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ORIGINAL PAPER

Enzymatic regulation of seasonal glycogen cycling in the freeze-tolerant wood frog, *Rana sylvatica*

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Abstract Liver glycogen is an important energy store in vertebrates, and in the freeze-tolerant wood frog, *Rana sylvatica*, this carbohydrate also serves as a major source of the cryoprotectant glucose. We investigated how variation in the levels of the catalytic subunit of protein kinase A (PKAc), glycogen phosphorylase (GP), and glycogen synthase (GS) relates to seasonal glycogen cycling in a temperate (Ohioan) and subarctic (Alaskan) populations of this species. In spring, Ohioan frogs had reduced potential for glycogen synthesis, as evidenced by low GS activity and high PKAc protein levels. In addition, glycogen levels in spring were the lowest of four seasonal samples, as energy input was likely directed towards metabolism and somatic growth during this period. Near-maximal glycogen levels were reached by mid-summer, and remained unchanged in fall and winter, suggesting that glycogenesis was curtailed during this period. Ohioan frogs had a high potential for glycogenolysis and glycogenesis in winter, as evidenced by large glycogen reserves, high levels of GP and GS proteins, and high GS activity, which likely allows for rapid mobilization of cryoprotectant during freezing and replenishing of glycogen reserves during thawing. Alaskan frogs also achieved a near-maximal liver glycogen concentration by summer and displayed high glycogenic and glycogenolytic potential in winter, but, unlike Ohioan frogs, started replenishing their energy reserves early in spring. We conclude that variation in levels of both glycogenolytic and glycogenic enzymes likely happens in response to seasonal changes in energetic strategies and demands, with winter survival being a key component to understanding the regulation of glycogen cycling in this species.

Keywords *Rana sylvatica* · Glycogen · Glycogen phosphorylase · Glycogen synthase · Protein kinase A

Introduction

Glycogen, a multibranched polymer of glucose residues, is the major storage form of carbohydrates in vertebrate animals (Bollen et al. 1998). In anurans, this carbohydrate not only fuels basal metabolism, but is also an important energetic substrate during the breeding season, and, in the case of freeze-tolerant species, a critical source of the cryoprotectant glucose (Feder and Burggren 1992).

Regulation of glycogen metabolism is achieved, in part, by changes in the activities of glycogen synthase (GS) and glycogen phosphorylase (GP), and these two opposing enzymes are regulated by the catalytic subunit of protein kinase A (PKAc) (Bollen et al. 1998). Whereas GP is a key enzyme in glycogenolysis that is activated by a phosphorylation cascade triggered by PKAc, GS is essential for glycogenesis and can be deactivated by PKAc. In anurans from temperate climates, the liver glycogen depot cycles prominently, reaching a minimum following the breeding season and a maximum during fall or winter (e.g., Fenoglio et al. 1992; Scapin and Di Giuseppe 1994), partly as a consequence of dynamics in GP and GS activity (Castiñeiras et al. 1977; Scapin and Di Giuseppe 1994; Dinsmore and Swanson 2008). Currently, it is not known how seasonal variation in enzyme abundance is involved in glycogen cycling.

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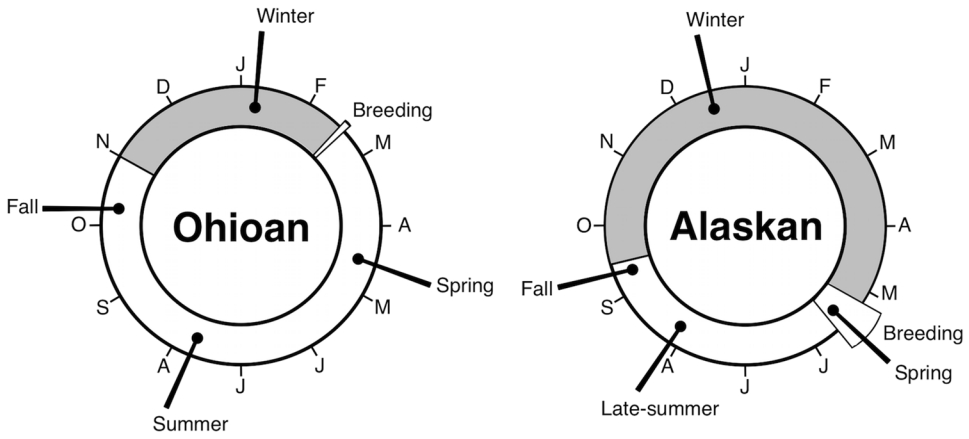

Fig. 1 Sample times and schematic representation of the annual activity cycle of *R. sylvatica* endemic to southern Ohio and Interior Alaska. Periods of hibernation (*shaded segment*) and activity (*light segment*), which begins with breeding (Costanzo 2013; Kessel 1965; Kirton 1974), are demarcated. Multiple cohorts were sampled to provide the liver tissue needed for this study. For Ohioan frogs, analyses of glycogen, PKAc, and GP were made using frogs collected in 2010 (sampled in October, 2010 and January, 2011) and 2011 (sampled in April and July, 2011); analysis of GS was made using frogs collected in 2011 (sampled in July and October, 2011, and January, 2012) and 2012 (sampled in April, 2012). For Alaskan frogs, analyses of glycogen, PKAc, and GP were made using frogs collected in 2010 [sampled in December, 2010 (depicted in diagram)] and 2011 (sampled in May, August, and September, 2011); analysis of GS was made using frogs collected in 2011 [sampled in May, August, September, and November, 2011 (not depicted)]

Many of the studies of liver glycogen cycling in frogs have focused on aquatic hibernators (e.g., Byrne and White 1975; Koskela and Pasanen 1975), while little is known of the phenomenon in terrestrial hibernators, including freeze-tolerant species, which have unique physiological needs for stored carbohydrate (Feder and Burggren 1992). The wood frog, *Rana sylvatica*, is widely distributed in North America, ranging from north of the Arctic Circle, to as far south as Georgia, USA (Martof and Humphries 1959). A terrestrial hibernator, it is one of several frogs that tolerate the freezing of their extracellular fluids to survive winter's cold. Freeze tolerance is achieved, in part, through a glycemic cryoprotectant system in which glucose, synthesized through liver glycogenolysis only after freezing begins, is distributed throughout the body and colligatively reduces ice content and cellular dehydration, and exerts specific protective effects on cellular membranes and proteins (Storey and Storey 2004; Costanzo and Lee 2013). Not only does *R. sylvatica* need an abundance of glycogen to produce cryoprotectant, but also it must meet metabolic demands during hibernation (which may last up to 8 months in subarctic populations; Costanzo et al. 2013; Larson et al. 2014) and have enough glycogen remaining to fuel reproductive activities, which begin immediately upon emergence (Fig. 1). It remains to be determined how the cycling of hepatic glycogen, and its associated enzymes, have adapted to meet the ecological and physiological demands faced by *R. sylvatica*.

Northern populations of anurans experience particularly long winters, which require larger glycogen reserves to sustain metabolism during dormancy (Pasanen and Koskela 1974). In Alaskan *R. sylvatica,* an exceptionally large glycogen depot also contributes to greatly enhanced freeze tolerance, which aids survival under relatively harsh conditions (Costanzo et al. 2013; do Amaral et al. 2015). Glycogen is markedly reduced by the end of winter (Costanzo et al. 2014); energetic substrates acquired during the brief activity season must be optimally allocated among reproduction, maintenance metabolism, somatic growth, and regeneration of nutrient stores (Fig. 1). It would be instructive to determine how temperate and subarctic frogs have evolved to regulate glycogen metabolism differently in response to their respective environmental pressures.

In this study we investigated seasonal variation in liver glycogen metabolism in *R. sylvatica* by examining three enzymes, PKAc, GP, and GS, that are essential in glycogen metabolism and determining how changes in these related to glycogen cycling. We focused on a well-studied population from southern Ohio, and for contrast also examined an Alaskan population. In samples representing each of the four seasons, we measured liver glycogen concentrations, as well as protein levels of liver PKAc and GP, and protein levels and enzyme activity of liver GS. Additionally, we measured transcript levels of PKAc and GP to determine if variation in PKAc and GP protein amounts could be attributed to changes in mRNA abundance. Finally, because recent work (do Amaral et al. 2013) suggested the occurrence of different isoforms of PKAc in Alaskan and Ohioan populations, we obtained the coding RNA sequence for both PKAc and GP to determine if there were population differences in the amino acid sequences of these enzymes.

Materials and methods

Experimental animals and acclimatization

We collected *R. sylvatica* from Adams County, Ohio, USA (38.8°N, 83.5°W), in February (2010, 2011, and 2012) following their emergence from hibernation. They were kept, unfed, on damp moss within darkened plastic boxes (4 °C) for 3 weeks after collection from the field. Thereafter they were kept outside in a 48-m^2 pen at the Ecology Research Center (39.5°N, 84.7°W), Miami University. Frogs freely accessed a pool of water and were fed vitamin-fortified crickets three times weekly; this diet was supplemented by various arthropods attracted to a "black light" hung in the pen. In April, a few frogs were sampled from the outdoor enclosure (hereafter "spring" frogs), shortly after they began feeding regularly. Other frogs were sampled from these quasi-natural conditions in late July (hereafter "summer" frogs) and in October (hereafter "fall" frogs). Feeding was suspended in late October, and in November, the frogs, by then on the verge of dormancy, were recaptured and kept at 4 °C, in darkness, in simulated hibernation until sampled in January (hereafter "winter" frogs). Additional sampling details are given in Fig. 1.

We collected additional *R. sylvatica* near Tok, Alaska, USA (64.8°N, 143.0°W), and from areas surrounding Fairbanks, Alaska, USA (64.8°N, 147.7°W), which have a similar climate. Frogs were shipped under refrigeration to Miami University and sampled promptly (see below) or housed in clean plastic cups with either damp paper or moss. A single group of frogs (hereafter "spring" frogs) was captured in May (2011), within 2 weeks following their emergence from hibernation, and kept at 4 °C, in total darkness, for 4 days before being sampled. Other frogs were captured in early August (2010 and 2011) and either sampled promptly (hereafter "late-summer" frogs), or winter acclimatized by housing them in a programmable environmental chamber (Percival, model I-35X; Boone, IA, USA) as described previously (Costanzo et al. 2013). Acclimatization required exposing frogs over 5 weeks to dynamic, diel cycles of temperature and ambient light, modeled from institutional long-term records of weather (obtained from the National Oceanic and Atmospheric Administration's National Climatic Data Center, NOAA NCDC), that were seasonal and appropriate to their origin. Initially, temperature varied daily from 17 to 8 °C and the photophase was 16.5 h, but by the end of acclimatization, in mid-September, temperature varied daily from 13 to 3 °C and the photophase was 13.3 h. During acclimatization, 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, a few frogs were sampled immediately (hereafter "fall" frogs), whereas remaining frogs were kept at 4 °C, in darkness, in simulated hibernation until sampled in mid-November (2011) or mid-December (2010) (hereafter "winter" frogs).

We intended to sample only adult males to eliminate potential gender- and age-based differences in physiology. This objective was largely achieved for Ohioan frogs and the spring sample of Alaskan frogs. However, as secondary sex characteristics of frogs captured in August were not evident, Alaskan frogs comprised about 20 % females randomly distributed among summer, fall, and winter groups.

Frogs were collected under permits from the Ohio Division of Wildlife and the Alaskan Department of Fish and Game. Rearing and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Miami University.

Tissue harvesting

Frogs were weighed to 0.1 g, double-pithed, and dissected at 4 °C. Their livers were excised and a small sample of each was blotted to remove excess moisture, weighed to 0.01 mg, and placed in a 65 °C oven for 3–5 days, to determine its dry mass. Initial water concentration in the tissue, expressed as mg water g^{-1} dry matter, was determined by dividing the mass lost upon drying the sample by its dry mass. The remaining liver, destined for metabolite, protein, mRNA, and enzyme analyses, was immediately frozen in liquid N_2. Samples were stored at −80 °C before assays and metabolite analyses were carried out.

Metabolite analyses

Deproteinized extracts of liver samples were prepared by homogenization in cold 7 % (w/v) perchloric acid. Samples were assayed for glycogen as previously described (do Amaral et al. 2013). Briefly, a portion of the whole-tissue homogenate (100 µl) was neutralized with KOH and incubated with amyloglucosidase (1 mg ml^{-1}) at 40 °C for 2 h in a 0.2 M sodium acetate buffer, pH 4.8. After incubation, the reaction was stopped by adding cold 7 % (w/v) perchloric acid and the free glucose in the sample was determined using a colorimetric assay kit (Pointe Scientific, Canton, MI); glycogen concentration was expressed as µmol glucosyl units g^{-1} dry tissue after subtraction of initial free glucose, assayed as above, in the initial homogenate.

Immunoblotting

Liver collected from spring, summer, fall, and winter-acclimatized frogs was homogenized whilst still frozen in

ice-cold RIPA buffer (1:10) containing a protease-inhibitor cocktail (P2714, Sigma-Aldrich Chemical Company, Saint Louis, MO). The homogenate was centrifuged (14,000 g for PKAc; 3000*g* for GP and GS) for 5 min at 4 °C. Following centrifugation, the supernatant was assayed for protein concentration using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), with bovine serum albumin as the standard. The soluble protein solution was aliquoted and frozen at −80 °C.

Soluble protein (15 μg for PKAc; 30 μg for GP and GS) was mixed with Laemmli sample buffer (containing 5 % β-mercaptoethanol) and heated at 95 °C for 5 min. SDS-PAGE of the protein samples was performed using a 4–15 % Tris–HCl gradient gel (Bio-Rad). To allow comparisons among different gels, we loaded into all gels a standard sample relative to which all protein bands were normalized.

Following electrophoresis, proteins were transferred to a nitrocellulose membrane (GE Healthcare, Waukesha, WI) and then each membrane was stained with 0.2 % (w/v) Ponceau S (Sigma-Aldrich) containing 5 % (v/v) acetic acid to verify uniformity of protein transfer; these membranes were then digitized for densitometry analysis of the total protein loaded. After the membranes were digitally scanned, they were destained using 0.1 M NaOH, rinsed for 3 min with ultrapure water, and blocked overnight in 10 % non-fat milk in TBS-T buffer (10 mmol l^{-1} Tris, 500 mmol l^{-1} NaCl, and 0.1 % Tween-20; pH 7.5). We used rabbit primary antibody for PKAc (Abcam, Cambridge, MA), goat primary antibody for GP (Santa Cruz Biotechnologies, Santa Cruz, CA), and rabbit primary antibody for GS (Aviva Biosystems, San Diego, CA) to detect liver PKAc, GP, and GS, respectively. Nitrocellulose membranes were incubated in anti-PKAc antibody solution (1:3000), anti-GP antibody solution (1:2000), or anti-GS antibody solution (1:7000) overnight, at 4 °C, with constant oscillation. Secondary antibodies were horseradish peroxidase-linked goat anti-rabbit for PKAc and GS (Sigma-Aldrich) and donkey anti-goat for GP (Santa Cruz Biotechnologies). All primary and secondary antibodies were diluted in a 5 % non-fat milk TBS-T solution. Following three 15-min washes in TBS-T, membranes were incubated in secondary antibody (1:3000 for PKAc and GP, 1:8000 for GS) for 2 h at room temperature with constant oscillation. Membranes were thrice washed in TBS-T for 15 min, incubated for 2 min in ECL (enhanced chemiluminescence) detection reagents (GE Healthcare), and exposed to autoradiography film.

GS antibodies were exposed to their respective antigenic peptides in a competition assay to determine the specificity of the bands visible in the blot. In the competition assay we obtained two bands: one with approximately 85 kDa and the other with approximately 40 kDa (results not shown). We analyzed only the band at 85 kDa, which is the predicted size for liver GS (Roach 1990).

Bands from digitally scanned radiography films were semi-quantified using AlphaView spot densitometry (ProteinSimple, Santa Clara, CA). Total protein was used as a loading control (Aldridge et al. 2008). In this method, a densitometric measurement was performed on the scanned membranes by selecting a thin strip in the center of each lane that encompassed all proteins in the lane. For densitometry of the probed proteins, target bands were selected using a uniform sampling area that encompassed the band of interest, and background optical density was determined individually for each band. Densitometry values determined for individual protein bands were standardized to total protein densitometry values. These ratios were divided by the optical density of the standard present in all gels so that comparisons could be made among all values. Membranes contained samples from all four seasons from either Alaskan or Ohioan frogs. All membranes were run in duplicate and the average of densitometry values were used in statistical analyses.

Quantitative real-time PCR

Total RNA was extracted from liver samples using Trizol reagent (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. Total RNA yield and quality were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Total RNA (1 μg) was treated with 4 U of DNase I (Life Technologies) following the manufacturer's instructions and the resultant RNA was further purified and concentrated using the GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Fisher Scientific, Waltham, MA), per the manufacturer's instructions. Total RNA (1 ng) was used in a one-step reverse-transcription polymerase chain reaction (one-step RT-PCR) using the iTaqTM Universal One-Step kit (Bio-Rad). Gene-specific primers were used for PKAc (sense 5′-CTACCTGCAGCAAGTTCCTTAG-3′, antisense 5′-GCCGATCAGCCCATACAAATA-3′) and GP (sense 5′-CAGGTTGAGGAAGCAGATGAT-3′, antisense 5′-GCTCCACCCTACCATAGAAATG-3′); these were designed from partial *R. sylvatica* PKAc and GP sequences obtained following the procedure described in "Isolation, cloning, and sequencing of PKAc and GP coding sequences". Each sample was run in triplicate, and the average cycle at threshold (CQ) value was determined. A control without Reverse Transcriptase was run for each sample to check for genomic DNA contamination, and a negative control, without RNA, was run to check for contamination from other sources. The expression levels for GP and PKAc were each normalized to two genes, 18S RNA and β-actin (Rosendale et al. 2015). The primer sequences for

18S RNA were designed from conserved regions of the 18S RNA mRNA sequences of several ranids (NCBI accession numbers: JQ511853, JQ511845, JQ511826, JQ511830, JQ511820, AF542043, and AY145522) (sense 5′-CAAGACGAACCAAAGCGAAAG-3′, antisense 5′-TCGGAACTACGACGGTATCT-3′). The primer sequences for β-actin mRNA were designed from a partial sequence of *R. sylvatica* β-actin (NCBI accession no KC438290) (sense 5′-TGGTTGGTATGGGACAGAAAG-3′, antisense 5′-GTTGGTTACAATGCCGTGTTC-3′). These reference genes show stable expression in some ectotherms during cold and warm acclimation (Lucassen et al. 2003; Zimmerman et al. 2006; Orczewska et al. 2010) and were also stably expressed in our samples as determined by the software qBase PLUS (Biogazelle, Zwijnaarde, Belgium). Data were analyzed using qBase PLUS and the 2[−Delta Delta C(T)] method as previously described (Schmittgen and Livak 2008). Normalization to the reference genes was performed in qBase PLUS, by using the geometric mean of the two reference genes as previously described (Vandesompele et al. 2002). As samples were run across multiple plates, we used two inter-run calibrators to account for differences between runs.

GS activity assays

GS activity was measured at 22 °C using a two-step assay that measures the liberation of UDP from UDP-glucose (Schwartz and Carter 1982). GS exists in a glucose-6-phosphate (G6P)-independent form, GSI, which is active in vivo, and a G6P-dependent form, GSD, which is inactive in vivo and is converted to GSI by protein phosphatase (Roach 1990). Activity of GSI was assayed in the absence of G6P, and total GS (GSI and GSD) activity was assayed in the presence of G6P (Russell and Storey 1995). Liver was homogenized (1:5) on ice in cold 20 mM imidazole–HCl buffer (pH 7.2) containing 100 mF NaF, 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF and 15 mM β-mercaptoethanol (Russell and Storey 1995). The homogenate was centrifuged (3000*g*) at 4 °C for 5 min, and the supernatant was transferred to a new tube. The first step of the assay, during which UDP is liberated from UDP-glucose during glycogen synthesis, was initiated by adding the supernatant to a solution containing 20 mM imidazole buffer (pH 7.0), 2 mg ml^{-1} glycogen, 5 mM $MgCl_2$, 5 mM UDPG, and, when measuring total GS activity, 5 mM G6P (Russell and Storey 1995). This reaction was incubated for 2 h at 22 °C and terminated by heating the solution at 98 °C for 5 min. The denatured solution was centrifuged (3000*g*) for 5 min to remove the denatured proteins and the supernatant was transferred to a new tube. In the second step, the denatured supernatant was assayed for UDP liberated by measuring the rate of decrease in absorbance caused by the oxidation of β-NADH. The supernatant was incubated in a 20 mM imidazole buffer (pH 7.0) with 5 mM $MgCl_2$, 20 mM KCl, 0.15 mM β-NADH, 1 mM PEP, and 5 units each of pyruvate kinase and lactate dehydrogenase. Controls lacking UDPG and the denatured supernatant were also assayed. Standard solutions of UDP were also assayed and used to build a standard curve to determine the amount of UDP liberated by GS in each sample (Schwartz and Carter 1982). The activity of GS is expressed as nmol of UDP formed min^{-1} mg^{-1} protein. For each liver extract, the supernatant was assayed for protein concentration using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin (BSA) as the standard.

Isolation, cloning, and sequencing of PKAc and GP coding sequences

Total RNA was extracted from liver using Trizol (Life Technologies, Carlsbad, CA) following the manufacturer's instructions, and its quality was confirmed by checking the optical density ratio at 260 and 280 nm. cDNA was reverse-transcribed using an oligo(dT)-adaptor primer (Integrated DNA Technologies, Coralville, IA) following the manufacturer's protocol (Reverse Transcription System; Promega, Madison, WI). The resulting cDNA was used in a PCR using degenerate, sense, and anti-sense primers designed from areas of conserved amino acid sequences from PKAc and GP of *Xenopus laevis*, *X. tropicalis*, and *Homo sapiens* (Table 1). PCR was performed in 25 µl GoTaq Green Master Mix (Promega) containing 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 0.625 units of GoTaq DNA polymerase, and 2 µl first-strand cDNA in a Veriti thermal cycler (Applied Biosystems, Foster City, CA). Reaction parameters were an initial denaturation step of 95 °C for 3 min, followed by 30 cycles of denaturation (95 °C, 1 min), annealing (54 °C, 0.5 min), and extension

Table 1 Sequences used for isolation, cloning, and sequencing of PKAc and GP

Primer	DNA sequence (5′–3′)	End
PKAc degenerate	TYAGTGARCCWCAYGCAMGVTTYT	5′
PKAc degenerate	TBGTRTCTCCDGGMCCYYKRCAYT	3′
GP degenerate	GATCMGVACSCAGCAGTAYT	5′
GP degenerate	GATASARRACACGGGAGATGTT	3′
PKAc 1	GCAGTCGATTGGTGGGCATTAG	3′
PKAc 2	CAGGTCTACCTGCAGCAAGTTC	5′
PKAc 3	GTCTGAACTGAAATGGGATG	5′
GP 1	GACTGGCAGCCTATGGCTACG	3′
GP 2	CCGAGCCGACCAGAGTCTCATTG	5′
GP 3	CATGTATCCAGGAACTGGAG	5′
T7	TAATACGACTCACTATAGGG	5′

(72 °C, 1 min), with a final extension step of 72 °C for 10 min. A PCR product of approximately 500 base pairs (bp) was ligated into the pGEM-T Easy plasmid cloning vector following the manufacturer's instructions (Promega), transformed into *Escherichia coli* JM109 cells, and plated on LB-ampicillin agar containing isopropyl β-D-1-thiogalactopyranoside (0.5 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (80 μg ml^{-1}). Plasmid DNA was isolated using High-Speed Mini Columns (IBI Scientific, Peosta, IA) and the sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and standard T7 sequencing primer (Promega) (Table 1). Sequences were analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). Partial GP and PKAc sequences were identified for each phenotype by comparing these to those published in the National Center for Biotechnology Information's (NCBI) GenBank database using nucleotide comparison (BLASTN).

Rapid amplification of cDNA end (RACE) techniques were used and performed by Norclone Biotech Laboratories (London, Canada) to obtain full PKAc and GP sequences. For 3′ RACE, cDNA was generated from total RNA using reverse transcriptase (RT) and an oligo-dT adapter primer. The 3′-unknown cDNA sequence was captured by PCR using a gene-specific primer (Table 1) and an adapter primer that targeted the poly(A) tail region. For 5′ RACE, cDNA was synthesized from total RNA by using RT and a gene-specific primer (Table 1). A homopolymeric tail was then added to the 3′-end of the cDNA using terminal deoxynucleotidyl transferase (TdT) and dCTP. The tailed cDNA was then amplified by PCR using a nested, gene-specific primer and a complementary homopolymer tail primer (Table 1).

Complete coding of cDNA sequences for GP and PKAc were further confirmed by Lofstrand Labs, Ltd. (Gaithersburg, MD, USA). One-step cDNA amplification was performed using the SuperScript III One-Step RT-PCR Platinum *Taq* HiFi kit (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions. Products of the RT-PCR reactions were run on agarose gels and target bands were excised with a minimum of UV exposure and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilgen, Germany). cDNA concentration was determined by picogreen analysis (Qubit dsDNA HS Assay, Invitrogen). DNA sequencing was performed using ABI 3730*xl* DNA Analyzers.

Analysis of sequences

DNA sequences were examined for quality in CodonCode aligner v 5.1.4 (CodonCode Corporation, Centerville, MA, USA). PKAc and GP cDNA from *R. sylvatica* were translated in silico and compared to known proteins using BLASTp with GenBank at NCBI. Molecular mass and isoelectric point were predicted through the Expasy server (http://web.expasy.org/compute_pi/). Amino acid sequences of the newly identified PKAc and GP sequences were aligned with PKAc and GP proteins from other vertebrate taxa in MEGA5 (Tamura et al. 2011) and analyzed for differences. Phylogenetic trees for PKAc and GP were constructed in MEGA5 (Tamura et al. 2011) using the neighbor-joining method (Saitou and Nei 1987) with Poisson correction and bootstrap analysis (10,000 replicates).

Statistical analysis

Mean ± standard error of the mean (SEM) are the descriptive summaries used for the variables measured. For each population, mean values were compared among samples of winter, spring, summer, and fall frogs using a one-way ANOVA; a post hoc Tukey's HSD test was used to detect pair wise differences among frogs from different seasons. Analyses were performed using SYSTAT (Cranes Software International Limited, Chicago, IL); significance was accepted at $P < 0.05$. To achieve consistency of presentation, all figures were plotted with seasons arranged sequentially along the abscissae, beginning with "spring"; however, the actual chronological sampling sequence may have differed (see "Experimental animals and acclimatization").

Results

Ohioan frogs

Liver glycogen concentration varied ($F_{3,24} = 50.4$, $P < 0.0005$) among seasons, being lowest in spring, when it averaged 714 μmol g^{-1} dry tissue. Values were approximately 3.5-fold higher in summer frogs. Concentrations in fall and winter frogs were also higher than those of spring frogs, but statistically indistinguishable from those of summer frogs (Fig. 2a).

PKAc protein amounts varied throughout the year ($F_{3,23} = 8.7$, $P < 0.0005$), being highest in spring and then decreasing by half in summer frogs; levels were comparable among summer, fall and winter frogs (Fig. 2b). Transcript levels of PKAc also varied seasonally ($F_{3,24} = 5.20$, $P = 0.007$), being lowest in summer, but higher in fall and remaining elevated in winter. GP levels varied among seasons ($F_{3,23} = 11.40$, $P < 0.0005$), with spring values being about half of those from winter frogs; in contrast, there was no variation ($F_{3,23} = 2.73$, $P = 0.067$) in transcript levels of this protein (Fig. 2c). Seasonal variation was found in GS protein levels ($F_{3,24} = 24.5$, $P < 0.0005$), with summer samples having protein amounts 90 % below those found in other seasons (Fig. 2d).

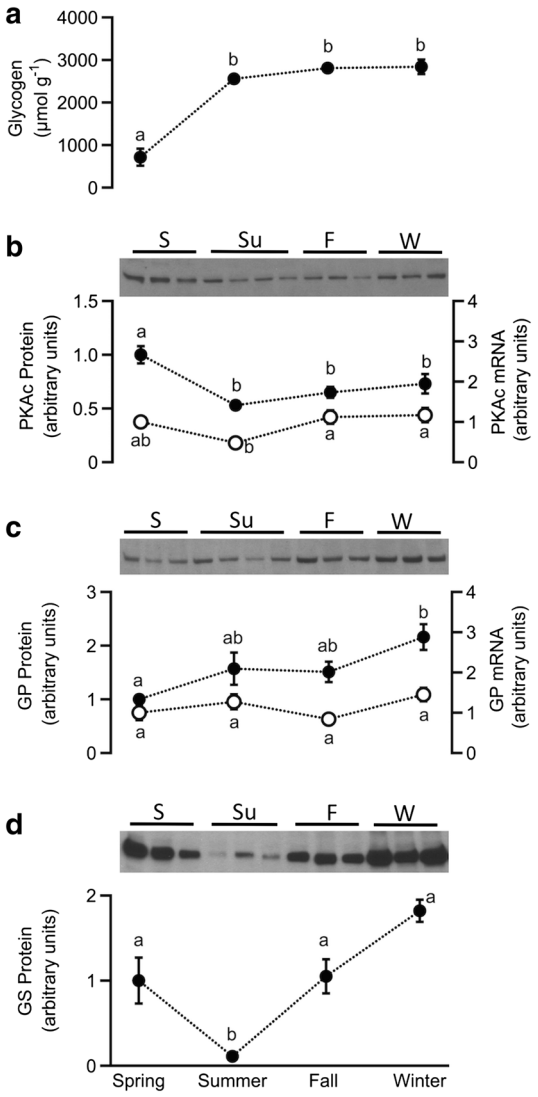

Fig. 2 Seasonal variation in liver **a** glycogen; **b** PKAc protein (*closed circle*) and mRNA (*open circle*); **c** GP protein (*closed circle*) and mRNA (*open circle*); and **d** GS protein in Ohioan *R. sylvatica*. Data are mean ± SEM (N = 5–7 frogs per group). Representative immunoblots are shown in **b**–**d** with 3–4 replicates per season (*S* spring, *Su* summer, *F* fall, *W* winter). Means identified by *different letters* were statistically distinguishable (ANOVA/Tukey; $P < 0.05$)

GSI activity varied ($F_{3,21} = 13.9$, $P < 0.0005$) seasonally, being lowest in the spring and equally high in summer, fall, and winter (Fig. 3a). Total activity of GS varied ($F_{3,23} = 3.77$, $P = 0.025$) in a similar fashion, with spring values being about 30 % of fall values (Fig. 3a). The percentage of total enzyme present as GSI varied ($F_{3,21} = 6.3$, $P = 0.003$) among seasons, with values in winter (28.0 ± 2.9 %) being lower than those in summer (63.7 ± 6.8 %) and fall (64.1 ± 6.7 %). The percentage of active enzyme in spring, 41.7 ± 6.3 %, did not differ from that in the other seasons.

Alaskan frogs

Glycogen concentration in liver varied markedly among seasons ($F_{3,20} = 6.9$, $P = 0.002$), being lowest in spring and about twofold higher in other seasons (Fig. 4a). Mean values ranged from 1500 ± 403 to 2902 ± 237 μmol g^{-1} dry tissue in spring and winter, respectively.

There was no seasonal variation in PKAc protein amount ($F_{3,21} = 2.10$, $P = 0.14$) or transcript level ($F_{3,21} = 2.44$, $P = 0.093$; Fig. 4b). However, GP protein levels varied ($F_{3,21} = 11.4$, $P < 0.0005$) throughout the year, with summer frogs having values 70 % below those of spring frogs and 90 % below those of winter frogs; means for spring and winter frogs were indistinguishable (Fig. 4c). Transcript levels of GP also varied ($F_{3,21} = 50.60$, $P < 0.0005$), but the pattern was roughly inverse to that seen with the protein. GS protein levels were generally high throughout the year, except for those of summer frogs, which were about 75 % lower ($F_{3,22} = 4.80$, $P = 0.01$) than those of other seasons (Fig. 4d).

Total activity of GS showed marked variation ($F_{3,23} = 14.32$, $P < 0.0005$) among seasonal samples, with winter values being about 4.5-fold higher than summer values, and 1.5-fold higher than spring values (Fig. 3b). This pattern could be qualitatively discerned for GSI, although the variation was not significant ($F_{3,22} = 2.46$, $P = 0.089$) (Fig. 3b). The percentage of total enzyme present as GSI, ranging from 38.9 ± 11.0 % in spring frogs to

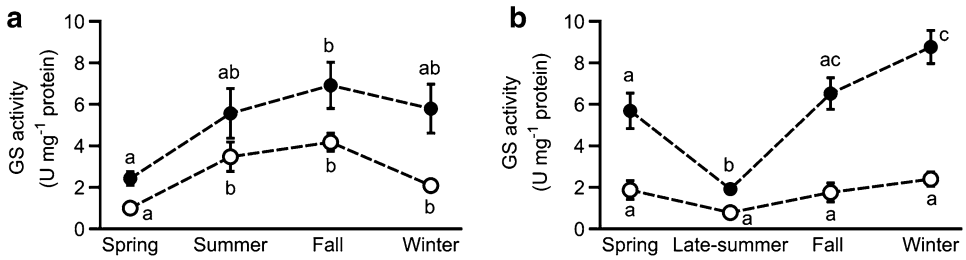

Fig. 3 Seasonal variation in liver GSI activity (*open circle*) and total GS activity (*closed circle*) in **a** Ohioan and **b** Alaskan *R. sylvatica*. Data are mean ± SEM (N = 4–7 frogs per group). Means identified by *different letters* were statistically distinguishable (ANOVA/Tukey; $P < 0.05$)

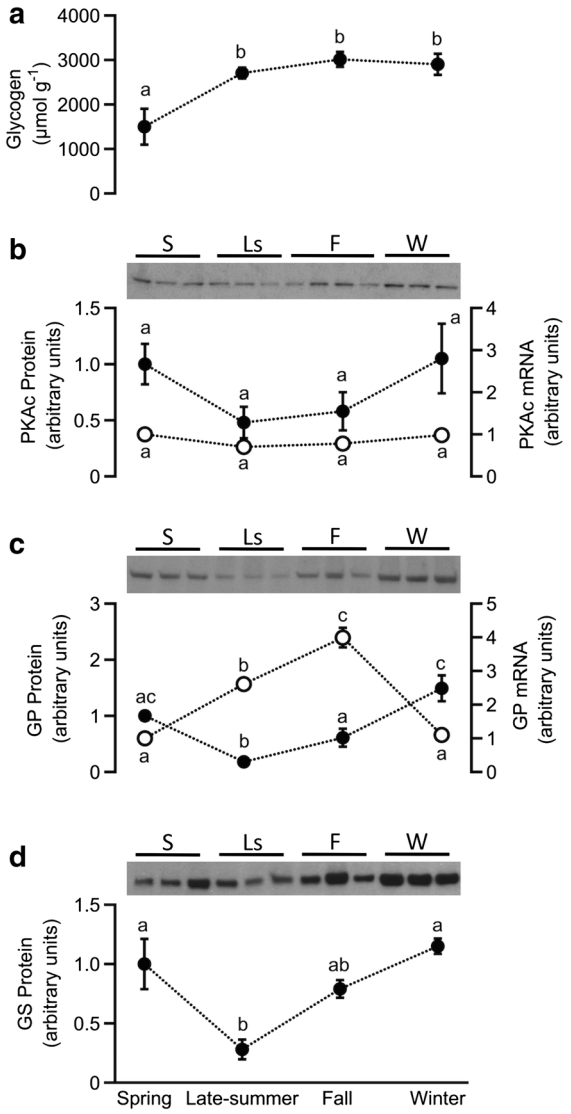

Fig. 4 Seasonal variation in liver **a** glycogen; **b** PKAc protein (*closed circle*) and mRNA (*open circle*); **c** liver GP protein (*closed circle*) and mRNA (*open circle*); and **d** GS protein in Alaskan *R. sylvatica*. Data are mean ± SEM ($N = 5$–7 frogs per group). Representative immunoblots are shown in **b**–**d** with 3–4 replicates per season (*S* spring, *Ls* late-summer, *F* fall, *W* winter). Means identified by *different letters* were statistically distinguishable (ANOVA/Tukey; $P < 0.05$)

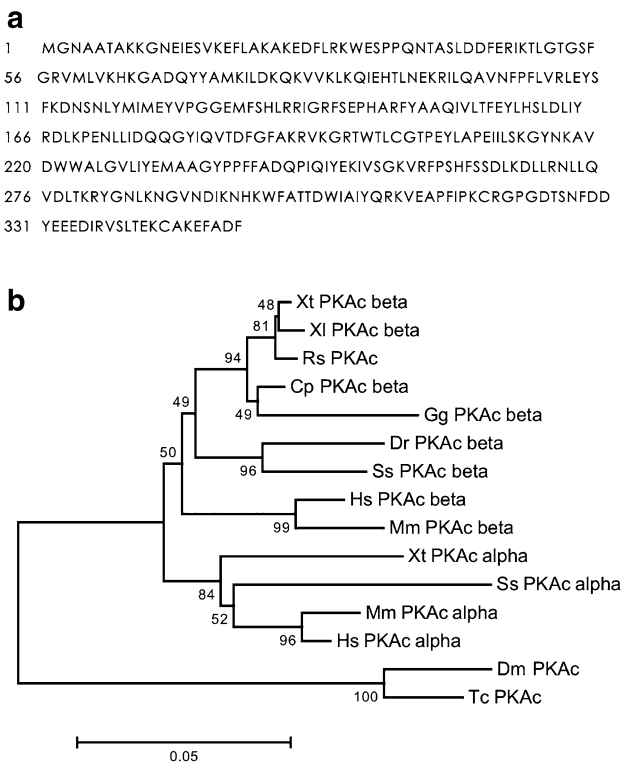

Fig. 5 Analysis of the newly-identified PKAc from *R. sylvatica*: **a** protein sequence obtained through in silico translation; **b** phylogenetic relationships among PKAc isoforms including *Homo sapiens* (Hs) (accession numbers NP_002721 and NP_002722), *Mus musculus* (Mm) (accession numbers NP_035230 and NP_032880), *Xenopus tropicalis* (Xt) (accession numbers NP_001164667 and NP_001016403), *Salmo salar* (Ss) (accession numbers ACN11388 and XP_014058693), *Xenopus laevis* (Xl) (accession number NP_001080696), *Chrysemys picta bellii* (Cp) (accession number XP_005294493), *Gallus gallus* (Gg) (accession number XP_422379), *Danio rerio* (Dr) (accession number NP_001030148), *Drosophila melanogaster* (Dm) (accession number AAA28412), *Tribolium castaneum* (Tc) (XP_088199609). Evolutionary history was inferred using the Neighbor-Joining method and the Poisson correction method. *Scale bar* represents the number of substitutions per amino acid site. Bootstrap proportions (10,000 replicates) are shown above the nodes

26.6 ± 2.4 % in winter frogs, did not vary ($F_{3,22} = 0.49$, $P = 0.69$) among seasons.

Isolation, cloning, and sequencing of PKAc and GP

For PKAc, the same cDNA sequence with high homology to mammalian PKAc was obtained in Ohioan and Alaskan *R. sylvatica*. The coding sequence was 1053 bp (Genbank accession number KU193794), and in silico-translated cDNA revealed a protein consisting of 351 amino acids (Fig. 5a) with a predicted molecular mass of 40.7 kDa and an isoelectric point of 8.65. Multiple-protein sequence analysis revealed that the liver *R. sylvatica* PKAc protein sequence shared a high degree of identity with the PKAc β isoform of *X. tropicalis* (99 %), as well as the PKAc β sequence of *D. rerio* (95 %) and *H. sapiens* (94 %). The phylogenetic tree built using the newly-identified PKAc protein sequence from *R. sylvatica* as well as the predicted and functionally characterized PKAc sequences available in the NCBI protein database showed three main clusters. The first cluster included all sequences of the PKAc β isoform (Fig. 5b). The novel sequence from *R. sylvatica* in this cluster was grouped with other amphibian sequences. The second group included all sequences of the α isoform of

PKAc, and two insect sequences of PKAc formed the third cluster (Fig. 5b).

For GP, two distinct cDNA sequences with high homology to mammalian GP were obtained for the Ohioan and Alaskan phenotypes of *R. sylvatica*. The coding sequences were 2573 bp (Genbank accession numbers KU193792 and KU193793), and in silico-translated cDNA revealed two proteins of 856 amino acids (Fig. 6a), with a predicted molecular mass of 98.4 and 98.5 kDa for Ohioan and Alaskan isoforms, respectively, and an isoelectric point of 6.86. The sequences differed between the phenotypes at three positions: 319, 402, and 718 (Fig. 6a). Two of these sites (402 and 718) are regions of conserved amino acid positions, whereas position 319 is a non-conserved position. Multiple-protein sequence analysis revealed that the GP protein sequences shared a high degree of identity with the GP homolog found in *X. laevis* (91 %) and *H. sapiens* (86 %). The phylogenetic tree built using the newly-identified GP protein sequences from the liver of *R. sylvatica*, as well as the predicted and functionally characterized GP sequences available in the NCBI protein database, showed four main clusters (Fig. 6b). The first cluster with high bootstrap support included the brain isoform of GP for various vertebrate species. The second group included sequences for the muscle isoform from various vertebrates. The two GP sequences for liver of *R. sylvatica* as well as the sequences for GP from the livers of other vertebrates formed the third cluster (Fig. 6b). Two insect sequences of GP formed the fourth cluster.

Discussion

Glycogen, the major storage form of carbohydrates in vertebrates, constitutes a key energetic substrate when metabolic demands are not met by feeding, or when feeding is suspended, such as during food shortage or dormancy (Feder and Burggren 1992). Liver glycogen levels are strongly affected by phenological events like breeding and dormancy, and depend on the dynamic balance between catabolic and anabolic processes determined, in part, by interplay among important hepatic enzymes (Bollen et al. 1998). Heretofore, few studies of anurans have comprehensively examined the relationship between hepatic enzymes and glycogen cycling. In our present study, *R. sylvatica* was an ideal subject because of its dependence on this carbohydrate as an energy store during hibernation and the breeding season, and as a source of cryoprotectant during episodes of corporeal freezing.

Liver glycogen metabolism in Ohioan frogs

Spring

Liver glycogen concentration was lower in spring than during other seasons, as has been reported previously (Storey and Storey 1987; Irwin et al. 2003), possibly as a result of glycogen being used both for hibernation and breeding (Storey and Storey 1987; Costanzo et al. 2014). Our frogs had been feeding regularly before they were sampled; unexpectedly, however, judging from the high level of PKAc and low GS activity in their livers, the assimilated nutrients were not being used in glycogenesis. Instead, net energy intake presumably supported metabolism and somatic growth, as is observed in other anurans in spring (Jørgensen 1983; Windlarsen and Jørgensen 1987). Despite the enzyme's low activity, GS was relatively abundant in this season. Thus, there must be additional regulatory mechanisms, such as glycosylation (Parker 2004), that effectively limit the activity of extant enzyme and thereby inhibit glycogenesis.

Small hepatic glycogen stores negatively impact freeze-tolerance capacity, as these result in reduced cryoprotectant mobilization and, consequently, lower survival (Storey and Storey 1987; Costanzo and Lee 1993; Jenkins and Swanson 2005; Costanzo et al. 2014). Spring *R. sylvatica* had low hepatic glycogen levels and low GP protein amounts, bolstering the notion that limited freeze tolerance in this season results, in part, from a decreased glycogenolytic capacity.

Summer

As early as late July, Ohioan frogs attained the high glycogen concentrations that would be carried into fall and winter. By contrast, other temperate anurans (particularly aquatic forms) usually reach peak levels only by mid-fall or winter (e.g., Castiñeiras et al. 1977; Fenoglio et al. 1992; Scapin and Di Giuseppe 1994). Apparently, a shift in energy allocation occurred in our frogs sometime between April and July, allowing them to accumulate large amounts of glycogen by mid-summer. We speculate that the need for an exceptionally large quantity of glycogen, both as an energy store and as a critical source of cryoprotectant, may protract the process in *R. sylvatica*. Early glycogen accumulation may be particularly advantageous to *R. sylvatica* when nutrient acquisition is hampered by poor environmental conditions and low prey abundance. Furthermore, nutrients acquired in the summer and fall may be used preferentially to support gamete synthesis and maturation (Delgado et al. 1989; Lofts 1964) instead of augmenting glycogen stores. Consequently, early accumulation of glycogen ensures *R. sylvatica* has the energetic reserve and cryoprotectant source necessary for overwinter survival without compromising other important physiological processes. Apparently this pattern of early glycogen accumulation is also seen in other freeze-tolerant anurans, such as the chorus frog (*P. triseriata*) (Edwards et al. 2004), although one study of this species suggests otherwise (Jenkins and

a

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1   MAKPLTDQEKRKQISIRGIVGVENVAELKKGFNRHLHFTLVKDRNVATTRDYYFA
56  LAHTVRDHLVGRWIRTQQYYYEKDPKRTYYLSLEFYMGRTLQNTMINLGLQNACD
111 EAIYQIGLDIEELEEMEEDAGLGNGGLGRLAACFLDSMATLGLAAYGYGIRYEYG
166 IFNQKIKDGWQVEEADDWLRHGNPWEKDRPEYMLPIHFYGRVEHHKTGVRWVDTQ
221 VVLAMPYDTPVPGYMNNTVNTMRLWSARAPNDFNLQDFNVGDYIEAVLDRNLAEN
276 ISRVLYPNDNFFEGKELRLKQEYFVVAASLQDIIRRFKASGLGCKDRIRTGFDSF
331 PEKVAIQLNDTHPALGIPELMRIFLDIEKLPWEKAWEITKKTFAYTNHTVLPEAL
386 ERWPVDLVEKLLPRHLSIIYEINQRHLDRIAALYPKDLDRARRMSLIEEDGIKRI
441 NMAHLCIVGSHAVNGVAKIHSDIVKNQVFKDFNDMEPDKFQNKTNGITPRRWLLL
496 CNPGLAELIAEKIGETYVKDLSQLTKLKKFVDDDVFIRDVSKVKEENKLKFIQYL
551 EKEYKMKLNPASMFDVHVKRIHEYKRQLLNCLHIITMYNRIRENPTKEFVPRTVI
606 IGGKAAPGYHMAKMIIKVITAVGDIVNNDPLVGNKLKVIYLENYRVSLAEKVIPA
661 TDLSEQISTAGTEASGTGNMKFMLNGALTIGTMDGANVEMAEEAGEENLFIFGMR
716 VEDVAEMDKKGYNARDYYEKLPELKKAMDQIQNGFFSPTKPDLFKDIVNMLFNYD
771 RFKVFADYEAYVKSQEKVSALYKNPKEWTKVVIKNIAASGMFSSDRTIKEYARDI
826 WGVEPTDLKIAPPNEPRNVVDVKAAAPAAKG
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b

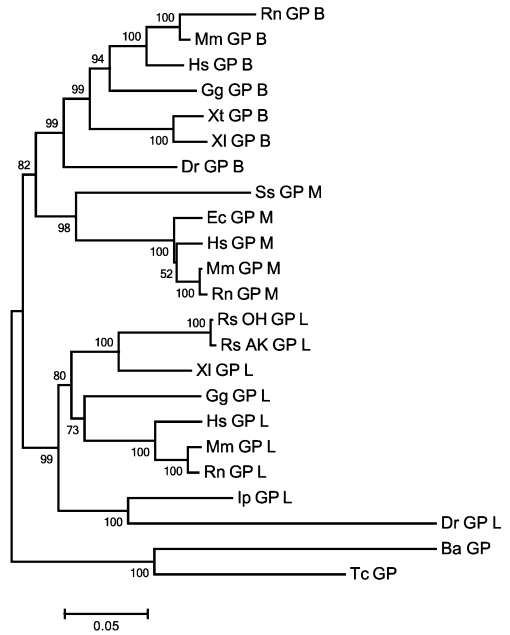

Fig. 6 Analysis of the newly identified GP from *R. sylvatica*. **a** Protein sequence from Ohioan *R. sylvatica* obtained through in silico translation. The sequence for Alaskan *R. sylvatica* is identical except at positions 319 (F), 402 (A), and 718 (E). **b** Phylogenetic relationships among GP brain (B), muscle (M), and liver (L) isoforms. Sequences used included Ohioan *R. sylvatica* (Rs OH), Alaskan *R. sylvatica* (Rs AK), *Homo sapiens* (Hs) (accession numbers NP_002853, AAI30515, CAA75517), *Rattus norvegicus* (Rn) (accession number AAI66475, EDM12601, NP_071604), *Mus musculus* (Mm) (accession numbers NP_722476, NP_035354, NP_573461), *Gallus gallus* (Gg) (accession number NP_001026205, NP_989723), *Xenopus tropicalis* (Xt) (accession number NP_001001904), *Xenopus laevis* (Xl) (accession numbers NP_001080170, AAH82952), *Danio rerio* (Dr) (accession number NP_997974, NP_001008538), *Equus caballus* (Ec) (accession number NP_001138725), *Salmo salar* (Ss) (accession number ACN10567), *Ictalurus punctatus* (Ip) (accession number AHH39573), *Belgica antarctica* (Ba) (accession number AFS17314), *Tribolium castaneum* (Tc) (accession number XP_968960). Evolutionary history was inferred using the Neighbor-Joining method and the Poisson correction method. *Scale bar* represents the number of substitutions per amino acid site. Bootstrap proportions (10,000 replicates) are shown above the nodes

Swanson 2005). Additional research is needed to determine if early glycogen accumulation in woodland frogs is directly related to freeze-tolerance capacity.

GSI activity increased and PKAc protein levels decreased concomitant with the increase in liver glycogen between spring and summer. The observed changes in these enzymes favor glycogen deposition (Bollen et al. 1998). However, by mid-summer, glycogenesis had stalled, as glycogen levels remained static throughout the reaming period of activity into dormancy. Downregulation of this process presumably was achieved by the observed reduction in GS protein, coupled with modest activity of the enzyme, during summer.

Fall and winter

Hepatic glycogen concentrations in Ohioan *R. sylvatica* remained at peak levels in fall and winter. This pattern, which is seen among other temperate anurans (Pasanen and Koskela 1974; Scapin and Di Giuseppe 1994; Dinsmore and Swanson 2008), presumably serves to sustain metabolism during winter and to fuel breeding following spring emergence. In *R. sylvatica*, however, in addition to meeting these demands, glycogen is a critical source of the cryoprotectant, glucose, which is mobilized coincident with the freezing of somatic tissues. During such periods of heightened glycogenolysis, enhanced PKAc function would allow for rapid activation of the glycogenolytic cascade (e.g., Pfister and Storey 2006). Although the abundance of this enzyme in fall and winter frogs was approximately 70 % of the seasonal maximum, its activity can be quickly raised through modulation of intracellular levels of cAMP or changes in subcellular localization of the catalytic subunits (Skalhegg and Tasken 2002). Furthermore, upregulation of other enzymes of the glycogenolysis cascade could also accelerate glycogenolysis. Accordingly, GP levels in winter frogs were double those of spring frogs, likely to support the glycemic cryoprotectant system, as has been proposed previously (Kiss et al. 2011).

Despite the fact that glycogen deposition was reduced during fall and winter, frogs sampled during these periods showed an abundance of GS protein and high activity of GSI. This result seems enigmatic, but probably is explained by the need to clear excess cryoprotectant from tissues following a freezing bout. Accordingly, reconversion of glucose to glycogen in the liver can occur within a few days (Costanzo et al. 2013) and is facilitated by a post-thaw increase in GS activity (Russell and Storey 1995).

Liver glycogen metabolism in Alaskan frogs

Spring

Liver glycogen concentrations in spring frogs were about 50 % of the values measured in other seasons. This pattern

of variation qualitatively resembles that seen in Ohioan frogs, although the northern phenotype retained a greater fraction of their maximal glycogen concentration. Because liver glycogen reserves decrease quickly following emergence from hibernation (Storey and Storey 1987; Costanzo et al. 2014), this disparity potentially could reflect differences in sample timing: Alaskan frogs were sampled within 2 weeks of hibernal emergence, whereas Ohioan frogs had been active for at least several weeks. The greater retention of glycogen by spring Alaskan frogs could also result if, by virtue of the lower winter temperatures they experience, their metabolic demands are reduced as compared to those of frogs from more temperate regions.

Whereas *R. sylvatica* from temperate regions usually do not feed whilst mating (Wells and Bevier 1997), we observed at least one Alaskan frog consuming prey while calling in a breeding pond as well as insect remains in the guts of five of the seven frogs comprising the spring group. Frogs adapted to the subarctic climate, which is characterized by an extremely brief activity season (Fig. 1), apparently must feed immediately and extensively following hibernal emergence (Martof and Humphries 1959). Presumably, energy assimilated during this period is partitioned among metabolism, somatic growth, and replenishment of energy reserves and, accordingly, levels of hepatic GS protein and activity were high in spring Alaskan frogs. Initiating glycogen deposition shortly following hibernal emergence is an energetic strategy distinct from that seen in temperate conspecifics (and also other anuran species; Jørgensen 1983; Windlarsen and Jørgensen 1987), but may be crucial to achieving the necessary levels within such a short activity season.

Abundance of GP protein was high in spring, possibly indicating that glycogen helps sustain metabolism following hibernation; however, given that these frogs were actively feeding, stored carbohydrate probably was a minor substrate. The springtime elevation in GP abundance, which would heighten the cryoprotectant mobilization response to corporeal freezing, could simply be a carryover from the winter condition. It would nevertheless benefit these frogs, which retain a modicum of freeze tolerance during this time (Costanzo et al. 2014).

Summer

As was also the case in Ohioan frogs, replenishment of hepatic glycogen reserves mostly occurred between spring and summer, suggesting that this pattern is characteristic of the species. That levels of GS protein and activity in liver of Alaskan frogs were greatly reduced in summer attests that glycogen deposition had been completed. During summer, as frogs allocate energetic stores to somatic growth and fat deposition, the observed low abundance of GP protein probably serves to diminish glycogenolysis and promotes reliance on other substrates to meet energetic needs.

Fall and winter

Livers of Alaskan *R. sylvatica* maintained maximal levels of glycogen in fall and winter, just as did those of Ohioan frogs. Concentrations determined for these frogs were slightly lower than those reported by Costanzo et al. 2013 for sympatric frogs (fall: 3719 ± 529; winter: 3540 ± 88 μmol g^{-1} dry tissue), the difference presumably being due to inter-study variability in diet, energy use, sex, and other factors. Nevertheless, the hepatic glycogen levels observed in Alaskan *R. sylvatica* are, to the best of our knowledge, the highest reported for any anuran species. Perhaps not coincidentally, these frogs also exhibit the most profound capacity for freeze tolerance of any anuran, readily surviving experimental freezing to at least −16 °C (Costanzo et al. 2013, 2015; Larson et al. 2014).

Alaskan frogs had high levels of GP protein during winter, which, as in Ohioan frogs, likely expedites cryoprotectant production during corporeal freezing. At least one enzymatic process important to freezing recovery was also advantaged during winter: as evidenced by their greater abundance of GS protein and elevated GS activity, Alaskan frogs, like Ohioan frogs, had a heightened potential for glycogen deposition in winter. Moreover, protein phosphatase-1, which activates GS through dephosphorylation, is reportedly elevated in winter *R. sylvatica*, perhaps for the purpose of promoting glycogen deposition following thawing (Kiss et al. 2011).

We observed no marked changes in PKAc protein levels in Alaskan frogs. Therefore, seasonal regulation of this enzyme, if it occurs, must be achieved through modulation of its activity by changes in cAMP availability and/or subcellular localization of PKAc (Skalhegg and Tasken 2002). On the other hand, regulation of GS in Alaskan frogs occurs mostly through changes in enzyme protein levels. In contrast, in Ohioan frogs, regulation of PKAc involved changes in protein levels, whereas GS was regulated through changes in both protein amounts and enzyme activation. Thus, despite the overall similarities in glycogen cycling between populations of *R. sylvatica,* regulation of hepatic enzymes apparently involves different mechanisms in temperate and subarctic frogs.

Relationship between PKAc and GP protein and transcript levels

We measured transcript levels of PKAc and GP in Ohioan and Alaskan frogs to determine if variation in protein levels mirrored changes in gene expression. Overall, this was not the case. Protein abundance in the cell results from a

dynamic balance among several factors, including transcript levels, stability of mRNA, translation rate, and protein turnover (Vogel and Marcotte 2012). Consequently, levels of transcript and protein do not necessarily change in concert (Tian 2004). The nature of the association between protein and its encoding mRNA apparently varied between the phenotypes, particularly in the case of GP (Figs. 2c, 3c), suggesting that Ohioan and Alaskan frogs use distinct strategies to regulate transcription and translation of at least some enzymes.

Sequence and phylogenetic analysis of PKAc and GP

PKA has an essential role in signal transduction in the cell and in regulating glycogen metabolism in particular (Bollen et al. 1998; Søberg et al. 2013). do Amaral et al. (2013) conjectured that variation in amino acid sequence accounted for observed differences between Alaskan and Ohioan *R. sylvatica* in catalytic activity of PKAc. However, because our results showed that the sequence for this protein was identical between the two phenotypes, the earlier reported variation in catalytic efficiency may instead be due to differences in post-translational modifications to this enzyme (Caldwell et al. 2012). As a consequence of a gene duplication event in a vertebrate ancestor, most extant vertebrates have at least two functional isoforms of PKAc (α and β) with a high degree of conservation among isoform sequences (Søberg et al. 2013). Our phylogenetic analysis revealed the single sequence for this enzyme in *R. sylvatica* to be more similar to PKAc β; thus, we consider this sequence to be the *R. sylvatica* ortholog of PKAc β. Holden and Storey (2000) purified PKAc from *R. sylvatica* liver and similarly found only one isoform, suggesting that, if the gene for PKAc α is present in this species, its transcript and protein are scarce or non-existent. A complete analysis of the *R. sylvatica* genome and transcriptome is needed to confirm the presence or absence of genes and transcripts for other PKAc isoforms.

Glycogen catabolism requires GP to effect phosphorolytic cleavage and release glucose-1-phosphate, and, in *R. sylvatica*, is key to mobilizing the cryoprotectant glucose during the early hours of freezing (Storey and Storey 2004; Costanzo and Lee 2013). Sequences of the GP liver ortholog in *R. sylvatica* from the two populations differed in three positions, two of which are in conserved regions of the protein. At position 718, the Ohioan sequence had aspartic acid whereas the Alaskan sequence had glutamic acid. As both amino acids are hydrophilic, charged, and polar, this (rather common) substitution probably bears little on the enzyme's shape (Betts and Russell 2003). Position 402 in the sequence from Ohioan frogs contained serine whereas alanine was present in that of the Alaskan frogs. Although these two amino acids are similar in structure and size, alanine is non-polar and hydrophobic, and serine is polar and hydrophilic; thus, these differences conceivably could impact the enzyme's shape (Betts and Russell 2003). do Amaral et al. (2013) detected no differences in catalytic activity of GP in liver homogenates from Alaskan and Ohioan frogs, but given our present findings additional research, perhaps using purified proteins, seems warranted.

Perspectives

Glycogen is an essential nutrient reserve in *R. sylvatica*. Hepatic levels of this carbohydrate vary seasonally in both temperate and subarctic populations of *R. sylvatica* in a manner reflecting periodic needs for glycogenesis and glycogenolysis. The phenotypes differed in enzymatic regulation of glycogen metabolism in part because Alaskan frogs must ensure that reproduction, somatic growth, and replenishment of nutrient reserves are completed during their extremely short activity season. In anurans, seasonal cycling of liver glycogen is accompanied by variation in glycogenolytic and glycogenic enzymes, likely as the result of changes in energetic strategies and demands, and in freeze-tolerant frogs, winter survival is particularly important to understanding the regulation of glycogen metabolism.

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