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Urea Production Capacity in the Wood Frog (*Rana sylvatica*) Varies With Season and Experimentally Induced Hyperuremia

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ABSTRACT Wood frogs (*Rana sylvatica*) can accumulate substantial amounts of urea during fall and winter. In this study, maximal urea production capacity was examined in *R. sylvatica* collected at various times of the year and in response to experimental hyperuremia and dehydration. Activity and expression of carbamoyl phosphate synthetase I (CPS I), the hepatic regulatory enzyme of the urea cycle, were used as indicators of urea production capacity. The high levels of CPS I activity in summer frogs were maintained through much of winter, a time when many metabolic processes are downregulated, suggesting that urea production is important during hibernation. In laboratory experiments, hyperuremia in fully hydrated frogs caused CPS I activity to decrease by ~41%, suggesting that urea functions as a feedback inhibitor. In contrast, CPS I activity was maintained in hyperuremic, dehydrated frogs. The significance of this response is unclear, although perhaps urea functions to counteract inhibitory effects of concentrated salts. Generally, changes in CPS I activity were not reflected by corresponding changes in CPS I quantity, indicating that this enzyme is not primarily regulated through transcription and translation; rather, control may be achieved by posttranslational modifications and/or feedback inhibition. Our findings suggest that maintenance of urea production capacity in hibernating *R. sylvatica* facilitates accumulation of this osmolyte, which has important roles in the winter biology of this species. *J. Exp. Zool. 309A:484–493, 2008.*

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Anurans are especially prone to dehydration because their skin is relatively permeable and by itself is an insufficient barrier to water loss. Therefore, these animals must rely on other means to limit desiccation. Urea accumulation is one such mechanism universally used by amphibians (and some other vertebrate ectotherms) to maintain water balance under dehydrating conditions (Gordon et al., '61; McBean and Goldstein, '70; McClanahan, '72; Ip et al., 2005). An organic osmolyte, urea replaces some of the ionic solutes that might otherwise concentrate and become detrimental to cellular functions during osmotic stress (Grundy and Storey, '94).

There are two known mechanisms by which organisms accumulate urea. One is simply to retain urea rather than excrete it (Balinsky, '81). Many organisms using this stratagem reduce urine production and excretion, thereby causing urea to accrue in tissues. Among some amphibians, retention is aided by reabsorption of urea across the walls of the urinary bladder (Chew et al., '72). Another mechanism, which may be used in addition to retention, is to increase urea synthesis through modulation of urea-cycle activity (Balinsky, '81).

Much of our current understanding of urea-cycle regulation comes from studies of mammals. These indicate that carbamoyl phosphate synthetase I (CPS I), the first enzyme of the urea cycle, which catalyzes the conversion of ammonium and bicarbonate to carbamoyl phosphate, is a key regulatory point (Morris, 2002). CPS I function requires *N*-acetyl-L-glutamate, but it is unclear whether

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this molecule is involved in regulation of urea-cycle activity. Substrate availability is often the major determinant of the rate of urea synthesis in the short term, whereas long-term regulation in response to changing demands for urea synthesis apparently occurs primarily by controlling the quantity of CPS I and other urea-cycle enzymes through transcription and/or translation (Morris, 2002). Few other mechanisms of regulation have been identified, although it is known that acylation of the active site of rat CPS I irreversibly inhibits its activity (Corvi et al., 2001). Because of its regulatory role, researchers have interpreted changes in CPS activity to be reflective of changes in urea production capacity (McBean and Goldstein, '70; Chew et al., 2003; Wright et al., 2004). Modulation of CPS activity reportedly occurs in several, though certainly not all, urea-accumulating species (Weng et al., 2004; Lee et al., 2006).

The wood frog (*Rana sylvatica*) has a geographic range that extends from the southeastern United States into Canada and Alaska. In fall, these frogs begin hibernation under leaf litter and seem to prefer upland forests with well-drained soils as opposed to more moist lowlands (Regosin et al., 2003). At this time, soil moisture can be scarce, which can create an osmotic challenge to hibernating frogs. In addition, these frogs may face increasingly desiccating conditions when the remaining soil water freezes and becomes biologically unavailable. Urea may be an important osmoprotectant at this time. Plasma urea levels of up to 50 mmol L^{-1} have been found in hibernating *R. sylvatica*, and in the laboratory these frogs can accumulate to at least 90 mmol L^{-1} (Costanzo and Lee, 2005). Recent studies indicate that modest physiological levels of urea reduce freezing damage to cells and tissues (Costanzo and Lee, 2005; Costanzo et al., 2008), and may also contribute to an energy-sparing metabolic depression (Muir et al., 2007, 2008). Efforts to elucidate these roles are ongoing, but it is unclear whether urea accumulation in fall and winter is owing to decreased excretion alone or whether it also involves increased rates of urea synthesis. We compared both the activity and quantity of CPS I in *R. sylvatica* sampled at various times of the year to determine the relative roles of decreased excretion and increased synthesis in urea production capacity. Additionally, we examined regulation of urea production capacity by measuring CPS I activity and quantity in frogs rendered hyperuremic via urea injections in both the fully hydrated and dehydrated states.

MATERIALS AND METHODS

Animal collection and maintenance

Male wood frogs (*R. sylvatica*) were collected from a vernal pool in February 2004 and 2006 in Adams Co., Ohio, OH. Frogs from the 2004 collection were placed in an outdoor enclosure at the Ecology Research Center (ERC), Miami University, where they were exposed to naturally varying environmental conditions. During spring and summer they were fed crickets three times a week; water was continuously available. In October, feeding was terminated. Four frogs were transported to the laboratory and euthanized to provide tissue for experimentation (see below), whereas the remaining animals were transferred to a separate facility at the ERC for overwintering. Sited in a deciduous woodlot, this enclosure contained sparse shrub cover and leaf litter and provided environmental conditions appropriate to hibernation (see Costanzo and Lee, 2005). Small groups of frogs were recaptured in January, February, and March, transported to the laboratory, and euthanized for tissue collection.

Frogs collected in February 2006 were divided into two lots. One group was placed in covered, opaque boxes containing damp moss and kept at 4°C for up to 6 weeks until used in laboratory experiments. The other group was maintained inside the summer enclosure at the ERC (as described above) until August, when several frogs were transported to the laboratory and euthanized to provide tissue for experimentation (see below). The care and experimental treatment of frogs were in accordance with protocols approved by Miami University's Institutional Animal Care and Use Committee (protocol 629).

Euthanization and tissue collection

Frogs were euthanized by double pithing and immediately dissected. A portion of the liver was dissected out, placed in a cryo-storage vial, and immediately frozen in liquid N_2. Samples were subsequently stored at −80°C until used in determinations of CPS I activity and western (immunoblot) blot assays (see below).

Seasonal variation in CPS I activity and expression

In the population of *R. sylvatica* under investigation, feeding wanes and ultimately eases in October and the ensuing period of hibernation lasts until the frogs emerge to breed, usually in

late February. To determine whether CPS I activity varies seasonally in *R. sylvatica*, we sampled frogs ($N = 4$) from the outdoor enclosures on October 1 in 2004 (fall), and on January 31 (winter), February 28 (late winter), and March 16 (spring) in 2005. We included in this experiment frogs ($N = 5$) that were sampled from the outdoor enclosure in August 2006 (summer). Liver tissue was sampled as described previously and used in determinations of CPS I activity and semi-quantitative western blot protein assays.

Effect of hyperuremia on CPS I activity and expression

We examined the influence of hyperuremia on CPS I activity and expression in fully hydrated *R. sylvatica* by comparing these variables in urea-treated and saline-treated frogs. Cold-acclimated frogs from the February 2006 collection were randomly assigned to either of two groups ($N = 5$ each) and injected with phosphate-buffered saline (PBS; in $g\,L^{-1}$: 6.10 NaCl, 0.15 KCl, 0.88 Na_2HPO_4, 0.15 KH_2PO_4; pH 7.4) or PBS containing $1.5\,mol\,L^{-1}$ urea. The injectant (volume, ~3% of standard body mass) was delivered into the dorsal lymph sac using a 27.5 gauge needle. From measurements of body mass and tissue water concentration, we estimated that administering this urea solution would elevate plasma urea concentration to $70\,mmol\,L^{-1}$, which is within the physiological range (Costanzo and Lee, 2005). After receiving the injection, each frog was returned to its box and held at 4°C, in darkness, for 24 hr before being euthanized and dissected. Approximately 250 μL of blood was collected from the dorsal aorta in heparinized capillary tubes and centrifuged (4,000*g*, 5 min), and the resulting plasma was stored at −80°C until analyzed for urea using a colorimetric BUN kit (Pointe Scientific, Canton, MI). Liver tissue was sampled as described previously and used in determinations of CPS I activity and western blot assays.

Effect of hyperuremia and dehydration on CPS I activity and expression

In a separate experiment, we examined the influence of hyperuremia coupled with dehydration on CPS I activity and expression in *R. sylvatica* by comparing these variables in urea-treated and saline-treated frogs subjected to gradual dehydration. Cold-acclimated frogs from the February 2006 collection were injected with PBS ($N = 5$) or PBS containing $1.5\,mol\,L^{-1}$ urea ($N = 5$) as described above. They were placed individually inside plastic tubes (which also served as respirometry chambers in the study by Muir et al., 2007) and held for 6 d at 4°C, in darkness, after which they received a second injection of PBS or PBS with $1.5\,mol\,L^{-1}$ urea as appropriate. Frogs were gradually dehydrated over the next 23 d by slowly passing a stream of air (4°C; relative humidity ~60%) through the tubes during a 14-hr period on alternating days. Finally, the frogs were weighed and euthanized, and blood and liver samples were collected as described above. Body water content, expressed as percentage of fresh mass, was determined by thoroughly drying the carcass (including residual liver) in a 65°C oven. Plasma was assayed for urea concentration and liver tissue was used in determinations of CPS I activity and western blot assays.

CPS I activity assay

Liver samples were thawed on ice, gently blotted on laboratory tissue, and weighed to 0.01 mg. We added homogenization buffer ($50\,mmol\,L^{-1}$ triethanolamine HCl, $15\,mmol\,L^{-1}$ magnesium acetate, $1\,mmol\,L^{-1}$ DL-dithiothreitol, $10\,mmol\,L^{-1}$ ATP; $10\,\mu L\,mg^{-1}$ tissue) and homogenized the sample on ice using a rotating-blade homogenizer (Tissue Tearor, Biospec, Bartlesville, OK). The homogenate was centrifuged (14,000*g*, 4°C, 15 min) and most of the resulting supernatant was passed through a Sephadex G25 column (20 cm high × 0.7 cm diameter) equilibrated with homogenization buffer. Small aliquots of the raw supernatant and residual filtrate were frozen in liquid N_2 and stored at −80°C for subsequent protein determination using an assay kit (Bradford protein assay, Bio-Rad Laboratories, Hercules, CA).

Two reaction tubes were prepared for each enzyme sample. A 10-μL volume of the enzymatically active filtrate was added to 165 μL of homogenization buffer in each tube. One tube in each pair was incubated at 95°C for 5 min to inactivate the enzyme, whereas the other was kept on ice. The enzymatic assay was carried out as described by Pierson ('80) except that our initial incubations were carried out at room temperature (~23°C). Before reading sample absorbances, reaction tubes were centrifuged (4,000*g*, 4°C, 6 min) to remove precipitated proteins. Absorbances were read at 458 nm zeroed on reagent blanks prepared by substituting an equal volume of homogenization buffer for filtrate.

To account for possible color formation owing to other components of the homogenate, we subtracted the absorbance of the inactivated solution (range 0.001–0.022) from that of the corresponding active solution before calculating the amount of carbamoyl phosphate produced. This step was necessary because urea also produces a yellow color with this assay. However, our preliminary studies showed that the Sephadex G25 column effectively removed high levels ($>150\,mmol\,L^{-1}$) of urea; thus, urea in the liver samples should not have contributed to color formation. Because the water content of liver samples varied in our study, we expressed CPS I activity as nmol carbamoyl phosphate produced $hr^{-1}\,mg^{-1}$ soluble protein.

Western blot analysis

A separate portion of each liver sample was prepared by mechanical homogenization for 45 sec in extraction buffer ($250\,mmol\,L^{-1}$ sucrose, $10\,mmol\,L^{-1}$ Tris, $1\,mmol\,L^{-1}$ dithiothreitol, $1\,mmol\,L^{-1}$ phenylmethylsulfonyl fluoride; pH 7.4) followed by sonication (10-sec pulses, 4 ×). Homogenates were frozen in liquid N_2 and stored at −80°C until used. After thawing, samples were centrifuged (7,000*g*, 4°C, 5 min) and the total protein concentration in the resultant supernatant was determined. Protein (1, 2, or 4 μg) was incubated with Lammelli buffer (Bio-Rad Laboratories) and loaded into 4–15% gradient Tris–HCl Ready Gels (Bio-Rad Laboratories); electrophoresis was run for 7 min at 120 V and then for 40 min at 180 V. Protein was transferred to a nitrocellulose membrane (Bio-Rad Laboratories) for 90 min at 80 V. We used Ponceau S staining (Sigma Chemical Company, St. Louis, MO) to confirm that protein was transferred properly and equally loaded.

Following destaining, the membranes were blocked overnight at 4°C in wash buffer ($10\,mmol\,L^{-1}$ Trizma, $100\,mmol\,L^{-1}$ NaCl; pH 7.5 with 0.1% Tween 20) containing 10% nonfat dry milk. The membranes were warmed to room temperature, incubated with rabbit anti-CPS I primary antibody (Abcam, Cambridge, MA) diluted 1:4,000 in wash buffer containing 5% milk for 1 hr, and then given four 10-min washes with wash buffer. Next, the membranes were incubated with goat anti-rabbit secondary antibody (Sigma, St. Louis, MO) diluted 1:1,000 in wash buffer containing 5% milk for 1 hr, followed by four 10-min washes with wash buffer. CPS I was visualized using an enhanced chemiluminescence system (Amersham ECL western blotting detection system, Amersham Biosciences, Piscataway, NJ). Bands were quantified with ImageQuant 5.2 software (Molecular Dynamics, Amersham Biosciences). Each sample was run in duplicate on two separate blots; densitometry volumes from the duplicate blots were averaged to provide the single value used in the statistical analysis.

Statistical treatment of data

Group means (reported ± SEM) were compared using Student's *t*-test or analysis of variance, followed by Fisher's protected least-significant difference. Percentage data were analyzed after arcsine-root transformation. Significance was accepted at $P \leq 0.05$.

RESULTS

Approximately 1–3 μg of protein was present in the 10-μL aliquot of filtrate used in the enzyme assays. Preliminary experiments showed that the relationship between protein concentration and absorbance remained linear at least up to 7 μg of protein; thus, enzyme activity was not limited by substrate or cofactor availability. We detected CPS I activity in all liver samples tested. Heat-inactivated preparations had very little or no color development over the reagent blanks.

Our preliminary western blot experiments showed that the primary anti-CPS I antibody cross-reacted equally well to *R. sylvatica* CPS I as it did to rat liver CPS I, against which the antibody had been raised. Western blots revealed an expected single band of 165 kDa that occurred just above the 150 kDa molecular-weight marker, which we identified as CPS I.

Seasonal variation in CPS I activity and expression

CPS I activity in *R. sylvatica* liver varied seasonally (Fig. 1A; $P = 0.04$). The activity in summer frogs was similar to that in frogs sampled in fall and winter, but was substantially higher than that found in frogs sampled in late winter and spring. Soluble protein concentration of the filtrate ranged from 3.53 ± 0.53 mg $100\,mg^{-1}$ wet tissue in winter frogs to 4.30 ± 0.24 mg $100\,mg^{-1}$ wet tissue in spring frogs, but there was no statistically significant ($P = 0.17$) variation among any of the groups.

Western blot analyses indicated that quantities of CPS I varied among frogs collected at different times of the year ($P = 0.002$). Summer frogs had ~1.8 times more CPS I than the quantity found in

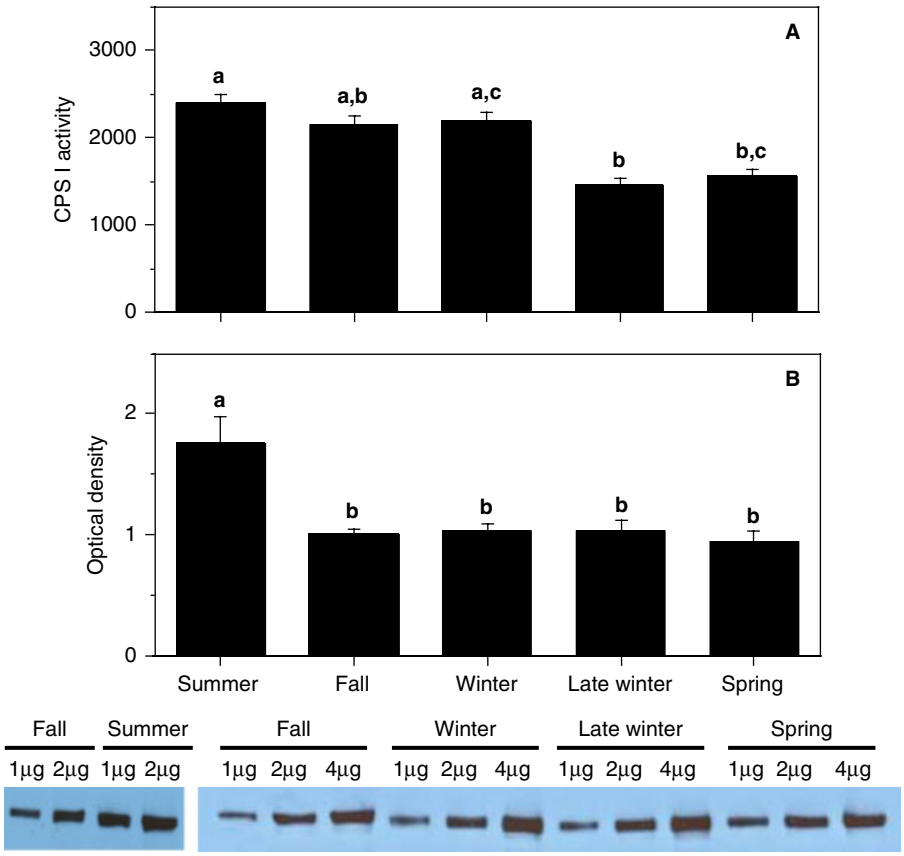

Fig. 1. Seasonal variation in CPS I activity and quantity in *R. sylvatica*. (**A**) CPS I activity expressed as nmol carbamoyl phosphate $hr^{-1} mg^{-1}$ soluble protein. (**B**) Western blot detection of CPS I showing densitometric analyses (arbitrary units) and representative blots. Samples from summer frogs were run on separate blots alongside additional samples from fall frogs against which the densitometric results were normalized. Bars represent mean $\pm$ SEM; $N = 4$ for all groups except summer, where $N = 5$. Within each panel, groups not sharing the same letter are significantly different (ANOVA/Fisher's PLSD; $P \leq 0.05$). CPS I, carbamoyl phosphate synthetase I; ANOVA, analysis of variance; PLSD, protected least-squares difference.

frogs sampled at other times, but no other differences among groups were detected (Fig. 1B).

Effect of hyperuremia on CPS I activity and expression

Experimental hyperuremia was associated with a decrease in CPS I activity, but not quantity, in *R. sylvatica* liver. At the time of euthanization, urea-treated frogs exhibited a blood urea concentration of 69.6 ± 2.5 mmol L^{-1} as contrasted to 9.8 ± 0.8 mmol L^{-1} for saline-treated frogs; both values are within the physiological range for hibernating *R. sylvatica* (Costanzo and Lee, 2005). Saline-treated frogs had 1.7-fold higher ($P = 0.01$) levels of CPS I activity relative to urea-treated frogs (Fig. 2A). This variation could not be attributed to differential amounts of enzyme, because western blot analyses indicated that the groups had similar ($P = 0.96$) quantities of CPS I (Fig. 2B). Neither was there a difference ($P = 0.61$) in total soluble protein concentration between urea-treated (4.7 ± 0.4 mg protein 100 mg^{-1} wet tissue) and saline-treated frogs (4.4 ± 0.4 mg protein 100 mg^{-1} wet tissue).

Effect of hyperuremia and dehydration on CPS I activity and expression

All frogs were alive at the end of the 23-d dehydration experiment, though they had lost ~27% of their initial body mass. Results from paired *t*-tests showed that body water content decreased from an estimated initial value of 78.5 ± 0.2 to 71.5 ± 0.4% in saline-treated frogs ($P < 0.0001$), and from 78.3 ± 0.6 to 69.3 ± 1.0% in urea-treated frogs ($P < 0.0001$). The final measurements did not differ ($P = 0.08$) between the groups, indicating that all frogs had lost approximately the same quantity of water. By the last day

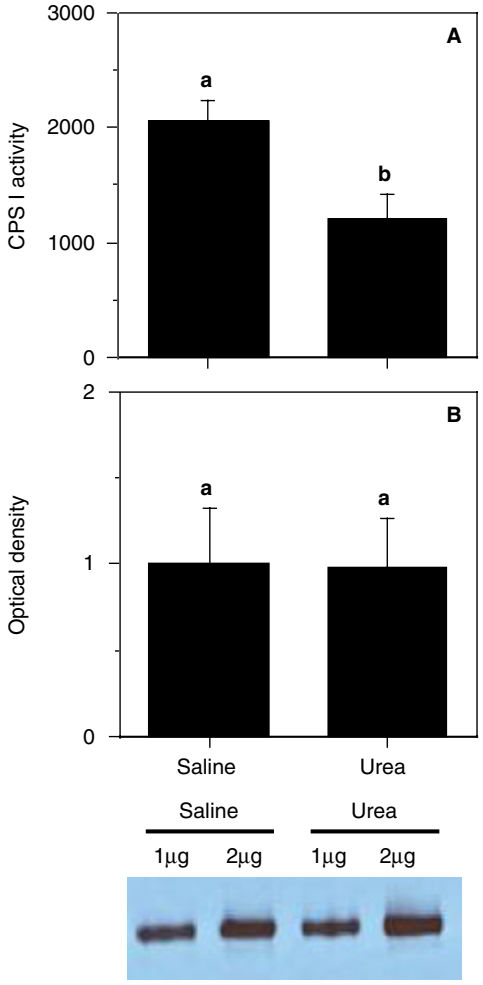

Fig. 2. Variation in CPS I activity and quantity in *R. sylvatica* treated with phosphate-buffered saline or phosphate-buffered saline containing 1.5 mol L^{-1} urea. (**A**) CPS I activity expressed as nmol carbamoyl phosphate hr^{-1} mg^{-1} soluble protein. (**B**) Western blot detection of CPS I showing densitometric analyses (arbitrary units) and representative blots. Bars represent mean ± SEM ($N = 5$). Within each panel, groups not sharing the same letter are significantly different (Student's *t*-test; $P \leq 0.05$). CPS I, carbamoyl phosphate synthetase I.

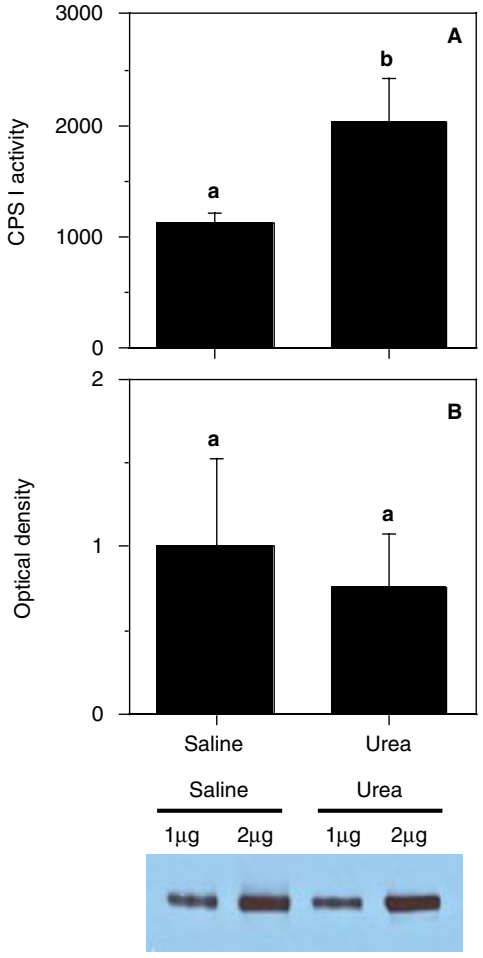

Fig. 3. Variation in CPS I activity and quantity in *R. sylvatica* treated with phosphate-buffered saline or phosphate-buffered saline containing 1.5 mol L^{-1} urea before being exposed to dehydrating conditions for 23 d. (**A**) CPS I activity expressed as nmol carbamoyl phosphate hr^{-1} mg^{-1} soluble protein. (**B**) Western blot detection of CPS I showing densitometric analyses (arbitrary units) and representative blots. Bars represent mean ± SEM ($N = 5$). Within each panel, groups not sharing the same letter are significantly different (Student's *t*-test; $P \leq 0.05$). CPS I, carbamoyl phosphate synthetase I.

of the experiment, plasma urea concentration had reached 38.3 ± 1.8 mmol L^{-1} in saline-treated frogs and 161.5 ± 7.6 mmol L^{-1} in urea-treated frogs. Urea levels in the latter group probably were higher than those usually occurring in natural populations of *R. sylvatica*, but were readily tolerable (Shpun et al., '92).

Hepatic CPS I activity in liver of urea-treated frogs, 2,032 ± 386 nmol carbamoyl phosphate hr^{-1} mg^{-1} protein, was 1.8-fold higher ($P = 0.05$) than that measured in saline-treated frogs (Fig. 3A). Western blot analyses indicated that CPS I quantity did not differ ($P = 0.70$) between these groups (Fig. 3B). Total soluble protein concentrations in filtrates prepared from saline-treated frogs (5.3 ± 0.5 mg soluble protein 100 mg^{-1} wet tissue) and urea-treated frogs (6.1 ± 0.2 mg soluble protein 100 mg^{-1} wet tissue) were statistically indistinguishable ($P = 0.14$).

DISCUSSION

Measurements of CPS I activity in *R. sylvatica* liver were consistent with the relatively

high values expected of terrestrial species (Jørgensen, '97) and comparable to those reported for the crab-eating frog, *R. cancrivora* (2,358 nmol carbamoyl phosphate $h^{-1} mg^{-1}$ protein; Wright et al., 2004), and *Xenopus laevis* (1,860 nmol carbamoyl phosphate $hr^{-1} mg^{-1}$ protein; Lindley et al., 2007) while under osmotic stress.

Many metabolic processes in amphibians (and other ectotherms) are downregulated in winter to conserve energy, as survival depends on the judicious use of finite endogenous energy reserves (Pinder et al., '92; Tattersall and Ultsch, 2008). Because urea synthesis is energetically costly, one might expect urea-cycle activity to decrease markedly during hibernation. To the contrary, hepatic CPS I activity in *R. sylvatica* was maintained in fall and winter at the levels found in summer animals with a decrease occurring only with the advent of spring. Maintenance of this relatively high capacity for urea production, coupled with anuria and urea retention, probably contributes to the urea accumulation observed in hibernating *R. sylvatica* (Costanzo and Lee, 2005), whereas its reduction in spring coincides with arousal from hibernation and diminishing levels of freeze tolerance (see discussion in Layne et al., '98). This finding supports our hypothesis that elevated urea is of physiological importance in hibernation owing to its functions as a cryoprotectant (Costanzo and Lee, 2005; Costanzo et al., 2008) and a metabolic depressant (Muir et al., 2007, 2008).

Current understanding of urea-cycle function suggests that CPS I activity is regulated primarily through transcription and translation (Morris, 2002). Apparently, this is true not only of mammals, but also of some nonmammalian vertebrates. For example, in one study, CPS I activity in *X. laevis* increased two-fold with a concomitant six-fold increase in mRNA (Lindley et al., 2007). In another study, the air-breathing catfish (*Clarias batrachus*) responded to high ambient ammonia by increasing CPS III (a glutamate-dependent CPS) activity two-fold, and this rise was reflected by a 1.75-fold increment in protein quantity (Saha et al., 2007). We anticipated finding that CPS I activity more or less matched the quantity of this enzyme in *R. sylvatica* liver. However, that this was generally not the case suggests that CPS I activity in *R. sylvatica* is also regulated at the protein level. Unfortunately, little is known about regulation of urea-cycle activity in nonmammalian vertebrates, although Anderson ('81) proposed that urea-accumulating species might use multiple mechanisms of control. Saha et al. (2007) suggested that, in addition to transcriptional regulation, mechanisms such as phosphorylation or regulation by *N*-acetyl-L-glutamate could influence control of urea-cycle activity in *C. batrachus*. In our experiments, *N*-acetyl-L-glutamate was present in the assay mixture; thus, the observed variation in CPS I activity cannot be ascribed to differential cellular concentrations of this activator. In principle, regulation of this enzyme could be achieved through feedback inhibition, phosphorylation, or allosteric modifiers. Reversible phosphorylation is a particularly efficient means to adjust metabolic functions in estivating animals (Storey, 2002), but whether or not this mechanism is also used by hibernating
R. sylvatica remains to be determined.

Although CPS I activity was comparable between fall and winter frogs and those sampled in summer, the latter had more of the enzyme. In summer, frogs undoubtedly experience intermittent periods when dietary protein is catabolized followed by periods of only basal levels of protein degradation and nitrogenous waste production. We suspect that protein-level regulatory mechanisms could keep CPS I activity at lower levels while frogs are not feeding, yet also allow for accelerated urea production when needed. This seems to be the case in the dogfish shark (*Squalus acanthias*), which also exhibits alternating periods of elevated protein metabolism, as activities of urea-cycle enzymes increase markedly upon feeding (Kajimura et al., 2006). The mechanism(s) by which *S. acanthias* modulates enzyme activities in this nitrogen-conserving response remains unknown. However, in feeding *R. sylvatica*, increasing blood ammonia levels could be a major regulatory signal to upregulate CPS I activity.

Experimentally treating *R. sylvatica* with exogenous urea apparently inhibited hepatic CPS I activity, which decreased by ~41% relative to saline-treated frogs. Because hyperuremia would serve no osmoregulatory purpose in these fully hydrated frogs, it is conceivable that enzyme activity was downregulated in order to retard urea production. Similarly, rats rendered hyperuremic (10 mmol L^{-1} urea vs. 4 mmol L^{-1} in controls) from partial nephrectomy show a transient decrease in urea synthesis as well as decreased levels of mRNA for all urea-cycle enzymes except ornithine transcarbamoylase (Nielsen et al., 2007). Because we found no difference in CPS I quantity between urea-treated and saline-treated frogs, it is unlikely that elevated urea influenced regulatory

pathways involving CPS I transcription and translation. It is more likely that elevated urea activated a separate control pathway or directly acted upon the enzyme to reduce its activity. Indeed, urea is a well-known protein destabilizer that can markedly alter enzyme function. Marine elasmobranchs, which maintain on the order of 400 mmol L^{-1} urea in their tissues, accumulate methylamines that serve to counteract urea's perturbing effects on proteins (Yancey et al., '82; Robertson, '89). In *S. acanthias*, lacking appropriate concentrations of these counteractants, elevated urea directly inhibits activity of CPS III (Anderson, '81). Given that anurans seemingly do not accumulate substantial amounts of methylamines (Wray and Wilkie, '95; Withers and Guppy, '96; Yancey, 2005), we suggest that elevated urea ($\sim$70 mmol L^{-1}) in our urea-treated frogs could have functioned as a feedback inhibitor by perturbing enzyme function. Whether or not CPS I levels would have changed had these frogs been sampled later than 24 hr after urea treatment is unknown; however, in the dehydration experiment, protein levels in hyperuremic frogs were unaltered even after $\sim$1 month (Fig. 3B).

Hibernating *R. sylvatica* can encounter low ambient water potential leading to dehydration (Costanzo and Lee, 2005), which is a potent stimulus of urea-cycle activity in some ectothermic vertebrates (Janssens and Cohen, '68; Wright et al., 2004). However, CPS I activity in frogs undergoing experimental dehydration was maintained (urea-treated frogs) or decreased (saline-treated frogs), rather than stimulated. This outcome was somewhat surprising, particularly because the kinetic response to hyperuremia in these frogs was contrary to that in fully hydrated frogs. Although we cannot provide a definitive explanation, the physiological states of the frogs used in the two experiments were markedly different. Besides having comparatively higher urea levels, frogs subjected to experimental dehydration undoubtedly had higher ion concentrations that, in principle, could have caused excessive stabilization of CPS I (Hochachka and Somero, 2002) and thereby reduced enzyme activity. Although this effect might explain our findings for saline-treated frogs, salt stabilization was not expressed in urea-treated frogs, which instead maintained levels of CPS I activity during dehydration. Speculation suggests that the much higher uremia in these frogs could account for the disparity, as elevated urea is known to counteract such salt inhibition (Dotsch et al., '95; Cerasoli et al., 2003; Pervushin et al., 2004). Similarly, urea treatment reduces salt toxicity and inhibition of enzyme activity in the feathered mosquitofern, *Azolla pinnata* (Mishra and Singh, 2006). In *R. sylvatica*, although the precise mechanism of protection was not discerned, urea pretreatment apparently preserved CPS I activity during dehydration. Perhaps urea accumulating in amphibians during osmotic stress serves not only to replace inorganic ions as osmolytes, but also to counteract the perturbing effects of salts on proteins.

Experimental dehydration of *R. sylvatica* failed to increase CPS I activity, such as that occurring during osmotic stress in some urea-accumulating species. However, we must consider that osmotic responses can be influenced by acclimatization and temperature (Jørgensen, '97) and, contrary to the case with many investigations, our findings pertain to cold-acclimated frogs. Upregulating urea production may be unnecessary, assuming that rates of water loss in hibernating frogs are lower than those experienced by frogs moving from fresh to saline water or from water to land. Moreover, because the maximal capacity of the urea cycle exceeds the usual demands for urea synthesis, the urea synthesis rate in dehydrating frogs could be accelerated by increasing substrate availability, even as CPS I activity remains constant (Weng et al., 2004; Lee et al., 2006). This process possibly contributed to urea accumulation in *R. sylvatica* in our dehydration experiment.

In conclusion, our findings suggest that urea production capacity is maintained at a high level in *R. sylvatica* during hibernation, because the advantages of sustaining urea-cycle activity during winter apparently outweigh the costs. The ability to accumulate urea is important not only because this osmolyte is functioning as an osmoprotectant, but also because urea assuages freezing injury (Costanzo and Lee, 2005; Costanzo et al., 2008) and contributes to energy conservation (Muir et al., 2007, 2008). Additional research is needed to determine the precise mechanism(s) of CPS I regulation and also to elucidate how urea and other solutes influence urea-cycle activity in this species.

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LITERATURE CITED

Anderson P. 1981. Purification and properties of the glutamine-dependent and *N*-acetyl-L-glutamate-dependent carbamoyl phosphate synthetase from liver of *Squalus acanthias*. J Biol Chem 256:2228–2238.

Balinsky JB. 1981. Adaptation of nitrogen metabolism to hyperosmotic environment in Amphibia. J Exp Zool 215:335–350.

Cerasoli E, Kelly S, Coggins J, Lapthorn A, Clarke D, Price N. 2003. Effects of salts on the function and conformational stability of shikimate kinase. Biochim Biophys Acta—Proteins Proteomics 1648:43–54.

Chew MM, Elliot AB, Wong HY. 1972. Permeability of urinary bladder of *Rana cancrivora* to urea in the presence of oxytocin. J Physiol 223:757–772.

Chew SF, Ong TF, Ho L, Tam WL, Loong AM, Hiong KC, Wong WP, Ip YK. 2003. Urea synthesis in the African lungfish *Protopterus dolloi*—hepatic carbamoyl phosphate synthetase III and glutamaine synthetase are upregulated by 6 days of aerial exposure. J Exp Biol 206:3615–3624.

Corvi M, Soltys C, Berthiaume L. 2001. Regulation of mitochondrial carbamoyl-phosphate synthetase 1 activity by active site fatty acylation. J Biol Chem 276:45704–45712.

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

Costanzo JP, Marjanovic M, Fincel EA, Lee RE. 2008. Urea loading enhances postfreeze performance of frog skeletal muscle. J Comp Physiol B 178:413–420.

Dotsch V, Wider G, Siegal G, Wuhrich K. 1995. Salt-stabilized globular protein-structure in 7-M aqueous urea solution. FEBS Lett 372:288–290.

Gordon MS, Schmidt-Nielsen K, Kelly HM. 1961. Osmotic regulation in the crab-eating frog (*Rana cancrivora*). J Exp Biol 38:659–678.

Grundy JE, Storey KB. 1994. Urea and salt effects on enzymes from estivating and non-estivating amphibians. Mol Cell Biochem 131:9–17.

Hochachka PW, Somero GN. 2002. Biochemical adaptation: mechanism and process in physiological evolution. New York, NY: Oxford University Press.

Ip YK, Tam WL, Wong WP, Chew SF. 2005. Marine (*Taeniura lymma*) and freshwater (*Himantura signifer*) elasmobranchs synthesize urea for osmotic water retention. Physiol Biochem Zool 78:610–619.

Janssens PA, Cohen PP. 1968. Biosynthesis of urea in the estivating African lungfish and in *Xenopus laevis* under conditions of water-shortage. Comp Biochem Physiol 24:887–898.

Jørgensen C. 1997. Urea and amphibian water economy. Comp Biochem Physiol A Physiol 117:161–170.

Kajimura M, Walsh P, Mommsen T, Wood C. 2006. The dogfish shark (*Squalus acanthias*) increases both hepatic and extrahepatic ornithine urea cycle enzyme activities for nitrogen conservation after feeding. Physiol Biochem Zool 79:602–613.

Layne JR, Costanzo JP, Lee RE. 1998. Freeze duration influences postfreeze survival in the frog *Rana sylvatica*. J Exp Zool 280:197–201.

Lee S, Wong WP, Hiong K, Loong A, Chew SF, Ip YK. 2006. Nitrogen metabolism and excretion in the aquatic Chinese soft-shelled turtle, *Pelodiscus sinensis*, exposed to a progressive increase in ambient salinity. J Exp Zool 305A: 995–1009.

Lindley TE, Laberge T, Hall A, Hewett-Emmett D, Walsh PJ, Anderson PM. 2007. Sequence, expression and evolutionary relationships of carbamoyl phosphate synthetase I in the toad *Xenopus laevis*. J Exp Zool 307A:163–175.

McBean RL, Goldstein L. 1970. Accelerated synthesis of urea in *Xenopus laevis* during osmotic stress. Am J Physiol 219:1124–1130.

McClanahan L. 1972. Changes in body fluids of burrowed spadefoot toads as a function of soil water potential. Copeia 1972:209–216.

Mishra A, Singh S. 2006. Protection against salt toxicity in *Azolla pinnata Anabaena azollae* symbiotic association by using combined-N sources. Acta Biol Hung 57: 355–365.

Morris SM. 2002. Regulation of enzymes of the urea cycle and arginine metabolism. Annu Rev Nutr 22:87–105.

Muir TJ, Costanzo JP, Lee RE. 2007. Osmotic and metabolic responses to dehydration and urea-loading in a dormant, terrestrially hibernating frog. J Comp Physiol B 177: 917–926.

Muir TJ, Costanzo JP, Lee RE. 2008. Metabolic depression induced by urea in organs of the wood frog, *Rana sylvatica*: effects of season and temperature. J Exp Zool 309A: 111–116.

Nielsen S, Gofte T, Gronbaek H, Tygstrup N, Vilstrup H. 2007. Opposite effects on regulation of urea synthesis by early and late uraemia in rats. Clin Nutr 26:245–251.

Pervushin K, Wider G, Iwai H, Wuthrich K. 2004. NMR structures of salt-refolded forms of the 434-repressor DNA-binding domain in 6 M urea. Biochemistry 43: 13937–13943.

Pierson DL. 1980. A rapid colorimetric assay for carbamoylphosphate synthetase-I. J Biochem Biophys Methods 3:31–37.

Pinder AW, Storey KB, Ultsch GR. 1992. Estivation and hibernation. In: Feder ME, Burggren WW, editors. Environmental physiology of the amphibians. Chicago, IL: The University of Chicago Press. p 250–274.

Regosin JV, Windmiller BS, Reed JM. 2003. Terrestrial habitat use and winter densities of the wood frog (*Rana sylvatica*). J Herpetol 37:390–394.

Robertson JD. 1989. Osmotic constituents of the blood plasma and parietal muscle of *Scyliorhinus canicula* (L.). Comp Biochem Physiol 93A:799–805.

Saha N, Datta S, Kharbuli Z, Biswas K, Bhattacharjee A. 2007. Air-breathing catfish, *Clarias batrachus* upregulates glutamine synthetase and carbamoyl phosphate synthetase III during exposure to high external ammonia. Comp Biochem Physiol B Biochem Mol Biol 147:520–530.

Shpun S, Hoffman J, Katz U. 1992. Anuran amphibia which are not acclimable to high salt, tolerate high plasma urea. Comp Biochem Physiol A Physiol 103:473–477.

Storey KB. 2002. Life in the slow lane: molecular mechanisms of estivation. Comp Biochem Physiol A Physiol 133: 733–754.

Tattersall GJ, Ultsch GR. 2008. Physiological ecology of aquatic overwintering in ranid frogs. Biol Rev 83:119–140.

Weng L, Wong W, Chew S, Ip Y. 2004. Excretory nitrogen metabolism in the Chinese fire-belly newt *Cynops orientalis* in water, on land, or in high concentrations of environmental ammonia. J Comp Physiol B 174:113–120.

Withers PC, Guppy M. 1996. Do Australian desert frogs co-accumulate counteracting solutes with urea during aestivation? J Exp Biol 199:1809–1816.

Wray S, Wilkie DR. 1995. The relationship between plasma urea levels and some muscle trimethylamine levels in *Xenopus laevis*: a ^{31}P and ^{14}N nuclear magnetic resonance study. J Exp Biol 198:373–378.

Wright P, Anderson P, Weng L, Frick N, Wong W, Ip Y. 2004. The crab-eating frog, *Rana cancrivora*, up-regulates hepatic carbamoyl phosphate synthetase I activity and tissue osmolyte levels in response to increased salinity. J Exp Zool 301A:559–568.

Yancey PH. 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol 208:2819–2830.

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. 1982. Living with water stress: evolution of osmolyte systems. Science 217:1214–1222.