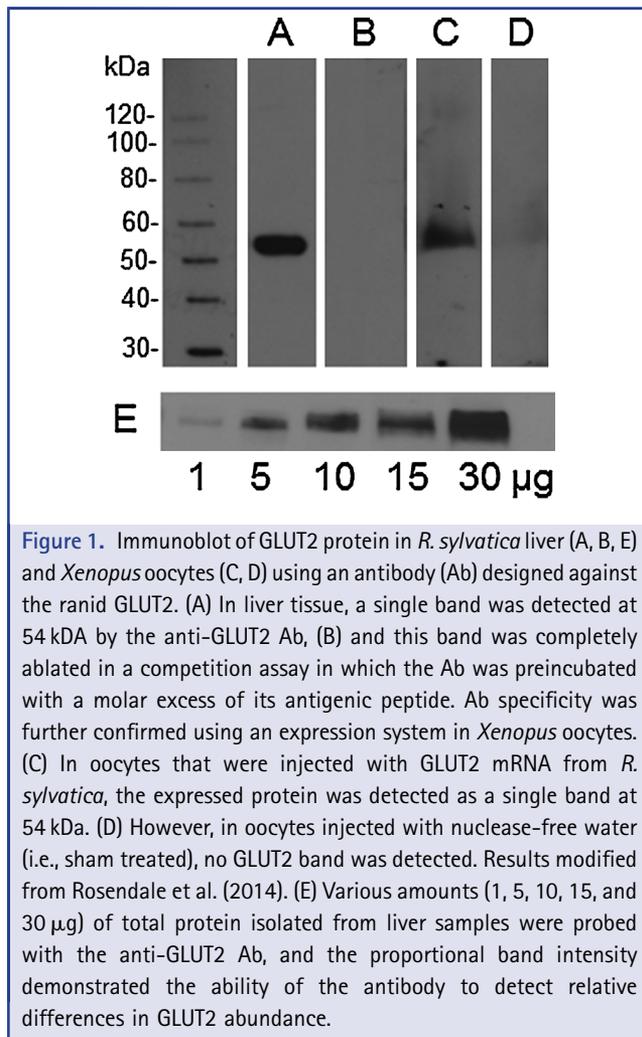


Figure 1. Immunoblot of GLUT2 protein in *R. sylvatica* liver (A, B, E) and *Xenopus* oocytes (C, D) using an antibody (Ab) designed against the randid GLUT2. (A) In liver tissue, a single band was detected at 54 kDa by the anti-GLUT2 Ab, (B) and this band was completely ablated in a competition assay in which the Ab was preincubated with a molar excess of its antigenic peptide. Ab specificity was further confirmed using an expression system in *Xenopus* oocytes. (C) In oocytes that were injected with GLUT2 mRNA from *R. sylvatica*, the expressed protein was detected as a single band at 54 kDa. (D) However, in oocytes injected with nuclease-free water (i.e., sham treated), no GLUT2 band was detected. Results modified from Rosendale et al. (2014). (E) Various amounts (1, 5, 10, 15, and 30 µg) of total protein isolated from liver samples were probed with the anti-GLUT2 Ab, and the proportional band intensity demonstrated the ability of the antibody to detect relative differences in GLUT2 abundance.

GLUT2 Expression by Quantitative Real-Time PCR

Total RNA was extracted from liver using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. To eliminate potential genomic DNA contamination, 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 by checking the optical density ratio at 260:280 nm and 260:230 nm. qPCR reactions (10 µL) consisted of iTaqTM Universal SYBR[®] Green reaction mix (BioRad, Hercules, CA, USA), 300 nM forward and reverse primers, 1 ng RNA, iTaq reverse transcriptase, and nuclease-free water. For these reactions, primers specific to *R. sylvatica* GLUT2 (accession # KF270880) were utilized (For; 5'-GCCAGAAAGCCCAAGATA-3', Rev; 5'-CTTCCCTCTCCCTCTCAATTTC-3'). Primers for the reference gene β -actin (For; 5'-TGGTTGGTATGGGACAGAAAG-3', Rev;

5'-GTTGGTTACAATGCCGTGTTTC-3') were designed from *R. sylvatica* β -actin (accession # KC438290). β -actin was chosen as a reference gene for its stable expression in other organisms during thermal acclimation, hypoxia exposure, and freezing (Shang et al., 2006; Zimmerman et al., 2007; Rey et al., 2008). Additionally, we confirmed that quantification cycle (Cq) values for β -actin did not differ significantly (ANOVA; $P = 0.365$) among treatment groups, suggesting the gene was stably expressed. In a validation experiment (Schmittgen and Livak, 2008), the optimal concentration of each primer set was determined over a 10 \times dilution series of total RNA, and we found that the amplification efficiencies for GLUT2 (98.2%) and β -actin (97.6%) were similar among primer sets. qPCR reactions were analyzed using a Rotor-Gene thermal cycler (Qiagen, Germantown, MD, USA). Reactions 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 sec and annealing/extension at 60°C for 30 sec, and denaturation 95°C for 1 min. Following amplification, a melt-curve analysis was performed from 65 to 95°C with 0.5°C increments every 10 sec. Following the qPCR reactions, background-corrected fluorescent intensities were collected with Rotor-Gene v. 6.1 software, and Cq values corresponding to the number of cycles at which the fluorescence exceeded the threshold limit were determined. Thresholds were manually set in the exponential phase of amplification well above the background level to avoid noise and also below the plateau phase (as described in Pflaffl, 2007). Each sample was run in triplicate, and the average Cq value was determined. A negative control (-reverse transcriptase) was run for each sample to check for genomic DNA contamination and a no-template control was run with each primer pair to check for primer-dimers and reagent contamination. GLUT2 mRNA levels were normalized to β -actin using the $\Delta\Delta C_T$ method as previously described (Schmittgen and Livak, 2008).

Plasma Membrane Isolation

GLUT2 abundance in the plasma membrane and cell homogenate was examined in the livers of unfrozen ($N = 7$) and frozen ($N = 7$) frogs that were treated and sampled as described above. Plasma membrane isolation was performed using a modification of an established method (Persson and Jergil, '92) that employs a two-phase affinity partitioning, which uses the surface properties of cellular components to separate particles in one of two polymer solutions, and also employs a ligand, wheat-germ agglutinin (WGA), that is specific to plasma membranes. Briefly, ~100 mg of liver tissue was homogenized using a shearing-type homogenizer in 0.5 mL of 0.25 M sucrose buffer (in 15 mM Tris/H₂SO₄ with 0.1 mM PMSF). The homogenate was centrifuged at 500g for 10 min to pellet large cellular debris. A portion of the supernatant (hereafter, "total homogenate") was stored at -80°C until use, whereas the remainder was added to a solution of 5.7% (w/w) each of Dextran T500 and polyethylene glycol 3,350 diluted in 15 mM Tris/H₂SO₄. This solution was centrifuged at 150g for 5 min and the resulting supernatant, containing the membranes of interest,

was mixed with a two-phase system containing 6% (w/w) of WGA in 20 mM boric acid (pH adjusted to 7.8 with Tris base). The solution was centrifuged at 200g for 5 min, and the resulting pellet was washed with a sucrose buffer (0.25 M sucrose in 5 mM Tris/HCl, pH 8.0) that contained 0.1 M N-acetylglucosamine to unbind the plasma membrane from the WGA. Plasma membranes were pelleted by centrifugation at 100,000g for 60 min, and the isolated pellet (hereafter, "membrane fraction"), was resuspended in 200 μ L of sucrose storage buffer (0.25 M sucrose in 5 mM Tris/HCl, pH 8.0) and stored at -80°C until use. All steps of the membrane isolation were performed at 4°C .

We evaluated the aforementioned preparations for plasma membrane yield by determining enrichment in protein-specific activity of alkaline phosphatase, a plasma membrane-bound enzyme, relative to that in the initial liver homogenates. A colorimetric assay for alkaline phosphatase was performed following Graham ('93). Protein concentration in the preparations was determined using the Bio-Rad protein assay with BSA as a standard. Total homogenate and membrane fraction were analyzed for GLUT2 protein abundance as described above.

Statistical Analysis

Summary statistics are reported as means \pm SEM. For most experiments, mean protein abundance, mRNA levels, and plasma metabolite concentrations were compared between control and treated frogs using Student's *t*-test; however, one-factor analysis of variance (ANOVA) with Dunnett's *post-hoc* test was used to compare frozen and thawed groups to unfrozen controls in the freezing experiment. For the localization study, alkaline phosphatase activity and GLUT2 protein abundance in distinct preparations were compared between control and frozen frogs using Student's *t*-test. Means were considered significantly different at $P < 0.05$.

RESULTS

Effect of Experimental Freeze/Thaw Cycle on GLUT2 Expression

Frogs reached an equilibrium T_b of -2.5°C during 54 hr of experimental freezing. Specimens sampled in the fully frozen state showed characteristics of substantial ice formation, including immobility, lack of heart function, and large ice crystals within the coelomic cavity. Frogs frozen under identical conditions and sampled 24 hr after thawing exhibited a regular cardiac rhythm, tissue perfusion, normal posture, and locomotor ability, indicating that they had survived the treatment.

The freeze/thaw cycle elicited typical physiological responses to freezing, including hyperglycemia and hyperlactemia. Freezing resulted in differences ($F = 36.1$, $P < 0.0001$) in plasma glucose levels among unfrozen, frozen, and thawed frogs (2.9 ± 0.2 , 221.9 ± 27.6 , and 93.8 ± 7.9 mM, respectively). Plasma lactate also varied ($F = 22.6$, $P < 0.0001$), as levels in frozen frogs (27.5 ± 4.0 mM) were elevated as compared to levels in unfrozen

and thawed frogs (3.1 ± 0.5 and 9.7 ± 1.2 mM, respectively). In frozen frogs, uremia (39.4 ± 5.0 mM) was higher ($F = 15.57$, $P = 0.0004$) than that in unfrozen controls (13.4 ± 0.3 mM) and thawed frogs (24.8 ± 1.2 mM). GLUT2 protein abundance varied ($F = 9.1$, $P = 0.003$) with treatment, being 2.4-fold higher in frozen frogs as compared to controls (Fig. 2A). GLUT2 mRNA expression also varied ($F = 9.3$, $P = 0.003$), as the level was 6.4-fold higher in frozen frogs and 4.6-fold higher in thawed frogs as compared to controls (Fig. 2B).

Effect of Experimental Dehydration on GLUT2 Expression

Body water content of frogs exposed to dehydrating conditions for 14 days was lower ($t = 10.8$, $P < 0.0001$) than that of fully hydrated

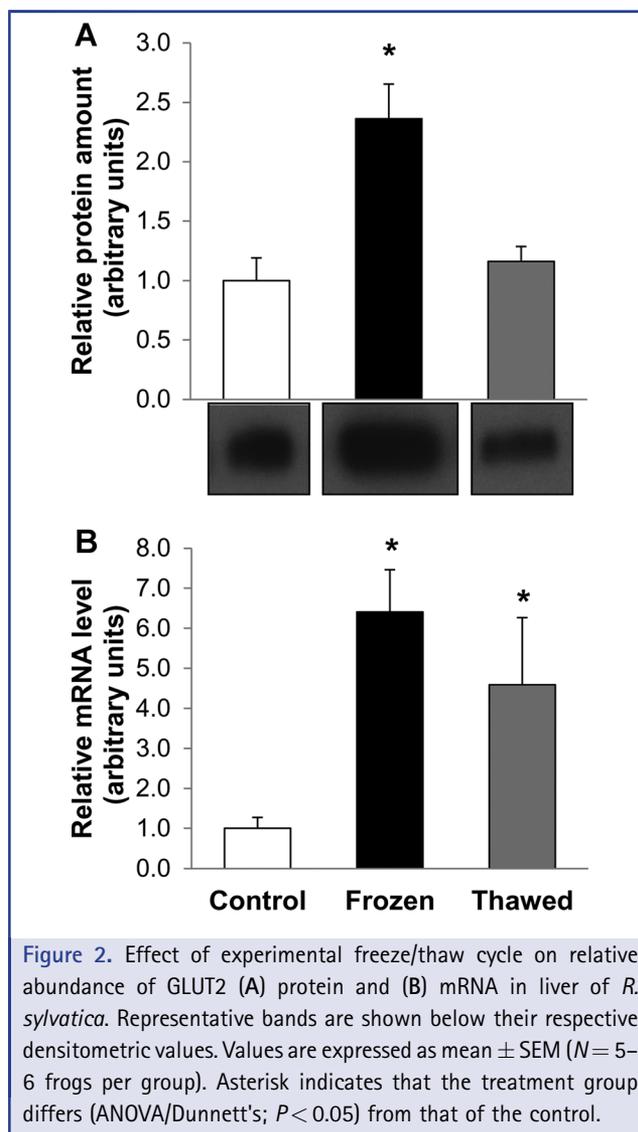


Figure 2. Effect of experimental freeze/thaw cycle on relative abundance of GLUT2 (A) protein and (B) mRNA in liver of *R. sylvatica*. Representative bands are shown below their respective densitometric values. Values are expressed as mean \pm SEM ($N = 5-6$ frogs per group). Asterisk indicates that the treatment group differs (ANOVA/Dunnett's; $P < 0.05$) from that of the control.

controls (1.93 ± 0.09 vs. 3.32 ± 0.09 g g⁻¹ dry, respectively). Assuming that dehydrated frogs started the treatment with a body water content similar to that of controls (~77% water by fresh mass), they lost an average of ~42% of their body water and attained a hydration state of ~66% water by fresh mass by the end of the trial. Expectedly, dehydration induced hyperuremia, as plasma urea concentration in dehydrated frogs (48.1 ± 3.0 mM) was higher ($t = 11.5$, $P < 0.0001$) than that in control frogs (13.4 ± 0.3 mM). However, it failed to induce a glycaemic response, as there was no statistically significant difference ($t = 2.0$, $P = 0.078$) in plasma glucose levels between control (2.9 ± 0.2 mM) and dehydrated frogs (34.2 ± 15.5 mM). GLUT2 protein abundance did not differ ($t = 0.03$, $P = 0.974$) between dehydrated and control frogs (Fig. 3A); however, GLUT2 mRNA expression was 3.1-fold greater ($t = 3.1$, $P = 0.010$) in dehydrated frogs as compared to controls (Fig. 3B).

Effect of Hypoxia Exposure on GLUT2 Expression

Frogs were exposed to hypoxic conditions for 34 hr, at which point impaired neurobehavioral functions (delayed righting reflex) were observed in several frogs; thus, the intended 48 hr trial was ended early to avoid any mortality. Hypoxia treatment elicited anaerobic metabolism, as evidenced by plasma lactate levels that were higher ($t = 5.3$, $P < 0.0002$) in hypoxic frogs (26.1 ± 4.6 mM) as compared to normoxic controls (1.5 ± 0.4 mM). Hypoxic frogs also had higher ($t = 5.6$, $P < 0.0001$) plasma glucose levels than controls (73.1 ± 12.6 vs. 2.4 ± 0.3 mM, respectively). GLUT2 protein abundance was 1.8-fold greater ($t = 3.1$, $P = 0.009$) in hypoxia-treated frogs than in controls (Fig. 4A); whereas, hypoxia-treated frogs had GLUT2 mRNA levels that were 7.7-fold higher ($t = 3.6$, $P = 0.004$) than those of controls (Fig. 4B).

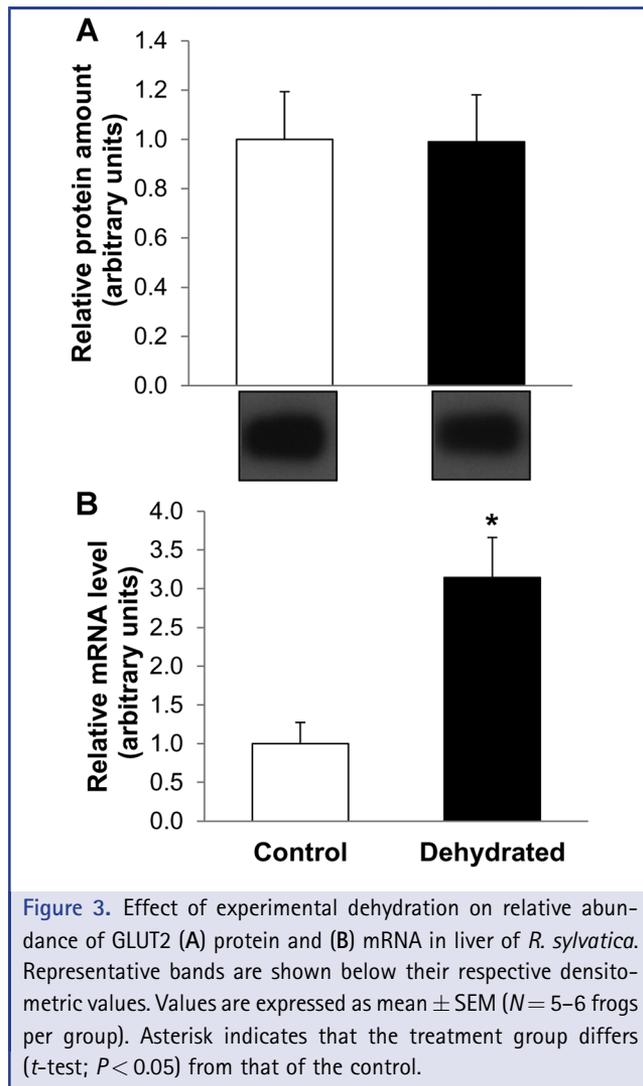


Figure 3. Effect of experimental dehydration on relative abundance of GLUT2 (A) protein and (B) mRNA in liver of *R. sylvatica*. Representative bands are shown below their respective densitometric values. Values are expressed as mean \pm SEM ($N = 5$ –6 frogs per group). Asterisk indicates that the treatment group differs (t -test; $P < 0.05$) from that of the control.

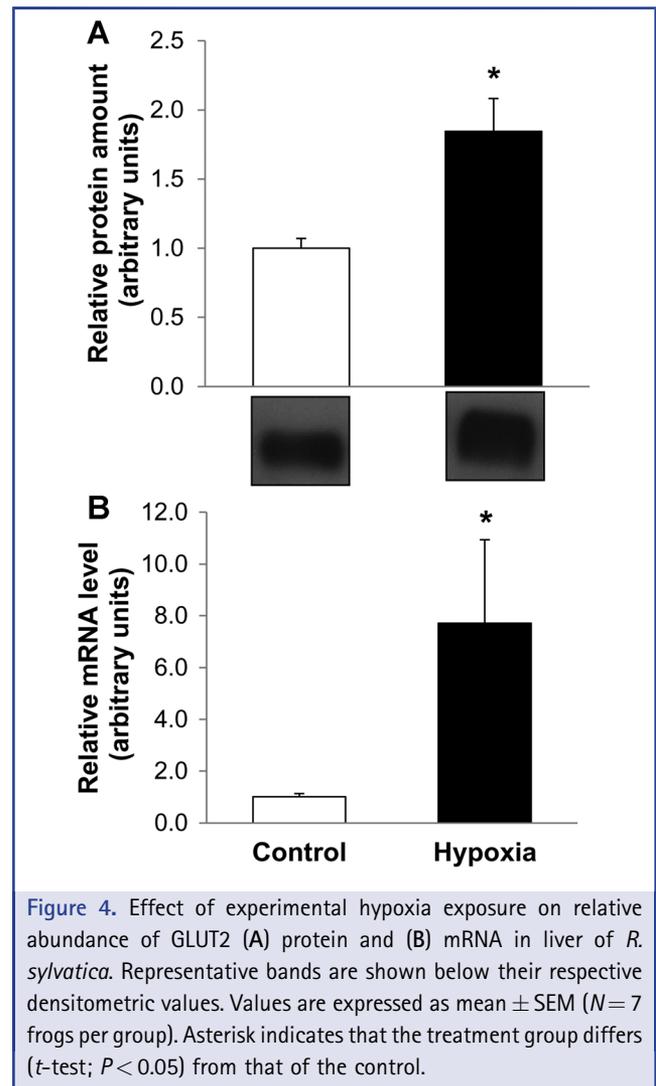


Figure 4. Effect of experimental hypoxia exposure on relative abundance of GLUT2 (A) protein and (B) mRNA in liver of *R. sylvatica*. Representative bands are shown below their respective densitometric values. Values are expressed as mean \pm SEM ($N = 7$ frogs per group). Asterisk indicates that the treatment group differs (t -test; $P < 0.05$) from that of the control.

Effect of Experimental Glucose Loading on GLUT2 Expression

To manipulate plasma glucose levels, frogs received two injections of a 1.5 M glucose solution over the course of 4 days. Glucose injections resulted in a glycemia of 214.5 ± 19.4 mM, which was markedly higher ($t = 10.9$, $P < 0.0001$) than that of control frogs (2.4 ± 0.3 mM). GLUT2 protein abundance was 2.0-fold greater ($t = 6.6$, $P < 0.0001$) in glucose-loaded frogs as compared to controls (Fig. 5A); whereas, mRNA expression was 4.2-fold higher ($t = 3.7$, $P = 0.003$) in glucose-loaded frogs as compared to controls (Fig. 5B).

Effect of Experimental Urea Loading on GLUT2 Expression

To manipulate plasma urea levels, frogs were injected with a 1.5 M urea solution. The injection resulted in a uremia of 84.5 ± 3.8 mM, which was greater ($t = 17.0$, $P < 0.0001$) than that of control frogs

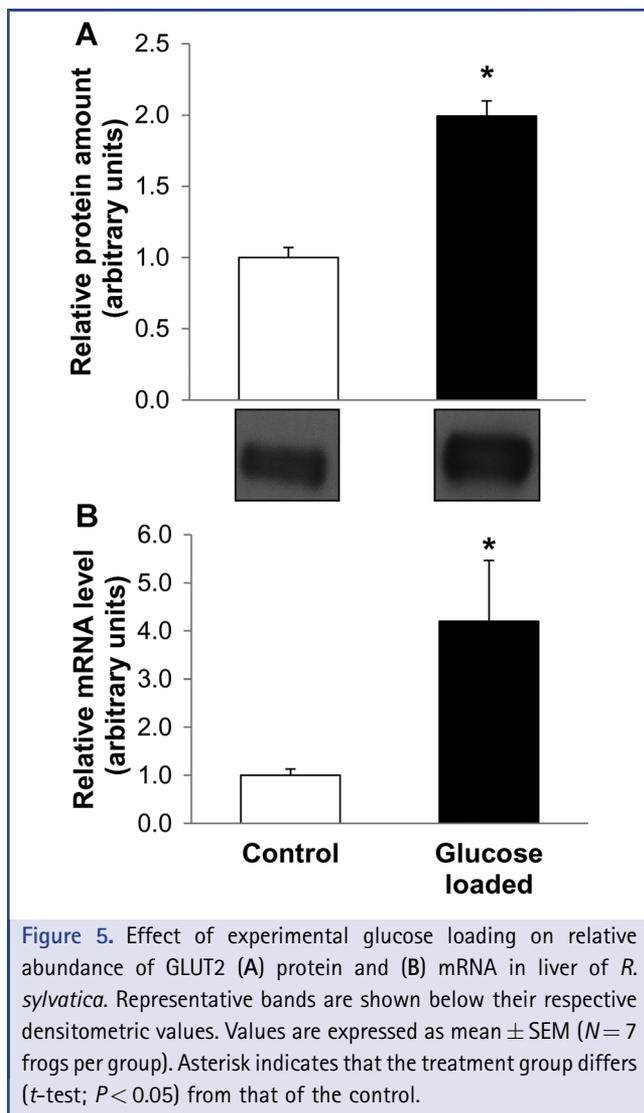


Figure 5. Effect of experimental glucose loading on relative abundance of GLUT2 (A) protein and (B) mRNA in liver of *R. sylvatica*. Representative bands are shown below their respective densitometric values. Values are expressed as mean \pm SEM ($N = 7$ frogs per group). Asterisk indicates that the treatment group differs (t -test; $P < 0.05$) from that of the control.

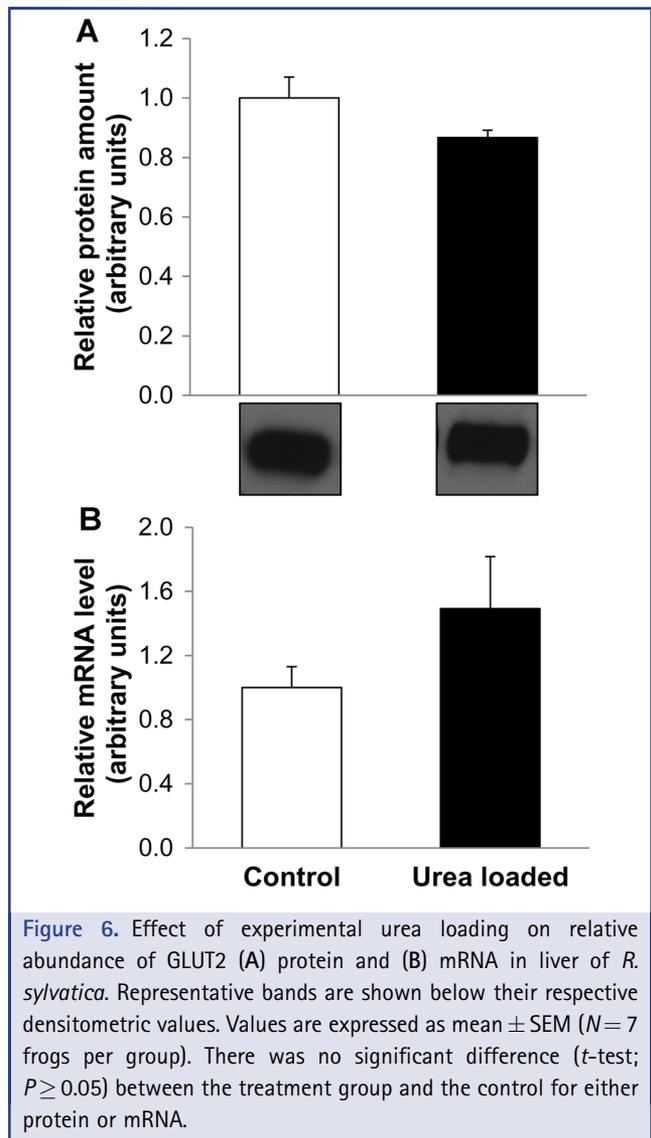


Figure 6. Effect of experimental urea loading on relative abundance of GLUT2 (A) protein and (B) mRNA in liver of *R. sylvatica*. Representative bands are shown below their respective densitometric values. Values are expressed as mean \pm SEM ($N = 7$ frogs per group). There was no significant difference (t -test; $P \geq 0.05$) between the treatment group and the control for either protein or mRNA.

(11.6 ± 1.9 mM). There was no difference ($t = 1.2$, $P = 0.264$) in plasma glucose concentration between urea-loaded and control frogs (3.0 ± 0.3 and 2.4 ± 0.3 mM, respectively). GLUT2 protein abundance did not differ ($t = 1.8$, $P = 0.101$) between urea-injected and control frogs (Fig. 6A), nor did GLUT2 mRNA expression ($t = 1.4$, $P = 0.094$; Fig. 6B).

Effect of Freezing on Total and Plasma Membrane Abundance of GLUT2

The average alkaline phosphatase activity in the membrane fractions was fourfold higher than that in the total homogenate, attesting that the preparations were substantially enriched in plasma membranes. There was no difference in alkaline

phosphatase activity between frozen and control frogs for the total homogenate ($t = 2.1$, $P = 0.056$) or membrane fraction ($t = 1.6$, $P = 0.126$), suggesting that our protocol was equally effective in partitioning plasma membrane sources of GLUT2 protein in both groups. GLUT2 abundance, as represented by arbitrary densitometric units, in the total homogenate from frozen frogs (10.8 ± 1.1) was 2.3-fold greater ($t = 3.5$, $P = 0.005$) than in controls (4.6 ± 1.5). In the membrane fraction, GLUT2 abundance was 1.7-fold greater ($t = 4.3$, $P = 0.001$) in frozen frogs (11.1 ± 0.9) as compared to controls (6.5 ± 0.6).

DISCUSSION

The wood frog overwinters in terrestrial hibernacula where it survives various physiological stresses in part through accumulation of glucose in tissues and body fluids (Churchill and Storey, '93; Holden and Storey, '97; Costanzo and Lee, 2013). Hepatic glycogen reserves are a major source of glucose during these stresses; therefore, it is likely that GLUT2, the primary glucose transporter in liver tissue, contributes to the stress-induced export of glucose from hepatocytes. In the present study, we found that GLUT2 protein abundance and mRNA expression increased in liver of *R. sylvatica* exposed to organismal freezing, hypoxia exposure, and glucose loading, thereby providing the first evidence for an important, general role for GLUTs in survival of physiological stress in amphibians.

Effect of Experimental Freeze/Thaw Cycle on GLUT2 Abundance

One of a few freeze-tolerant vertebrates, *R. sylvatica* survives the freezing of its body fluids in large part through the accumulation of cryoprotective solutes, particularly glucose (Costanzo and Lee, 2013). Following the initiation of freezing, glucose is rapidly mobilized from hepatic glycogen stores and transported to corporal tissues prior to the cessation of circulation (Storey and Storey, 2004). In our study, frozen frogs had plasma glucose levels that were 77-fold higher than those in unfrozen controls, reflecting the typical glycemic response to freezing. Once circulation ceases, a limited capacity for gas exchange leads to ischemia (Costanzo and Lee, 2013), which was evidenced by the ninefold higher plasma lactate levels in our frozen frogs as compared to controls. Upon thawing, glucose is gradually returned to the liver, where it is reconverted to glycogen (Costanzo and Lee, 2013). In our frogs, most physiological variables had returned to pre-freeze levels 24 hr post-thaw, although plasma glucose levels remained 32-fold higher than in unfrozen frogs.

When freezing begins, glycogen phosphorylase in the liver is activated, promoting glycogenolysis and greatly increasing glucose production capacity (Storey and Storey, 2004); nevertheless, freezing survival depends on the distribution of the glucose from the liver to tissues throughout the body. In our frogs, freezing resulted in a 2.4-fold greater abundance of hepatic GLUT2, which presumably enhances glucose export capacity. This upregulation would complement increases in glucose production, resulting in a

heightened glucose mobilization. The importance of glucose transport capacity in freeze tolerance becomes apparent when considering rapidly frozen frogs, which mobilize less glucose and thus have a more limited freezing survival as compared to frogs cooled more slowly (Costanzo et al., '92). In addition to GLUT2 in the liver, it is likely that other GLUT isoforms are involved in the process of moving glucose from the liver to tissues throughout the body (Storey and Storey, 2013).

GLUT2 abundance returned to pre-freeze levels within 24 hr after thawing began. This presumed reduction in transport capacity could limit the return of glucose to the liver, potentially contributing to the sustained hyperglycemia observed in thawed frogs (see also Costanzo et al., 2013). Conceivably, persistence of elevated glucose levels could facilitate recovery and/or enhance cryoprotectant levels in subsequent freezing events. Further study is needed to elucidate the post-freeze regulation of GLUT2 and its implications for winter survival of *R. sylvatica*.

In addition to glucose, urea serves as a cryoprotectant in *R. sylvatica*, with high urea levels contributing to winter survival through colligative, metabolic, and other effects (Costanzo and Lee, 2013). Urea typically accrues in response to seasonal changes in temperature and hydric conditions (Costanzo and Lee, 2005). However, recent evidence suggests that *R. sylvatica* also synthesizes urea in liver in response to freezing (Costanzo et al., 2013), and this notion is substantiated by our finding that uremia in frozen frogs was threefold higher than that in unfrozen controls; indeed, assuming that ~50% of their water had frozen (cf., Costanzo et al., 2013), freeze concentration would have raised uremia only by twofold.

Effect of Experimental Dehydration on GLUT2 Abundance

R. sylvatica is one of the most terrestrial of the North American ranids and can survive the loss of ~50% of its body water (Churchill and Storey, '93). Dehydration tolerance in amphibians is supported by various physiological responses, including urea accumulation. Urea accrual improves survival by aiding in the maintenance of proper hydration and by replacing harmful ionic solutes (Grundty and Storey, '94; Jørgensen, '97). Our dehydrated frogs, which lost 42% of their initial water, showed a robust uremia response, as their plasma urea concentration approached 50 mM.

In the case of severe dehydration, *R. sylvatica* also mobilizes glucose from hepatic stores (Churchill and Storey, '93; Muir et al., 2007). Although this relatively modest rise in glycemia contributes little to water conservation, the additional glucose supports metabolism when substantial body water loss (~50%) reduces cardiac output and perfusion, and causes ischemia (reviewed in Hillman et al., 2009). Although glycemia was relatively high (e.g., 34 mM) in our dehydrated frogs, and in line with values for *R. sylvatica* treated similarly (Muir et al., 2007), glucose levels were not significantly elevated above those of control frogs. Possibly, a more intense dehydration, in which frogs approach their tolerance limit (50–60% body water loss; Churchill

and Storey, '93), would be needed to elicit a significant glycemic response. Nevertheless, in contrast to the case with mammals (Vannucci et al., '94), water deprivation had no effect on GLUT2 abundance in our frogs. Pronounced hyperglycemic and ischemic conditions, which independently increase GLUT2 expression (discussed below) in *R. sylvatica*, presumably must accompany dehydration before transporter abundance is altered.

Effect of Experimental Hypoxia on GLUT2 Abundance

Exposure to hypoxia and/or anoxia is tolerated by several species of North American ranids, including *R. sylvatica* (Holden and Storey, '97). To survive oxygen lack, amphibians enter a hypometabolic state and rely more heavily on anaerobic metabolism (Hillman et al., 2009); accordingly, we noted a 17-fold increase in lactemia in frogs exposed to <1% O₂ for 32 hr. Glycogen reserves are an important substrate during hypoxia, and mobilization of glucose from the liver ensures that tissues have a sufficient supply of glucose (Hillman et al., 2009). In our hypoxia-treated frogs, glycemic levels were 30-fold higher than those in controls, indicating that the stress was sufficient to strongly mobilize glucose.

Hypoxic conditions elicit an upregulation of various GLUT isoforms in mammals (Bunn and Poyton, '96) and fish (Terova et al., 2009). Such changes in expression are initiated by the binding of hypoxia inducible factor-1 (HIF-1), a transcription factor, to hypoxia-responsive elements that are present on GLUT genes (Ebert et al., '95; Iyer et al., '98). In mammals, increased GLUT abundance facilitates utilization of glucose, which serves as a metabolic fuel and protects cells from hypoxic injury (Bunn and Poyton, '96; Lin et al., 2000). It is likely that the approximately twofold increase in GLUT2 abundance in our hypoxic frogs is similarly important in exploiting glucose as a fuel for anaerobic metabolism.

Effect of Experimental Glucose Loading on GLUT2 Abundance

Regulation of GLUT2 in mammals is affected by glucose levels, independent of other variables (Leturque et al., 2009). In our glucose-loaded frogs, hyperglycemia (~215 mM glucose) was associated with a twofold greater abundance of GLUT2, indicating that changes in circulating glucose levels can influence GLUT2 expression in *R. sylvatica*. Similarly, elevated glucose levels cause an increase in GLUT2 expression in mammalian hepatocytes (Asano et al., '92). GLUT2 is a glucose-sensitive gene, with metabolism of glucose inducing transcription of GLUT2 through ChREBP (carbohydrate-responsive element-binding protein), a transcription factor that interacts indirectly with a promoter region on the GLUT2 gene (reviewed in Leturque et al., 2009). Sensitivity of GLUT2 to its substrate enables this protein to function in a signaling cascade that alters cell metabolism and gene expression in response to changes in extracellular glucose (Leturque et al., 2009). In glucose-loaded frogs, increased GLUT2 abundance could facilitate uptake of glucose by the liver (to be

stored as glycogen), as well as enhance the ability of hepatocytes to sense glucose levels and make any requisite changes to metabolism and/or gene expression.

Effect of Experimental Urea Loading on GLUT2 Abundance

Accumulation of urea is a common response to osmotic challenge among amphibians, although *R. sylvatica* routinely accumulates urea in the fall (Costanzo and Lee, 2005) even in the absence of osmotic stress (Costanzo et al., 2013). Injecting *R. sylvatica* with a solution containing 1.5 M urea had no effect on glucose levels but raised their uremia to ~85 mM, about sevenfold over that of control frogs. Urea level attained by these frogs are within the range reported for hibernating *R. sylvatica* (Costanzo et al., 2013).

High levels of urea constrain glucose transport, with urea acting as a non-transportable, competitive inhibitor, likely by binding to the substrate site of GLUTs (Krupka, '71). Such inhibition occurs in *Xenopus* oocytes expressing GLUT2 from *R. sylvatica* (Rosendale et al., 2014); therefore, a compensatory increase in GLUT2 abundance might be expected to occur during urea accumulation. However, this was not the case. Possibly, uremic levels in our frogs remained below the threshold needed to upregulate transporter numbers. Alternatively, the abundance of extant transporters may have been sufficient to meet transport needs despite urea inhibiting glucose transport.

Stress-Induced Regulation of GLUT2

Changes in protein abundance commonly reflect corresponding changes in mRNA levels, and this pattern is seen in *R. sylvatica* with respect to various proteins that respond to freezing, dehydration, and/or anoxia (Storey and Storey, 2013). In the present study, changes in protein abundance mirrored adjustments in mRNA levels in experimental freezing, hypoxia exposure, and glucose loading. In thawed frogs and dehydrated frogs, mRNA levels rose, but a significant increase in the protein's abundance could not be detected. Overall, these results indicate that changes in GLUT2 abundance in *R. sylvatica* liver are regulated at the level of the transcript, although other forms of regulation cannot be ruled out.

Aside from adjusting transporter abundance, regulation of glucose transport can be achieved by shuttling extant proteins between the plasma membrane and intracellular vesicles (Leturque et al., 2009). As much as half of the available GLUT2 can be internalized in this manner, and moving these internal stores to the plasma membrane can quickly and substantially increase transport capacity (Eisenberg et al., 2005; Tobin et al., 2008). In our study, the amount of membrane-associated GLUT2 increased markedly with freezing. The corresponding upregulation of transporter abundance in the whole-cell homogenate suggests that de novo synthesis of GLUT2 contributes to the observed freezing response of membrane-associated GLUT2. However, this rise potentially could derive from a translocation of proteins from an internal pool, and further study using

immunohistochemistry techniques is needed to definitively determine if regulation of hepatic GLUT2 through subcellular localization occurs in *R. sylvatica*.

Perspectives

GLUT2 factors importantly in the ability of *R. sylvatica* to endure physiological stresses, including freezing, experienced during winter. During glucose mobilization, a myriad of changes within the liver serve to enhance glucose production. Increasing GLUT2 abundance likely ensures that glucose transport does not constrain glucose delivery to tissues, where it functions as a cryoprotectant and metabolic fuel. Hypothetically, freeze tolerance evolved largely from modifications of existing adaptations developed to cope with dehydration and hypoxia (Churchill and Storey, '93). Our results suggest that hypoxia, but not dehydration, may contribute to freezing-induced changes in GLUT2 abundance. Further study is needed to determine if the increase in transporter numbers with freezing is a result of higher glucose levels (as in glucose-loaded frogs), is a hypoxia-related response, or if freezing has an independent regulatory pathway.

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