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Adaptations of the reed frog *Hyperolius viridiflavus* (Amphibia, Anura, Hyperoliidae) to its arid environment

III. Aspects of nitrogen metabolism and osmoregulation in the reed frog, *Hyperolius viridiflavus taeniatus*, with special reference to the role of iridophores

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Summary. Reed frogs of the superspecies *Hyperolius viridiflavus* occur throughout the seasonally very dry and hot African savannas. Despite their small size (300–700 mg), estivating reed frogs do not avoid stressful conditions above ground by burrowing into the soil, but endure the inhospitable climate relatively unprotected, clinging to mostly dry grass stems. They must have efficient mechanisms to enable them to survive e.g. very high temperatures, low relative humidities, and high solar radiation loads. Mechanisms must also have developed to prevent poisoning by the nitrogenous wastes that inevitably result from protein and nucleotide turnover. In contrast to fossorial amphibians, estivating reed frogs do not become torpid. Reduction in metabolism is therefore rather limited so that nitrogenous wastes accumulate faster in these frogs than in fossorial amphibians. This severely aggravates the osmotic problems caused by dehydration. During dry periods total plasma osmolarity greatly increases, mainly due to urea accumulation. Of the total urea accumulated over 42 days of experimental water deprivation, 30% was produced during the first 7 days. In the next 7 days rise in plasma urea content was negligible. This strong initial increase of urea is seen as a byproduct of elevated amino acid catabolism following the onset of dry conditions. The rise in total plasma osmolarity due to urea accumulation, however, is not totally disadvantageous, but enables fast rehydration when water is available for very short periods only. Voiding of urine and feces ceases once evaporative water loss exceeds 10% of body weight. Therefore, during continuous water deprivation, nitrogenous end products are not excreted. After 42 days of water deprivation, bladder fluid was substantially depleted, and urea concentration in the remaining urine (up to 447 m*M*) was never greater than in plasma fluid. Feces voided at the end of the dry period after water uptake contained only small amounts of nitrogenous end products. DSF (dry season frogs) seemed not to be uricotelic. Instead, up to 35% of the total nitrogenous wastes produced over 42 days of water deprivation were deposited in an osmotically inert and nontoxic form in iridophore crystals. The increase in skin purine content averaged 150 µg/mg dry weight. If urea had been the only nitrogenous waste product during an estivation period of 42 days, lethal limits of total osmolarity (about 700 mOsm) would have been reached 10–14 days earlier. Thus iridophores are not only involved in colour change and in reducing heat load by radiation remission, but are also important in osmoregulation during dry periods. The selective advantages of deposition of guanine rather than uric acid are discussed.

Key words: Hyperolius viridiflavus – Estivation – Osmoregulation – Nitrogen metabolism – Iridophores

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In general, amphibians seem unable to cope xeric environments. Nevertheless, some anurans have successfully invaded semiarid and arid habitats with a prolonged and severe dry season. Most of these species escape the very demanding dry-season conditions by burrowing into the ground (e.g. Shoemaker et al. 1969; McClanahan 1972; Degani et al. 1984). Some species of the Rhacophoridae (*Chiromantis* spp.), Hylidae (*Phyllomedusa* spp.), and Hyperoliidae (*Hyperolius* spp.), in contrast, expose themselves relatively unprotected to the harsh dry season climate above ground (Loveridge 1970; Shoemaker et al. 1972; Loveridge 1976; Withers et al. 1982a).

Most unusual in this respect are species of the genus *Hyperolius*, especially members of the superspecies *Hyperolius viridiflavus* (Schiøtz 1971) which are common in many African savannas. At the beginning of the dry season most of these frogs are half grown with body lengths averaging only 1.7 cm and body weights within the range 300–700 mg. Because of their small size these frogs can store only small amounts of water and energy. Therefore, *H. viridiflavus* must have special adaptations in order to tolerate the dry season climate, especially high temperatures, low relative humidities, and heavy solar radiation loads. Such adaptations include avoiding overheating by solar radiation and preventing damage caused by UV radiation (Kobelt and Linsenmair 1986; Kobelt 1987), lowering evaporative water loss (EWL), and reducing energy consumption to survive with small amounts of stored reserves (Geise and Linsenmair 1986; Geise 1987). Voiding of urine and feces ceases once EWL has reached more than 10% of the body weight (Geise 1987), thus reducing water loss. This restriction in urine production, however, would allow ammonia nitrogen, continuously released from protein and nucleotide turnover, to accumulate. Because ammonia in high concentrations causes severe cell damage (Schmid 1968), it must be detoxified. Most terrestrial anurans store nitrogenous wastes as urea. In contrast to semi-terrestrial anurans, these species tolerate high urea concentrations (McClanahan 1967; Degani et al. 1984). Urea cannot be accumulated in

unlimited amounts because it is osmotically active and aggravates the osmotic problems already caused by desiccation.

In addition, for estivating *H. viridiflavus* energy reserves are also very limited. Fat reserves are the main energy source, but proteins may be important in regenerating carbohydrates (Jungreis 1976), and as an energy source after consumption of the fat pads. *Rana* sp. starved for long periods use proteins as their main energy source. After glucose injection their nitrogen output drops by two-thirds (Balinsky 1970). Protein catabolism results in a high output of nitrogen end products. If these were stored as urea, the maximum estivation period would be reduced considerably, because osmotic pressure would reach lethal levels before total depletion of water and energy reserves. The storage of nitrogenous wastes in a nontoxic and osmotically inert form, such as uric acid, would reduce osmotic problems.

In contrast to the larger tree frogs, *Chiromantis* and *Phyllomedusa*, excretion of uric acid has not so far been found in *Hyperolius* (Withers et al. 1982b; Geise and Linsenmair 1986). However, Kobelt and Linsenmair (1986) showed that after metamorphosis, and especially during the transition from wet to dry season conditions, the number of purine-crystal-containing iridophores in the skin of *Hyperolius viridiflavus* greatly increases. These iridophores play an important part in temperature regulation (Withers et al. 1982a; Kobelt and Linsenmair 1986; Kobelt 1987); but purine deposition could also play an essential role in dry-season osmoregulation by storing nitrogen end products in an osmotically inert and nontoxic form. The iridophores contain mainly the purines guanine and hypoxanthine, which provide a better N:C (see discussion) ratio than uric acid with regard to nitrogen elimination. Guanine excretion has previously been described in *Helix pomatia* and in various spiders by Peschen (1939). Spiders also store guanine in the abdominal cavity, and such "internal excretion" could also occur in *Hyperolius*.

In this study, nitrogen metabolism and osmoregulation in the reed frog *Hyperolius viridiflavus taeniatus* were investigated, with special reference to the quantitative nitrogen storage capability of the iridophores and their significance in reducing osmotic problems caused through production of nitrogenous wastes during long dry periods.

Materials and methods

To simulate wet season or transitional conditions, frogs (*H. v. taeniatus*) were maintained in terraria at 28°/24° C and 70%/100% R.H., (day/night), with a light period of 11 h. Wet terraria were sprayed daily, transitional terraria 3 times weekly. The frogs were fed flies of various sizes (*Lucilia, Musca, Drosophila*) according to the same schedule. Dry season conditions were simulated in an incubator at 30°/20° C and 30%/70% R.H. (day/night) with an identical light period (11 h/13 h LD).

The experimental animals (=group 1) were removed from their wet season terrarium 5–7 weeks after metamorphosis, maintained for 2 weeks under transitional conditions and then kept in 8 × 2.5-cm plastic tubes under dry season conditions. A second group (=group 2, still subadult) first lived for 5–6 months under transitional conditions and was then treated like group 1. As controls, frogs of the same age remained in transitional terraria during the entire experiment.

The experimental animals were double pithed and dissected in precooled petri dishes. After blood and urine samples had been taken, the liver was removed and homogenized in a precooled glass homogenizer with 0.1% CTB N-Cetyl-N,N,N-trimethyl-ammonium bromid solution. This mixture was immediately centrifuged for 20 min at 4000 RPM at 4° C. The activities of glutamate dehydrogenase (GIDH) and arginase were measured by the method of Radke (1976).

To measure the urine volume, frogs were shock frozen at −70° C, the frozen bladder contents removed, and urine volume measured. Plasma and urine concentrations were determined with a vapor pressure microosmometer (model 5100 C, Wescor Inc.). Plasma was obtained by centrifugation (10000 RPM) of blood samples. Urea and uric acid determinations were performed photometrically (Sigma diagnostic kits), and for total nitrogen determination of feces the Kjehldahl method was used.

Rates of water uptake were measured by placing the frogs in plastic tubes containing 500 µl double-distilled water for 15 min (=500 µl/15 min). The water was then removed and the frogs were reweighed 2 h later, after having been placed in an incubator at 30° C and about 20%–30% R.H. to make sure that water adhering to the skin had evaporated. Individuals that excreted urine during rehydration were discarded. To decide whether a frog had excreted urine during rehydration the water remaining after 15 min was tested for urea. All rates of uptake are given as percent of the body weight prior to rehydration per hour.

For purine determinations, the dorsal and ventral skin of the dissected animals was removed, dried at 60° C until a constant dry weight was reached, homogenized with 10% phosphoric acid (2 washings), and then kept at room temperature for 24 h. The samples were then exposed to ultrasonic sound for 60 min and then centrifuged for 10 min at 10000 RPM. The supernatant was buffered with Na_2HPO_4 to pH 2. Analysis was performed with a Kontron HPLC analyser. An SI 100:Polyol:Sulfopropyl 5 µm column (Serva) served as the stationary phase. As the mobile phase an ammonium dihydrogen phosphate buffer (pH 2) was used. The flow rate was 1.8 ml/min. Synthetic standards were chromatographed as identification markers. Because with two further separation columns of the same specification purchased at a later date separation was not obtained under otherwise identical conditions, another stationary phase had to be used. Therefore, additional investigations mentioned in the discussion (detailed description in prep.) were performed with a Sulfopropyl:Daltosil:100 column (Serva) using triethylamine pH 7.5 as mobile phase.

Results

Dry season frogs (DSF) have four compartments in which nitrogen end products could be stored: the body fluid, the bladder fluid, the intestinal contents, and the guanophores. All four compartments were investigated for possible increases in the amounts of stored nitrogen over a 42-day period of water deprivation.

Changes in composition and osmolarity of plasma and bladder fluid

During the first 7 days of water deprivation, urine and feces egestion were suppressed (see next section), and the

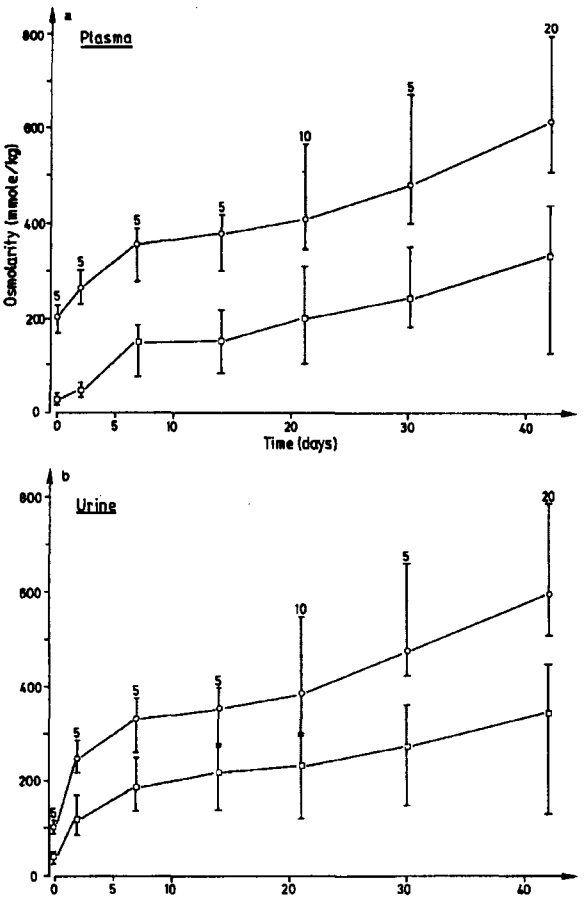

Fig. 1 a, b. Increase in total osmotic concentration of plasma and urine (o) and the partial osmotic effect of the increasing urea concentration in plasma and urine (□) during water deprivation in *Hyperolius viridiflavus taeniatus*. At day 0 all specimens were given water ad libitum. Prior to day 0 test specimens were kept under transitional conditions. *Vertical lines* indicate ranges, *numerals* indicate sample sizes

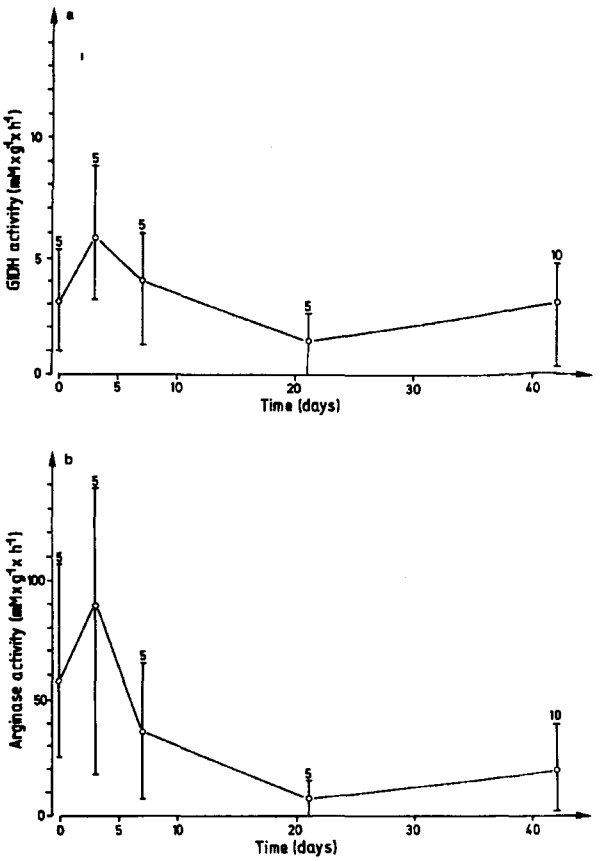

Fig. 2 a, b. Changes in the activities of the liver enzymes glutamate dehydrogenase (GIDH) and arginase in *Hyperolius viridiflavus taeniatus* during water deprivation. *Vertical lines* indicate ranges, *numerals* indicate sample sizes

total plasma osmolarity of the frogs (both groups 1+2) increased strongly. This increase was mainly due to urea accumulation, (Fig. 1). In the following days and weeks total plasma osmolarity continued to rise, but at a significantly lower rate ($p<0.001$; U test). After 14 days, the rate at which total plasma osmolarity increased had risen again but still remained significantly lower than in the first 7 days ($p<0.001$; U test). At the end of the experimental dry period, BUN (blood urea nitrogen) had reached 8 times the original concentration, with a mean concentration of 335 mM urea stored in the plasma.

Changes in the osmotic concentration of the bladder fluid followed a similar pattern. Differences of more than 20% in total osmolarity between plasma and bladder fluid occurred for a short time (about 24 h) after water uptake (on day 0 in Fig. 1). Then bladder urea concentration increased sharply and total urine osmolarity remained only slightly hypoosmotic to plasma during the rest of the dry period (see Fig. 1). During the first 14 days of water deprivation, urea was more concentrated in the bladder fluid than in the plasma ($p<0.01$; U test; mean difference 46 ± 28 mM). This increase in bladder urea concentration resulted mainly from water reabsorption across the bladder wall. Urine was not formed under conditions of water deprivation. This was demonstrated in other specimens (shock-frozen at $-70°$ C), in which the total urea content of the bladder fluid remained unchanged between day 1 and day 30. At the end of the dry period urea concentrations in urine (354 ± 73 mM; $n=20$) and plasma (335 ± 86 mM; $n=20$) did not differ significantly ($p>0.05$; U test).

After the onset of the dry period the rate of urea synthesis, measured as activity of the urea-cycle enzyme arginase, was significantly higher during the first 7 days than later ($p<0.01$; U test) (Fig. 2). Change in the activity of GIDH (glutamate dehydrogenase), the key enzyme in amino acid metabolism, showed a similar pattern (Fig. 2).

Within 7 days, however, 'transitional' frogs given water on alternate days showed a higher rate ($p<0.01$; U test) of urea synthesis (317 ± 24.2 µg N_2/g day; $n=10$) than frogs deprived of water (236.9 ± 32.8 µg N_2/g day; $n=5$) kept under the same conditions. Most of the urea synthesized, however, was excreted ($87.4\%\pm4.2\%$) by the 'transitional' frogs during prolonged water uptake (500 µl/45 min).

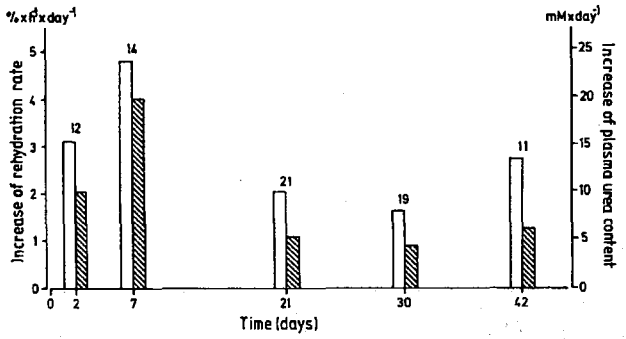

Fig. 3. Rehydration rate (*unshaded columns*) and plasma urea content (*shaded columns*) in *Hyperolius viridiflavus taeniatus* during water deprivation. *Numerals* indicate sample sizes

Table 1. Decrease in relative amount of urine of *Hyperolius viridiflavus taeniatus* during a 42-day dry season (Data from these individuals are not included in Figs. 1 and 2)

Number of individuals tested	Length of dry period	Body weight loss (%)	Urine (% body weight) mean	range
15	1 day	0%	15% ± 7.8%	(5–25%)
10	30 days	35.4% ± 3.8%	5% ± 1.9%	(0–12%)

Although the high initial rate of urea synthesis does not seem to be a specific response to desiccation stress, the resulting rise in total plasma concentration could be important in replenishing water loss. Once water loss had reached a value of more than 10% of the body weight, urea excretion during water uptake (500 µl/15 min) was greatly reduced, despite the fact that the water taken up was more than sufficient to compensate all losses. Specimens with EWL below 10% body weight, without elevated plasma urea levels, excreted about 138 ± 43 µg N_2/g body weight ($n=25$), whereas those which had lost more than 10% body weight before rehydration excreted negligible amounts of urea (27 ± 23 µg N_2/g body weight; $n=14$). This indicates that urea is not always treated as a waste product and eliminated whenever possible. Indeed the rate of water uptake is strongly correlated with the increase in BUN (Fig. 3).

Nitrogen excretion in urine and feces

Under our experimental conditions excretion of urine occurred exclusively during water uptake.

The nitrogen storage capacity of the bladder is very limited in *H. viridiflavus*. First, bladder water content never exceeded 25% of the total body weight (Table 1) and decreased greatly during the dry period. Secondly, total osmolarity of the urine never exceeded that of the plasma. At the end of the dry season, urea concentration of bladder fluid was not significantly different from that of the plasma (see page 355 above). Uric acid was present only in trace amounts. If water was available (500 µl/15 min) after the dry season, large amounts of urea were excreted. Three days after dry season frogs were returned to wet season conditions, they did not show significantly higher BUN values than frogs that had remained permanently in transi-

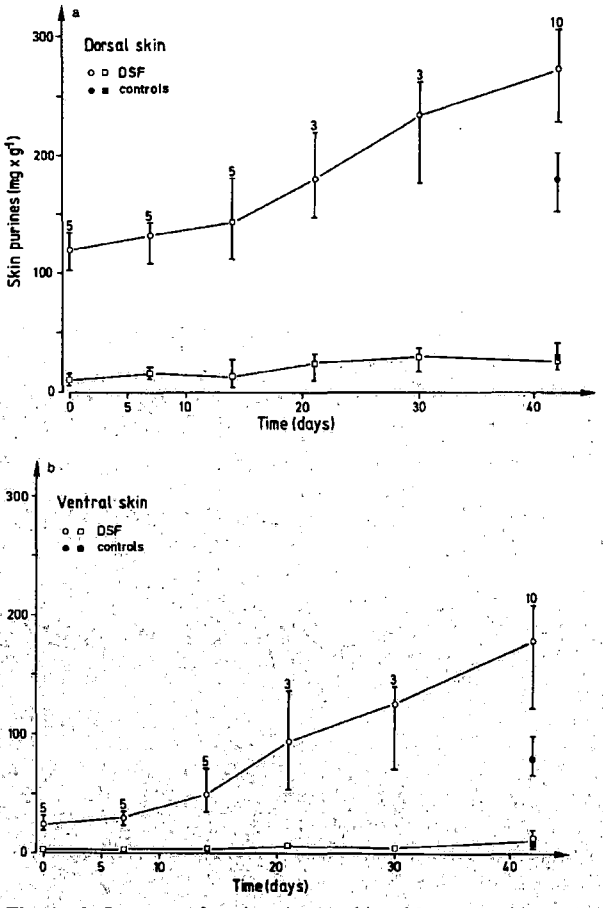

Fig. 4a, b. Increase of purines in the skin of 2-month-old *Hyperolius viridiflavus taeniatus* during a 42-day dry season. The purines were identified as (o) guanine and (□) hypoxanthine. *Open symbols*, dry season frogs (DSF) *closed symbols*, controls (frogs of the same age maintained in transitional terraria throughout the experiment). *Vertical lines* indicate ranges, *numerals* indicate sample sizes

tional terraria, which had BUN levels no higher than those at the beginning of the experiment.

No voiding of feces was observed during the dry period. Only after repeated water uptake (water was given on alternate days after the dry season) did defecation occur. Feces contained 46.0 mg N_2/g dry weight (35.6–66.9 mg N_2/g; $n=8$). About 20% originated from protein, the remainder predominantly from the indigestible cuticle of arthropods fed shortly prior to onset of the dry season. Uric acid excretion could not be demonstrated.

Nitrogen storage in iridophores

The number of skin iridophores increases greatly during the dry season, lowering radiation absorption in the visible and near infrared range (Kobelt 1987). Two or three layers of iridophores, corresponding to about 100–150 µg/mg dry weight, increased the skin's reflective properties greatly, but additional iridophore synthesis made little further difference. Since more iridophores are in fact produced, it seems reasonable to suggest that these chromatophores could have an additional function in the elimination of nitrogenous wastes. To find out whether enough guanine is thus stored

Table 2. Urea concentration in various compartments of *Hyperolius viridiflavus taeniatus* after 42 days of water deprivation. Data from the same individuals as in Figs. 1, 2, and 3 and Tables 3 and 4

Individual	Plasma fluid (m*M*)	Carcass homogenate (m*M*)	Liver homogenate (m*M*)
1	353	343	350
2	433	425	462
3	219	221	236
4	215	186	228
5	124	117	119
6	410	363	433
7	352	327	346
8	333	328	352

to be of quantitative significance in nitrogen elimination, the amount of purines from the skin of dry-adapted *Hyperolius viridiflavus taeniatus* was measured.

In both the dorsal and the ventral skin, about 150 µg purine/mg skin dry weight was synthesized over a water deprivation period of 42 days (Fig. 4). On average, the amount of purines increased from 136.3 to 298.0 µg/mg in the dorsal and from 32.1 to 192.1 µg/mg in the ventral skin ($n=10$), which corresponds to a mean of about 90 m*M* nitrogen stored in the skin pigments. Of the total purine content 80%–90% was guanine; the rest was identified as hypoxanthine. During the dry period, this proportion seemed to change still more in favor of guanine. The level of hypoxanthine, which was 12.5% at the beginning of the dry period, never exceeded 10% ($\bar{x}=8.9\%$; $n=10$) at the end of the dry period. No uric acid could be found in the iridophores. Over the same period, the increase in total purine content averaged only 60 µg/mg in controls.

In contrast to group 1 (7–9-week-old frogs), the increase in skin purine content in group 2 (5–6-month-old frogs) did not show a consistent pattern. Group 2 frogs dissected at the beginning of the dry period had considerably higher and much more variable skin purine contents than 7–9-week-old frogs (range 192–271 µg/mg dorsally, 87–139 µg/mg ventrally). This might be because iridophore production is a continuous process, (see next section) and the rate of purine synthesis apparently differs from individual to individual (this might result from individual preferences in the terraria for certain perching places differing in their microclimate). Therefore, interindividual variations increase in the older individuals. For this reason, nitrogen balance was calculated for group 1 only.

Nitrogen balance

Group 1 frogs showed a 37%–55% loss of weight after 42 days without water, which is close to or even beyond the lethal limit (45%–52%; Geise 1987). The plasma concentration increased to 568–758 mOsm.

Nitrogenous wastes were not stored at constant rates in the plasma and guanophores over the whole period (Figs. 1 and 3). Plasma urea content increased during the first 7 days up to 4 times its original value, whereas skin pigment purines did not rise significantly over 7 days after the onset of water deprivation.

Although cell membranes are usually highly permeable to urea, long-term compartmentation of urea has been described in *Bufo viridis* (Degani et al. 1984). To test whether this had also occurred in *H. viridiflavus*, after blood and urine samples had been taken the remaining carcasses were homogenized and their urea content measured. An identical test was performed using homogenized liver. The data (Table 2) demonstrate that in *H. v. taeniatus* urea was evenly distributed over all body fluids at the end of the dry period.

The distribution of the nitrogenous wastes in skin iridophores and body fluid is shown in Table 3. At the end of the dry season, only one Group 1 frog still had fat pads. This individual also differed from all others in its low plasma urea concentration. Test specimens with lower levels of BUN (frog 3, 4, and 5 in Table 3) stored about the same amount of purines in skin guanophores per day as individuals with high BUN, and therefore the relative amounts of nitrogen stored as purines are higher in the former. In group 2, only two frogs had fat pads. Compared to the rest of the animals (240–385 m*M*), these two also had unusually low plasma urea concentrations (186 m*M* and 158 m*M*).

After 42 days of water deprivation the total purine content of the skin of group 1 individuals was 246–344 µg/mg in the dorsal and 137–232 µg/mg in the ventral skin, corresponding to an increase of 100–170 µg of purine per mg skin dry weight. Similar levels of purine storage may be assumed for group 2. These animals reached levels of 285–397 µg/mg in the dorsal and 209–292 µg/mg in the ventral skin (Table 4). If the frogs had accumulated this nitrogen as urea the plasma concentration would have been about 86 ± 30 m*M* higher.

Approximately 5–7-month-old *H. v. nitidulus*, taken from the field in April 1986 (4 weeks after the first rainfall), had purine levels of 412–472 µg/mg dry weight in the dorsal and 243–287 µg/mg in the ventral skin ($n=5$). Seven-to-eight month old *H. v. taeniatus* kept in the laboratory under

Table 3. Increase in body urea and skin purine contents, in *Hyperolius viridiflavus taeniatus* during 42 days of water deprivation

Individual	Initial body weight (g)	Body weight loss (%)	Fat pads (mg)	Final body urea concentration (m*M*)	Increase of body urea content (µg N_2/g/day)	Increase of total purine content of the skin		Final plasma concentration (mOsm)
						(µg N_2/g/day)	(% N_2)	
1	0.458	47.7	0	353	98.5	29.6	23.1	648
2	0.480	53.3	0	433	115.6	13.6	10.5	758
3	0.412	54.8	0	219	56.6	28.9	33.8	648
4	0.415	51.2	0	215	60.0	23.2	27.9	628
5	0.350	49.1	0.2	124	36.1	18.9	34.4	568
6	0.235	40.3	0	410	154.9	24.6	13.7	691
7	0.413	37.4	0	352	108.9	28.4	20.7	628
8	0.646	42.5	0	333	94.8	27.9	22.7	619

Table 4. Purine content of the iridophores under different conditions in *Hyperolius viridiflavus taeniatus* of differing ages (±S.D., range given in parentheses). Values in each group were tested for significance against 7–8-month-old wet season frogs. G=Guanine, Hyp=Hypoxanthine

Maintenance conditions	Ages of animals (months)	Number of individuals tested	Purine content of iridophores			$p<$ (U test)
				Dorsal (µg/mg)	Ventral (µg/mg)	
42-day dry period after transitional conditions	2–3	10	G	273 ±41 (225–306)	175 ±39 (123–211)	0.002
			Hyp	25 ±7.8 (21– 38)	17 ± 2.9 (14– 21)	n.s.
Wet season frogs	7–8	7	G	207 ±26 (177–248)	108 ±19 (76–137)	–
			Hyp	23.5± 5.2 (16– 30)	14.2± 1.6 (13– 16)	–
Transitional conditions	7–8	5	G	231 ±38 (198–272)	121 ±18 (98–146)	n.s.
			Hyp	21.7± 7.2 (14– 32)	17.1± 3.2 (15– 22)	n.s.
42-day dry period after transitional conditions	7–8	10	G	342 ±35 (260–365)	212 ±53 (196–270)	0.001
			Hyp	28.5± 4.3 (25– 32)	18.3± 2.6 (13– 22)	0.05
Adult frogs, after maintenance under transitional conditions and then wet season conditions for 1 month	12	5	G	263 ±27 (240–300)	200 ±14 (180–231)	0.005
			Hyp	31.2± 3.1 (26– 36)	21.0± 1.8 (17– 24)	0.005

transitional conditions had purine levels of 212–304 µg/mg and 113–168 µg/mg dry weight in the dorsal and ventral skin, respectively ($n=5$). If these subspecies have comparable purine storage capacities the additional storage capacity in the skin pigments, used under dry season conditions only, is about 200 µg purine/mg skin. In addition, the liver of *H. v. nitidulus* from the field was tightly packed with iridophores as were many other connective tissues such as the heart sac.

Purine crystals are produced to some extent over the whole juvenile stage independent of maintenance conditions. To investigate how the rate of production is influenced by environmental and growth conditions, frogs maintained under different conditions were dissected and total skin purine analyses were performed.

All juveniles of the same age had approximately the same body size and weight. Skin purine content is clearly strongly dependent upon water availability (Table 4). There were small differences between frogs given water daily and those given water 3 times a week. After a 42 days of water deprivation, skin purine content was significantly ($p<0.001$; U test) higher than in the controls, independent of the age of these controls. Adults had lower levels than juveniles. However, this may be due to the accelerated body growth rate after the dry period when the young frogs grow to adult size.

Discussion

Accumulation of nitrogenous wastes during long periods of water deprivation causes severe osmotic problems, predominantly in amphibians which do not retreat to subterraneous hides. *Chiromantis spp.* as well as *Phyllomedusa spp.* have solved this problem by evolving uricotelism (Loveridge 1970; Shoemaker and McClanahan 1982). In the third genus of "waterproof" frogs, *Hyperolius,* however, uricotelism has not been found.

Under experimental conditions juveniles of *H. v. taeniatus* tolerated water and food deprivation for about 6 weeks. In the field, frogs are probably able to tolerate longer periods than in the laboratory where disturbance resulting from general handling may induce elevated metabolic and EWL rates. Besides this there are great interspecific differences in maximum estivation times. *H. v. nitidulus* shows a much lower rate of EWL (Geise and Linsenmair 1986) and a lower rate of urea accumulation (Schmuck, unpubl. data) than *H. v. taeniatus*. Like most terrestrial and semiterrestrial anurans during estivation *H. v. taeniatus* stores nitrogenous wastes primarily as urea which accounts for about two-thirds of the rise in total plasma osmolarity (Table 3). After water deprivation for 42 days the mean BUN had risen from 34 mM in hydrated juveniles to about 305±106 mM ($n=6$) in DSF (group 1) (Table 3), indicating that urea accumulated at a rate of 13.8±9.8 mM/g/day, which is considerably lower than in dehydrated adult *H. nasutus* (about 23 mM/g/day; Withers et al. 1982b). These differences, however, might result from the high variations in the urea accumulation rate between the initial phase of dry period conditions and afterwards. During the first 14 days, all that were considered for *H. nasutus* in the above-mentioned study, *H. v. taeniatus* accumulated urea at a rate of 19.5 mM/g/day which is not greatly different from that for adult *H. nasutus*. Urea tolerance of other amphibians that tolerate high plasma urea levels is about 400 mM (Cleworth 1967, cited in Withers et al. 1982b), which was not significantly exceeded in the present study. Degani et al. (1984) have demonstrated that even at 900 mM BUN the intracellular concentration does not go beyond 500 mM in *Bufo viridis*.

Of the total urea content accumulated over 42 days of experimental water deprivation, 30% is produced during the first 7 days. This initial elevated urea accumulation rate is well correlated with high activity of the enzymes GIDH and arginase. Similar results have been found in both amphibians estivating above ground and in fossorial amphibians, (e.g. Delson and Whitford 1973; Jones 1982). In fossorial amphibians reduction in water loss to the ground by increased urea formation has been found (McClanahan 1972; Katz and Gabbay 1986). In above-ground estivating frogs, however, this cannot apply. It has been suggested that, following dehydration, elevated levels of urea result from increased amino acid metabolism. This increase in amino acid metabolism is seen as a primary pathway for regenerating carbohydrate reserves and is thus suggested

to be a specific response to starvation rather than dehydration stress (Jungreis 1976). This is further supported by the fact that urea synthesis in the first 7 days of adaptation to the dry season conditions did not exceed that of starved individuals that received water on alternate days (500 µl/45 min). Similar results were obtained in *Bufo woodhousei* and *Hyla cadaverina* (Jones 1982). Elevated amino acid catabolism, however, unavoidably results in a higher plasma ammonia concentration. This ammonia is removed most effectively by increasing the activity of the urea-cycle enzymes. Therefore, the high rate of urea formation in the first 7 days is primarily an unavoidable consequence of the reactions to incipient starvation. Nevertheless, the resulting rise in total plasma osmolarity could be advantageous in replenishing water losses. This is supported by the fact that after desiccation had reached more than 10% of the body weight, urea excretion was greatly reduced even though sufficient water was given for full rehydration. Thus, as acclimation to dry season conditions progresses, urea apparently gains physiological importance and is retained until a critical value is reached. In the present study, it was shown that rehydration rate was strongly affected by plasma urea levels. If water is available for a short time only, as is certainly often the case for *H. v. taeniatus* in its natural habitat, it should be highly advantageous to absorb it very rapidly.

Urea accumulation should cease after reaching a level sufficient to enable rapid rehydration, thus avoiding lethal plasma urea concentrations when water and/or energy reserves are not yet used up. Results agreed with this prediction. Between day 7 and day 14, no significant increase in plasma urea ($p > 0.05$; U test) could be detected, and the activities of glutamate dehydrogenase and arginase were clearly lower.

Around day 21, plasma urea increased again, presumably due to bladder water was greatly resorbed. This is supported by the sharp increase in urea concentration in the bladder fluid during the first days after water deprivation without changes in total urea content, indicating a net water efflux from the bladder fluid. Bladder water is assumed to replenish water lost from the plasma via evaporation. In fossorial anurans a large portion of total body water is stored in the bladder (up to 50% of the body water in *Cyclorana platycephalus*; Bentley 1966) and is important in regulating plasma osmolarity (McClanahan 1967; Loveridge 1976; Katz and Gabbay 1986). In *H. v. taeniatus* a significant portion of the total body water is stored in this compartment, but never more than 25% of the body weight. This might be because the estivating *H. v. taeniatus* retains its ability to become active at any moment and a large amount of stored bladder water might restrict its mobility. After water deprivation for 30 days, about 80% of the bladder fluid is resorbed (Table 1). If water were not resorbed from the bladder for 30 days, under our dry season conditions water loss from body fluids would be 35% rather than 25% on average, increasing osmotic problems and shortening life expectancy of an estivating *Hyperolius*. Beside its importance in restoring water losses from body fluids, bladder fluid might be also important in ionic regulation. At the end of the dry period the remaining bladder fluid contains urea isoosmotic to the plasma fluid, but K^+ at significantly higher concentration ($p > 0.001$; t test) (Schmuck, unpubl.).

Although it would be greatly advantageous to increase BUN only up to the optimum level for rapid rehydration, a further increase in plasma urea osmolarity during water deprivation is unavoidable. Protein and nucleotide turnover and especially protein catabolism for energy supply, which occurs mainly during the first days after water deprivation and after depletion of fat pads, cause a further production of ammonia that has to be detoxified. *Chiromantis spp.* (Loveridge 1970; Drewes et al. 1977) and *Phyllomedusa spp.* (Shoemaker and McClanahan 1982) predominantly excrete uric acid under dry season conditions. These species, however, continue to feed during the dry season. This results in a high nitrogen intake in a short time, which must be eliminated very rapidly. Uric acid excretion also solves the problem of the salt load caused by food uptake. Cations are precipitated as insoluble urate salts (Shoemaker and McClanahan 1975). In the dry season state, *H. v. taeniatus* and *H. v. nitidulus* have not been observed to feed during water deprivation unless R.H. exceeds 90% (Geise 1987). Therefore, it may be assumed that rates of salt and nitrogen accumulation are lower than in e.g. *Chiromantis*.

Nevertheless, urea accumulation probably can limit length of survival during water deprivation. During water deprivation nitrogen is deposited in the platelets of iridophores. After 42 days skin iridophores contain about 25% of the total stored nitrogenous end products. If urea were the only nitrogenous waste product its faster accumulation would lead to a 10–14 day reduction in maximum survival time under our conditions (taking 700 mOsm as the tolerable limit). In the field, guanine deposition may be even more important because water deprivation periods may greatly exceed 42 days (at least in *H. v. nitidulus*).

Although the ratio of purine nitrogen to total nitrogenous wastes in DSF is much higher than in WSF, the latter produce substantial amounts of purine. The establishment of a metabolic pathway that requires high energy expenditure and its maintenance during periods of optimal energy supply may be an important adaptation allowing energy conservation in periods of very limited energy resources. A similar mechanism would be expected in uricotelic anurans, and these amphibians too excrete part of their nitrogenous wastes as uric acid, even when water is superabundant (Shoemaker and McClanahan 1975). The possession of an enzymatic system that is already available when it is needed enables these amphibians to counteract rapidly the rise in plasma osmolarity under conditions of sudden water deprivation.

Compared to uric acid excretion, guanine excretion provides three advantages: (i) Guanine has a better N:C ratio than uric acid, i.e. per mole guanine, more nitrogen can be stored: (ii) Guanine can be remobilized and used in anabolism after the dry season: In *Rana* spp. a long-term high level of MSH (melanophore stimulating hormone) lowered skin purine content (Bagnara 1976), and if this is also true for *H. viridiflavus*, purine degradation probably takes place during the transition from juvenile to adult coloring when melanophores increase in number about fourfold, which is surely mediated by a high level of MSH (Richards 1982): (iii) Guanine is not only a nitrogen store, but also increases the light-reflecting ability of the skin (Kobelt and Linsenmair 1986) more than uric acid could do because of its higher refractive index and better crystalline qualities (Kobelt 1987). Light remission by the iridophores is important in temperature regulation (Withers et al. 1982a; Kobelt and Linsenmair 1986; Kobelt 1987), but total iridophore synthesis far exceeds the amount necessary for effective radia-

tion reflection, and the additional synthesis probably serves to eliminate nitrogen end products in an osmotically inactive and nontoxic form. This is supported by the observation that in *H. v. nitidulus* taken from the field 4 weeks after the first rainfall, the liver epithelium was tightly packed with iridophores, which surely do not help to reduce the radiation load. (In *H. v. taeniatus*, this phenomenon was only observed in frogs maintained under mild temperature conditions (25° C/15° C). Under these conditions, *H. v. taeniatus* survives up to 3 months without access to water.) In the field, total water deprivation may last for 3 months, and over this time *H. v. nitidulus* had used, besides the skin, other connective tissues to deposit nitrogenous wastes. Similarly, *H. nasutus* stores large amounts of purines around the intestine in iridophore layers the thickness of which is affected by age and water availability (Schmuck, unpublished data). This reed frog is sympatric with *H. viridiflavus* and has a very similar pattern of estivation.

Apparently, in contrast to uric acid excretion in *Chiromantis* or *Phyllomedusa*, the rate of purine deposition is not sufficient to prevent a rise in plasma urea level during dry season conditions (Fig. 1). Since the enzymatic pathway for guanine synthesis is the same as for uric acid synthesis, we suggest that the rate-limiting process is not the formation of guanine crystals but the synthesis of iridophore cells. Probably this is due to the high energy expenditure necessary for the production of cell membranes and organelles. This is supported by the fact that the rapid increase in iridophore production in the first 7 days after metamorphosis is accompanied by a high consumption of energy (i.e. of protein and fat deposits) (Schmuck et al. unpublished work).

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