ABSTRACT

UREA PRODUCTION CAPACITY IN THE WOOD FROG (*RANA SYLVATICA*) VARIES WITH SEASON AND EXPERIMENTALLY INDUCED HYPERUREMIA

by Tamar Marie Schiller

Wood frogs (*Rana sylvatica*) can accumulate substantial amounts of urea during the winter. In this study maximal urea production capacity is examined in wood frogs collected at various times of the year and in response to experimental hyperuremia and dehydration. Activity and expression of carbamoyl phosphate synthetase I (CPSI), the regulatory enzyme of the urea cycle, are used as indicators of urea production capacity in the wood frog. CPSI activity and expression did change seasonally, though it did not increase in winter. Hyperuremia decreased CPSI activity in hydrated frogs but maintained activity in dehydrated frogs. Changes in CPSI activity were not reflected by similar changes in CPSI quantity suggesting CPSI activity in the wood frog is not primarily being regulated through transcription and translation. Maintenance of urea production capacity in hibernating *R. sylvatica* probably facilitates accumulation of this osmolyte, which has important roles in the winter biology of this species.

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INTRODUCTION

Anurans are especially prone to dehydration stress as their skin is relatively permeable and by itself is an insufficient barrier to prevent water loss. As such these animals must rely on other means to limit desiccation. Urea accumulation is one such mechanism used by numerous species, amphibian as well as non-amphibian, to maintain water balance in dehydrating conditions (Gordon et al., '61; McBean and Goldstein, '70; McClanahan, '72; Ip et al., 2005). As an organic osmolyte urea can replace some of the ionic solutes that might otherwise concentrate and become detrimental to cells during osmotic stress (Grundy and Storey, '94).

There are two known mechanisms by which an organism may accumulate urea. One is simply to retain urea rather than excrete it (Balinsky, '81). Many organisms that use this strategy reduce urine production resulting in the accrual of urea in the body. Anurans can potentially reabsorb urea across the walls of the urinary bladder as well (Chew, '72). Another manner in which urea is accumulated, and which may be used in addition to retention, is to increase urea synthesis through modulation of urea cycle activity (Balinsky, '81).

In terrestrial vertebrates the ornithine-urea cycle in the liver is responsible for the conversion of ammonium ions into urea. Much of the current understanding of urea cycle regulation comes from mammalian studies. 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 and rate limiting enzyme of the urea cycle (Morris, 2002). CPS I requires N-acetyl-L-glutamate in order to function, but it is unclear whether 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. However long-term regulation in response to changing productivity demands is thought to occur primarily by controlling the quantity of CPS I and other urea cycle enzymes through transcription and/or translation (Morris, 2002). Few other mechanisms of the urea cycle regulation have been identified, though acylation of the active site of rat CPS I irreversibly inhibits its activity (Corvi et al., 2001). Because of its regulatory role many researchers have focused on changes in CPS activity to indicate possible changes in urea production capacity (McBean and Goldstein, '70; Chew et al., 2003; Wright et al., 2004). Changes in CPS activity are known in several but not all urea-accumulating species (Lee et al., 2006; Weng et al., 2004).

The wood frog (*Rana sylvatica*) has a range which extends from the southeastern United States into Canada and Alaska. In late 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 at a seasonal low potentially creating an osmotic challenge for hibernating frogs. In addition to soil moisture already being low, the frogs may face increasingly dehydrating conditions if the soil water freezes, rendering it biologically unavailable to them. Winter-caught wood frogs can have plasma urea levels up to 50 mmol l⁻¹ and laboratory animals can accumulate up to 90 mmol l⁻¹ suggesting that they may be using urea to maintain osmotic balance (Costanzo and Lee, 2005). This same research also showed that urea could function as a cryoprotectant in wood frogs as it reduces freeze damage in their tissues. Furthermore, urea could serve as a metabolic depressant during hibernation due to the perturbing affect it has on proteins (Costanzo and Lee, 2005; Muir et al., 2007). While information concerning the roles of urea in this amphibian continues to grow, it is not yet known whether the elevated urea levels seen in winter wood frogs are due to decreased excretion alone or also involve high urea synthesis rates.

The aim of this study was to test the hypothesis that urea synthesis capacity in *R. sylvatica* varies seasonally in order that hibernating frogs accumulate urea, which appears to play important roles in the winter biology of this species. To test this idea, wood frogs were collected at various times of the year and analyzed for the activity and relative quantity of CPS I. In laboratory experiments, regulation of urea production capacity was examined by analyzing CPS I activity and quantity in wood frogs experimentally dehydrated and/or rendered hyperuremic.

MATERIALS AND METHODS

Animal collection and maintenance

Male wood frogs (*Rana sylvatica*) were collected from a vernal pool in February 2004 and 2006 in Adams County, Ohio. Frogs from the 2004 collection were placed in an outdoor summer enclosure at the Ecological Research Center (ERC), Miami University, where they were exposed to changing environmental conditions. During spring and summer they were fed crickets three times a week and had constant access to water. In October 2004 feeding was suspended. At this time four frogs were collected and transported to the laboratory where they were immediately euthanized and their tissues collected (see below). The remaining frogs were

transferred to a separate outdoor enclosure (winter enclosure) at the ERC. This lot contained sparse shrub cover and leaf litter from nearby trees, which provided natural conditions for the frogs to hibernate. Frogs were recaptured in January, February, and March 2005, transported to the laboratory, and euthanized for tissue collection.

Frogs from the 2006 collection were placed in covered, opaque boxes containing damp moss and kept at 4°C in darkness until experimentally treated in the laboratory within six weeks of capture. The remaining untreated frogs were transferred to the summer enclosure at the ERC where they received the same feeding schedule as described above. Over the course of a week in August 2006 five frogs were caught from the summer enclosure and sampled for liver tissue. 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

Prior to experimental treatment or euthanization the animals' bladders were drained by gently inserting a polished glass cannula in the cloaca. The frogs were weighed and, if experimentally treated, were randomly assigned to treatment groups. After treatment, frogs were euthanized by double pithing and were dissected. Liver samples were immediately frozen in liquid nitrogen and stored at -80°C until used for CPS I activity and western blot assays.

Seasonal CPS I activity and expression

To test whether frogs modulate CPS I seasonally, frogs were collected on October 1 in 2004 (fall), and on January 31 (winter), February 28 (late winter), and March 16 (spring) in 2005 ($N=4$) from the ERC where they were exposed to naturally changing environmental conditions throughout the seasons. Measurements from summer frogs ($N=5$) gathered from the summer ERC enclosure in August 2006 were also included in the comparison. All frogs were euthanized for tissue collection without further treatment. Portions of the livers were used for CPS I activity and western blot analysis.

Effect of hyperuremia on CPS I activity and expression

The effects of hyperuremia on CPS I in fully-hydrated wood frogs was tested by injecting cold-acclimated frogs with either phosphate buffered saline (PBS; in g/l: 6.10 NaCl, 0.15 KCl, 0.88 Na₂HPO₄, 0.15 KH₂PO₄; pH 7.4) (saline-injected, $N=5$) or PBS containing 1.5 mol l⁻¹ urea (urea-injected, $N=5$) in volumes equaling 3.3% of the standard body mass. The frogs were returned to their boxes at 4°C in the dark for 24 h before double pithing and dissection. Blood

was collected from the aorta in a capillary tube and centrifuged. The plasma was stored at -80°C until analyzed for urea using a colorimetric BUN kit (Pointe Scientific, Canton, MI, USA).

Liver samples were used in CPS I activity assays and western blot analysis.

Effects of dehydration and hyperuremia on CPS I activity and expression

To see if dehydration stimulates urea cycle activity and if urea inhibits that stimulation, cold-acclimated frogs were injected in the dorsal lymph pad with either PBS ($N=5$) or PBS with 1.5 mol l⁻¹ urea ($N=5$) in volumes equaling 3.3% of standard body mass. These frogs were kept in plastic tubes (which served as metabolic chambers in a separate study; Muir et al., 2007) at 4°C in the dark for 6 d after which they received a second injection of PBS or PBS with 1.5 mol l⁻¹ urea. The frogs were dehydrated by passing 4°C air through the chambers at a low rate of flow for 14 h every other day over the course of 23 d. On the final day of the experiment frogs were weighed and then euthanized. Blood and liver samples were collected as described above. Plasma was assayed for urea concentration. Body water content expressed as percentage of fresh mass was determined by the change in carcass mass after drying. Liver samples were used for CPS I activity assays and western blot analysis.

Carbamoyl phosphate synthetase I activity assay

Enzyme samples for the CPS I activity assay were prepared by the following procedure. Liver samples were thawed on ice and gently blotted on laboratory tissue. A portion of each sample was weighed and 0.5 ml of homogenization buffer (50 mmol l⁻¹ triethanolamine HCl, 15 mmol l⁻¹ magnesium acetate, 1 mmol l⁻¹ dithiothreitol, 10 mmol l⁻¹ ATP) was added per 50 mg of tissue before homogenization on ice using a rotating blade homogenizer (Tissue Tearor, Biospec, Bartlesville, OK, USA). The homogenates were centrifuged at 14,000g at 4°C for 15 min. The supernatants were passed through a Sephadex G25 column (20 cm high x 0.7 cm diameter) equilibrated with homogenization buffer. Portions of the supernatants prior to column treatment as well as the filtrate remaining after use were frozen in liquid nitrogen and stored at -80°C for protein and hemoglobin analysis (see below).

Two tubes were prepared for each enzyme sample. A 10-μl aliquot of the enzymatically active column filtrate was added to each tube along with 165 μl of homogenization buffer. One tube from each sample was placed in a 95°C water bath for 5 min to inactivate the enzyme while the other tubes were left on ice. Reagent blanks were prepared by substituting an equal volume of homogenization buffer for filtrate. The rest of the assay was carried out as described by

Pierson ('80) except that initial enzyme incubations were conducted at room temperature. Prior to reading sample absorbances on the spectrophotometer, tubes were centrifuged at 14,000g (4°C, 6 min) to remove precipitated proteins. Absorbances were read at 458 nm zeroed on the reagent blanks.

Absorbances of inactivated solutions were subtracted from those obtained for their matching active solutions before calculating the amount of carbamoyl phosphate produced in order to account for possible color formation due to other components of the homogenate. Urea also produces a yellow color with this assay. However, preliminary studies showed that the Sephadex G25 column effectively removed high levels ($>150 \text{ mmol l}^{-1}$) of urea from samples so urea should not have contributed to color formation (data not shown). The supernatants and column filtrates, which had been frozen, were later analyzed for protein concentration using an assay kit (Bradford Protein Assay, Bio-Rad, Hercules, CA, USA). Enzyme activity was expressed as nmol carbamoyl phosphate produced $\text{h}^{-1} \text{ mg}^{-1}$ soluble protein. CPS I activity was not reported in μmol carbamoyl phosphate produced $\text{h}^{-1} \text{ g}^{-1}$ wet tissue as in some other studies since the hydration states of the tissues along with the amount of stored glycogen and lipids could vary among groups.

Western blot analysis

Remaining liver samples were homogenized as before 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) for 45 s followed by sonication using four 10 s pulses. Homogenized samples were snap frozen in liquid nitrogen and stored at -80°C. After samples were thawed and centrifuged at 7,000g (4°C, 5 min) protein concentrations in the supernatants were measured as before. Protein (1, 2, and in some cases 4 μg) incubated with Lammelli buffer (Bio-Rad) was loaded into 4-15% gradient TrisHCl Ready Gels (Bio-Rad). 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) for 90 min at 80 V. Ponceau S staining was used to confirm that protein was transferred properly and that protein was equally loaded. After 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% non-fat dry milk. The membranes were warmed to room temperature and incubated with rabbit anti-CPS I primary antibody (Abcam, Cambridge, MA, USA) diluted 1:4,000 in wash buffer containing 5% milk for 1 h followed by four 10 min washes with wash buffer.

Membranes were then incubated with goat anti-rabbit secondary antibody (Sigma, St. Louis, MO, USA) diluted 1:1,000 in wash buffer containing 5% milk for 1 h 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, USA). 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 replicate blots were averaged to obtain one value per animal for statistical analysis.

Statistical treatment of data

All values are reported as means $\pm$ SEM. Results were square-root transformed prior to analysis. Group means were compared with Student's *t*-test or ANOVA followed by Fisher's PLSD. Results were considered statistically significant if $P \leq 0.05$.

RESULTS

CPS I activity was present in all samples tested. Inactivated enzyme solutions had extremely little color development over reagent blanks. Approximately 1-3 μ g of protein was present in the 10 μ l aliquot used in the enzyme assay. Preliminary tests charting the amount of protein added vs. measured absorbance showed that more than 7 μ g of protein was still within the linear range, so substrate and cofactor availability did not limit enzyme activities in our assays (data not shown).

Preliminary results showed that the primary anti-CPS I antibody bound equally as well to wood frog CPS I as it does to rat CPS I against which the antibody had been raised. Western blots revealed a single band that occurred above the 150 kDa molecular weight marker where CPS I (165 kDa) is expected to be found.

Seasonal responses of CPS I

CPS I activity varied seasonally (Table 1; $P=0.01$). Summer activities were similar to fall and winter. However, late winter and spring CPS I activities declined by about 35-39% compared to summer. There were no significant variations ($P=0.20$) in the soluble protein concentration among any of the groups (Table 1).

Quantities of CPS I varied among frogs collected at different times of the year ($P=0.002$). Summer frogs had about 1.8 times more CPS I than other seasons, but no other differences among groups were apparent (Fig. 1).

Effect of hyperuremia on CPS I

Hyperuremia was associated with a decrease CPS I activity but not quantity in wood frog livers. At the time of euthanization the urea-injected group exhibited a mean blood urea concentration of $69.6 \pm 2.5 \text{ mmol l}^{-1}$ versus $9.8 \pm 0.8 \text{ mmol l}^{-1}$ for the saline-injected frogs. The saline-injected group showed a significant difference ($P=0.01$) in CPS I activity compared to the urea-injected group (2065 ± 172 vs. $1209 \pm 212 \text{ nmol h}^{-1} \text{ mg}^{-1}$ soluble protein, respectively). The change in activity was not reflected by a significant change ($P=0.99$) in CPS I quantity on western blots (Fig. 2). There was no difference ($P=0.16$) in total soluble protein concentration between urea-injected and saline-injected groups (4.7 ± 0.4 vs. $4.4 \pm 0.4 \text{ mg protein/100 mg wet tissue}$, respectively).

Effects of dehydration and hyperuremia on CPS I

At the termination of the 23-d dehydration experiment saline-injected frogs had accrued a mean blood urea concentration of $38.3 \pm 1.8 \text{ mmol l}^{-1}$, while urea-injected frogs had a mean value of $161.5 \pm 7.6 \text{ mmol l}^{-1}$. The mean body water content in saline-injected frogs decreased from $78.5 \pm 0.2\%$ to $71.5 \pm 0.4\%$. Urea-injected frogs had body water contents that decreased from $78.3 \pm 0.6\%$ to $69.3 \pm 1.0\%$. There was not a significant difference in body water content between the two groups at the end of the experiment ($P=0.08$). There also was not a significant difference ($P=0.61$) in total soluble protein concentration between urea-injected and saline-injected groups (6.1 ± 0.2 vs. $5.3 \pm 0.5 \text{ mg soluble protein/100 mg wet tissue}$, respectively).

The CPS I activities of saline-injected and urea-injected frogs differed ($P=0.05$). The urea treatment had 1.8-fold more activity than the saline group (2032 ± 386 vs. $1126 \pm 97 \text{ nmol carbamoyl phosphate h}^{-1} \text{ mg}^{-1}$ protein, respectively). Western blots did not reveal a difference ($P=0.76$) in CPS I quantity between the two treatment groups (Fig. 3).

TABLE 1. CPS I activities and soluble protein levels in livers of R. sylvatica collected at various times of the year from outdoor enclosures

Collection Time	CPSI Activity (nmol carbamoyl phosphate h ⁻¹ mg ⁻¹ soluble protein)	Soluble Protein (mg soluble protein per 100 mg wet tissue)
Summer	2384 ± 360 ^a	3.42 ± 0.16 ^a
Late Fall	2137 ± 209 ^{ab}	3.39 ± 0.14 ^a
Winter	2179 ± 124 ^{ab}	3.53 ± 0.53 ^a
Late Winter	1462 ± 210 ^c	3.67 ± 0.08 ^a
Early Spring	1551 ± 0.24 ^{bc}	4.30 ± 0.24 ^a

Values are means ± SEM ($N=4$ for all collection times except summer where $N=5$). Within a column means not sharing the same letter are statistically different (ANOVA/Fisher's PLSD: $P \leq 0.05$).

Fig. 1. Western blot detection of CPS I in livers of *R. sylvatica* collected at various times of the year showing representative blots and densitometric analyses of CPS I expression. Summer frogs were run on separate blots with fall frogs. Densitometric results for summer frogs were normalized to other seasons prior to statistical analysis using the values for fall frogs. Bars represent means $\pm$ SEM ($N=4$). Groups not sharing the same letter are significantly different (ANOVA/Fisher's PLSD: $P \leq 0.05$).

Fig. 2. Western blot detection of CPS I in livers of hydrated *R. sylvatica* injected with either PBS (saline) or with PBS containing 1.5 mol l^{-1} urea (urea) showing a representative blot and densitometric analyses of CPS I expression. Bars represent means $\pm$ SEM ($N=5$). The two groups were not statistically different (Student's *t*-test: $P=0.99$).

Fig. 3. Western blot detection of CPS I in livers of *R. sylvatica* injected with either PBS (saline) or PBS containing 1.5 mol l⁻¹ urea (urea) and dehydrated for 23 d showing a representative blot and densitometric analyses of CPS I expression. Bars represent means $\pm$ SEM ($N=5$). The two groups were not statistically different (Student's *t*-test: $P=0.76$).

DISCUSSION

Under the conditions in this study wood frogs collected during the fall and winter did not significantly alter CPS I activity when compared to summer. Retention of urea along with preservation of urea production rates may be sufficient to cause the accumulation of urea as seen previously in field and laboratory experiments (Costanzo and Lee, 2005). Alternatively, since the maximal capacity of the urea cycle exceeds regular demands for urea synthesis, it is possible that the rate of synthesis could still be accelerated, if necessary, by increasing the quantity of available substrates while the maximal CPS I activity remains constant (Weng et al., 2004; Lee et al., 2006). However, the fact that CPS I activity was maintained through the fall and majority of the winter may still imply an essential role for urea synthesis in the wood frog during hibernation. Many metabolic processes in ectotherms are downregulated in winter to conserve energy, when the animals must rely solely on endogenous energy reserves. Urea synthesis is an energetically expensive process utilizing 4 mol of ATP to make 1 mol of urea. As such, one might expect that urea cycle activity would decrease during dormancy. The fact that CPS I activity was not significantly reduced until late winter could be because of the continued importance of urea for winter survival.

Why summer frogs had greater amounts of CPS I protein than at other collection times but not significantly higher CPS I activities than in fall and winter is unknown. Perhaps the quantity of CPS I is upregulated during the summer to be able to respond readily to greater metabolic and feeding activities. Being sit and wait predators, the frogs may have sporadic intervals where large amounts of dietary protein are catabolized followed by periods when only basal levels of protein degradation and nitrogenous waste production occur. Regulation of CPS I activity may be further modulated at the protein level during these different periods. If CPS I activity was reduced during times when summer frogs are not assimilating dietary protein, this could account for CPS I activity remaining only slightly (though not statistically) higher than during other months. The dogfish shark (*Squalus acanthias*), another species exhibiting alternating periods of protein metabolism, increases activities of urea cycle enzymes upon feeding in order to conserve nitrogen (Kajimura et al., 2006). Only enzyme activity data were available in that study so how enzyme activities were modulated in the dogfish is not known. In summer wood frogs it is likely that increased ammonia levels from feeding would be a major regulatory signal to control CPS I activity.

Current understanding of the urea cycle suggests that CPS I activity is regulated primarily through transcription and translation (Morris, 2002). Our study did not find corresponding changes in CPS I quantity when there were changes in its activity. This suggests that there could be controls on wood frog CPS I at the protein level rather than solely at transcription and translation levels. While many studies have found changes in urea production capacity in response to various stresses, very few studies have addressed the regulatory mechanisms of urea cycle activity in non-mammalian vertebrates. One

study of *Xenopus laevis* found that CPS I activity increased two-fold concurrent with a six-fold increase of mRNA (Lindley, 2007). Another found that in response to hyper-ammonia stress the air-breathing catfish (*Clarias batrachus*) increased CPS III (a glutamate-dependent CPS) activity by two-fold; this rise was reflected by a 1.75-fold change in protein quantity (Saha et al., 2007). While this result indicates that transcriptional regulation is occurring, these authors suggested that other mechanisms such as phosphorylation or regulation by N-acetyl-L-glutamate may also control urea cycle activity. Anderson ('81) proposed that species using urea for purposes in addition to ammonia detoxification might be expected to regulate the urea cycle differently. Many times changes in urea cycle activity in mammalian systems are related to changes in protein ingestion or starvation (Morris, 2002). Mammals would not typically encounter large fluctuations in blood urea concentrations and do not tolerate dehydration to the extent as amphibians. Under the conditions used in this study, CPS I activity in the wood frog could be regulated through mechanisms such as feedback inhibition, phosphorylation or other allosteric modifiers. Since N-acetyl-L-glutamate was an added component of the activity assay mixture, differing cellular concentrations of this activator were not possible causes for the alteration in CPS I activity we observed. Reversible phosphorylation is a fast and convenient way of altering metabolic functions in estivating animals (Storey, 2002). Similar strategies may be used in the wood frog during hibernation or stressful conditions to regulate CPS I activity but allow for quick reversal when conditions become favorable. Further studies are needed to determine the exact way CPS I activity is regulated in the wood frog.

Our data suggest that acute hyperuremia has an inhibitory effect on CPS I activity in hydrated wood frogs. In fully-hydrated frogs it is likely that urea production mainly serves to detoxify ammonia. Under these conditions hyperuremia is probably not advantageous and urea production may be temporarily downregulated to avoid accumulating excess urea. Rats made experimentally hyperuremic (10 mmol l⁻¹ urea vs. 4 mmol l⁻¹ for the control) from partial nephrectomy showed a short-term decrease in urea synthesis as well as decreased levels of mRNA for all urea cycle enzymes except ornithine transcarbamoylase (Nielsen et al., 2007). Because there was no difference in CPS I quantity in our experiments it would seem that urea is not triggering a regulatory pathway involved with CPS I transcription and translation in the wood frog. However, urea could be activating a separate control pathway or directly acting upon CPS I to reduce its activity. Urea is well known for its ability to perturb proteins. Elasmobranchs, which accumulate on the order of 400 mmol l⁻¹ urea in their tissues, also accumulate methylamines in a specific ratio to urea in order to counteract disturbances to proteins (Yancey et al., '82; Robertson, '89). Activity of purified CPS III from the spiny dogfish (*S. acanthis*) is directly inhibited by urea at concentrations physiologically relevant to this species when methylamines are not in the appropriate concentrations (Anderson, '81). However, co-accumulation of methylamines with urea has not been observed in amphibians (Yancey, 2005). Generally, effects on enzyme activities

are seen at urea concentrations of $>100 \text{ mmol l}^{-1}$; however, some proteins become perturbed at concentrations as low as 25 mmol l^{-1} (Yancey, '94). As urea-injected wood frogs had approximately 70 mmol l^{-1} blood urea, which is 8-20 fold greater than concentrations found in hydrated frogs, it is possible that urea could be acting as a feedback inhibitor by perturbing enzyme function.

CPS I activity (approximately $1130\text{-}2380 \text{ nmol carbamoyl phosphate h}^{-1} \text{ mg}^{-1} \text{ protein}$) in *R. sylvatica* was somewhat similar to that reported for the crab-eating frog (*Rana cancrivora*), which had activities from $1602\text{-}2358 \text{ nmol carbamoyl phosphate h}^{-1} \text{ mg}^{-1} \text{ protein}$ (Wright et al., 2004). *Xenopus laevis* has CPS I activities of about $900\text{-}1860 \text{ nmol carbamoyl phosphate h}^{-1} \text{ mg}^{-1} \text{ protein}$ (Lindley et al., 2007). It should be noted that the lower values for both *R. cancrivora* and *X. laevis* represent CPS I activities from animals held in tap water while the upper values are from animals under osmotic stress in saline water. Interestingly, in the wood frog most of the untreated, hydrated groups had activities around $2000 \text{ nmol carbamoyl phosphate h}^{-1} \text{ mg}^{-1} \text{ protein}$ which is roughly the same as is found in these other two amphibians when they already upregulated urea production. It may be that *R. sylvatica* already has a relatively high urea production capacity due to its terrestrial nature, and CPS I activities need not be elevated during the winter or in response to dehydration to produce urea at higher rates. *Xenopus laevis* is primarily aquatic and facultatively switches from ammoniotelism to ureotelism when osmotically challenged. As such one would expect CPS I levels of *X. laevis* in tap water to be lower than a terrestrial amphibian such as the wood frog. The crab-eating frog may hunt in brackish or sea waters but may also spend time outside of water, which would explain why its CPS I activities are higher than *X. laevis* and more comparable to *R. sylvatica* (Jorgensen, '97).

In some species dehydration is a stimulator of urea cycle activity (Janssens and Cohen '68; Wright et al., 2004). In the wood frog dehydration did not raise urea production capacity. Rather, dehydration decreased CPS I activity of the saline-injected group while activity was maintained in the urea-injected group compared to hydrated frogs. This is somewhat puzzling as it is the exact opposite situation of when frogs were hydrated and received saline and urea injections. The decrease in saline-injected dehydrated frogs could be caused by an overall reduction in metabolism (Muir et al., 2007). It could also be that salt concentrated during dehydration caused excessive stabilization of CPS I, resulting in reduced activity (Hochachka and Somero, 2002).

In the urea-injected dehydration treatment the destabilizing effects of urea may counteract any salt effects on CPS I activity. Several examples of urea and salt counteraction exist. Often in protein folding studies investigators denature proteins with urea and then refold the proteins by adding salts (Dotsch et al., '95; Cerasoli et al., 2003; Pervushin et al., 2004). Urea treatment reduced the effects of salt toxicity and inhibition of enzyme activity in the plant, *Azolla pinnata* (Mishra and Singh, 2006). Other studies showed pretreatment of rat renal medullary cells with urea protected the cells against salt toxicity

and vice versa (Tian and Cohen, 2001), though the proposed means of protection were not by direct counteraction of the salt and urea perturbations of proteins. In one study of renal medullary cells it was suggested that pretreatment with salts induces heat shock proteins which could protect against urea stress (Neuhofer et al., '98). In another study the protective effects of urea pretreatment in renal cells were attributed to inhibition of salt induced apoptosis (Zhang et al., 2000). In our experiments, while the exact method of protection is not known, urea pretreatment preserved CPS I activity during dehydration that otherwise did not occur in the saline-injected group. Urea might afford two means of protection during dehydration: replacing salts as osmolytes in urea accumulators as well as counteracting the perturbing effects of salts on proteins.

Another potential explanation is that urea recycling may have caused CPS I activity to be maintained in the urea-injected/dehydrated group. In some mammals about 20% of blood urea is hydrolyzed to ammonia by bacteria residing in the gut which is then reabsorbed and converted again to urea (Fuller and Reeds, '98). Increased ammonia levels from injected urea could possibly serve as a stimulus to maintain urea cycle activity in wood frogs. However, this scenario requires that ammonia be a strong enough stimulus to override other conditions that caused activity to decrease in the saline-injected/dehydrated group as well as any inhibitory effects that urea may have. It is unknown whether amphibians undergo urea cycling. The large intestinal flora of the leopard frog (*Rana pipiens*) was reported to be similar to those of mammals and birds which might make it plausible for this process to occur in amphibians (Gossling et al., '82). The bacterial populations in the leopard frog were maintained at low temperatures. Even assuming that wood frogs harbor bacteria with urease activity, the rate at which urea is hydrolyzed at 4°C may be insufficient to have a significant stimulatory effect on the urea cycle.

While other processes are depressed in hibernating wood frogs, it appears that urea production is an important function to maintain. Since urea can lessen freeze damage and may aid in reducing metabolism in the wood frog (Costanzo and Lee, 2005; Muir et al., 2007), the advantages of sustaining urea cycle activity when the frog is susceptible to freezing and is surviving on endogenous energy reserves may outweigh the costs. Experimental dehydration did not stimulate urea cycle activity as it does in some urea-accumulating species. The rate of water loss that the wood frog experiences with the onset of cold, dry weather probably is not as rapid as other amphibians encounter when moving from fresh to saline waters or from water to land. As such, increased urea production capacity may not be necessary when dehydration is gradual. Responses to dehydration may also be influenced by temperature and seasonal acclimation. Our experiments used cold-acclimated spring frogs held at 4°C while similar dehydration studies of other species are typically conducted at higher temperatures. Retention along with sustained urea cycle activity are probably sufficient to account for elevated blood urea in winter *R.*

sylvatica (Costanzo and Lee, 2005). Our findings also indicate that transcription and translation do not appear to be the primary ways regulating CPS I in the wood frog in these conditions. Urea apparently depresses CPS I activity in hydrated wood frogs and may be involved in feedback mechanisms. Further research is needed to determine the exact ways CPS I is regulated in the wood frog as well as how urea influences urea cycle activity. It is likely that other species of ectotherms also exhibit means of modulating urea production that are different from traditional mammalian models.

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