

## CHANGES IN BODY FLUID OSMOLALITY, ION CONCENTRATION AND NITROGEN BALANCE IN *PELOBATES SYRIACUS* DURING ONTOGENESIS

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The larvae of *Pelobates syriacus* have a long developmental period to metamorphosis. This is atypical of xeric-inhabiting anurans and suggests that the populations of this species in Israel are relicts from the time when this region enjoyed a milder climate. In contrast, metamorphic climax is rapid. In the course of a few days the external appearance changes from that of tadpole to toadlet. During the same short period, together with other internal reorganizations, the kidney completes its maturation from pronephros to mesonephros. The production of urine, a fluid which is almost identical to plasma in the tadpoles, ceases, and when it is resumed in post-metamorphic toadlets, it is dilute and adult-type with nitrogen as urea replacing ammonia. There is a simultaneous increase in plasma osmolality. In *P. syriacus* a long period of development followed by the emergence of a relatively large metamorph may be an adaptation to a hostile environment. Coupled with the rapid metamorphic climax, these life history features may increase the opportunities to disperse and burrow into soil that remains moist, at the beginning of a long hot and dry summer.

*Key words:* *Pelobates*, ontogenesis, osmolality, ion-balance, nitrogen excretion

### INTRODUCTION

*Pelobates syriacus* (Boettger, 1889) is a rare and endangered amphibian in Israel. Its tadpoles are much larger than those of most anurans. Individual tadpoles may attain a gross weight of more than 25g. Development from hatching to metamorphosis is slow, taking 4-5 months. Maximum size is achieved as the first limb-buds appear and is maintained until all four legs have erupted. Weight decreases over a variable period of 1 to 2 months and the total body water content: weight of dry matter ratio falls from about 10:1 to about 5:1. Urine, which accounts for 15% or more of the gross weight in the less mature tadpoles, is reduced to a barely measurable volume as tail-length shortens.

Unlike the extended developmental period, the metamorphic climax is rapid. About a week after feeding ceases, the already shortened tail is resorbed, the skin develops the markings typical of an adult and the toadlet seeks dry land – all within a further 3-4 days. The external morphological changes are accompanied by maturation of the kidney to a functional mesonephros (Warburg & Gealekman, in prep.), an increase in plasma osmolality and a change from ammonotelism to ureotelism (Degani & Nevo, 1986). Many changes occur within a very short period; however, external features are not an accurate enough indication of the stage of internal reorganization.

*P. syriacus* females require fairly deep (at least 0.5 m) water in which to lay their eggs (personal observations, MRW). In years when the first heavy winter rains are late, slow development poses a serious problem so

that an occasional breeding season may occur in which no tadpoles metamorphose successfully before the ponds begin to dry out. The large size and slow development of the tadpoles provide a rare opportunity to collect both blood and urine from them at several different stages and to compare the composition of those fluids with those of both newly metamorphosed toadlets and adults.

The purpose of this study was to follow the changes taking place in the osmolality and ion content of plasma and urine as well as in mode of nitrogen excretion throughout the metamorphic cycle of this rare toad. We wished to compare the pattern of stage-related changes in the plasma composition with those previously determined in the similarly large tadpoles of *Rana catesbeiana* (Just *et al.*, 1977) and to determine the composition of tadpole urine.

### METHODS

A limited number of larval *Pelobates syriacus* were collected from breeding ponds in Upper Galilee in the springs of the years 1990-1993 by special permit from the Nature Conservation Board, as this toad is on the Red List of species for Israel. They were maintained in the laboratory for up to three months in aged tap-water, which was changed every 1-2 days, and were fed on washed lettuce leaves. Toads which had metamorphosed in previous years were kept, individually, in moist soil and fed mealworms. Tadpoles were selected for assay at various stages (according to Zabroda & Ilyenko, 1981) from the appearance of the first leg bud (Stage 11), through attainment of maximum size (Stage 14), to reduction of the tail until considerably shorter than the hind legs (Stage 17). Four toadlets within two weeks post-metamorphosis (Stage 19), three second

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year juveniles and five adults 3-8 years old were also included.

The animals that were used to determine water-content were measured (snout-vent and snout-tail-tip), lightly blotted and weighed. Bladder urine was obtained with a fine catheter through the cloaca. In toads and toadlets, gentle pressure was applied to the abdomen to expel the bladder contents but in tadpoles, several millilitres of a clear fluid were frequently voided when the external opening of the cloaca was very lightly touched with the tip of the catheter. This fluid proved to be identical in osmolality and electrolyte composition to that obtained when the catheter was inserted. As a precaution against possible faecal contamination, samples were centrifuged for 5 min at 2000 rpm but no sediment was visible. All animals were killed rapidly to avoid water influx across the skin by immersion in 0.02M MS222 (Sandoz). This is a higher concentration than that employed for anaesthesia. The carcasses were blotted and re-weighed before being dried to constant weight at 95°C.

Animals used to assay major osmolytes in the body fluids were treated similarly, but after killing and blotting, a ventral incision was made and blood from the heart and/or fluid from the body cavity was collected. The liver of some specimens in the later stages of development was removed for the assay of the ornithine urea cycle enzymes, carbamyl phosphate synthetase (CPS) and arginase.

Osmolality was determined immediately after sample collection (Wescor 5500 Vapour Pressure Osmometer) and the remainder of the sample frozen at -20°C for later analyses. Urea and ammonia concentra-

tions were determined colorimetrically (Sigma bulletin No. 640). Sodium and potassium were measured by flame photometry (Corning 480), and chloride on a Radiometer CMT10 chloride titrator. Ornithine urea cycle enzymes were assayed by the method of Brown & Cohen (1959). Results are expressed as mean values  $\pm$  SD. Mean values were compared by Student's *t*-test.

## RESULTS

Between stages 11 and 17, mean plasma osmolality increased from about 185 to about 215 mOsm/kg (Fig. 1) ( $t=2.853$ ,  $df=13$ ,  $P<0.01$ ) correlating with a decrease in percentage body water content from 93.5 to 80.2 (Fig. 2;  $t=5.882$ ,  $df=13$ ,  $P<0.001$ ). This was found to have decreased further, to 75.2%, in a single 8-year old adult. While this reflects the concentrating effect of water loss, the constant levels of potassium and chloride over the same period (Fig. 1) indicate some regulation of these ions. The electrolyte composition of body fluid and urine was almost identical in each individual tadpole throughout this period of development during which weight (3.7-25.4 g; Fig 2) and total length (5.0-16.5 cm) both fluctuated, peaking around stage 14.

At stage 11, urine accounted for about 6% of total body weight. This increased as the hind-legs developed and by stage 14 had reached about 15%. It decreased to about 3% (stage 16) as the fore-legs erupted, and very little urine remained at stage 17. Within two weeks post-metamorphosis, the urine volume of a single specimen was within the normal adult range (Fig. 3).

The osmolality of the toads' plasma was significantly higher than that of the tadpoles' body fluid

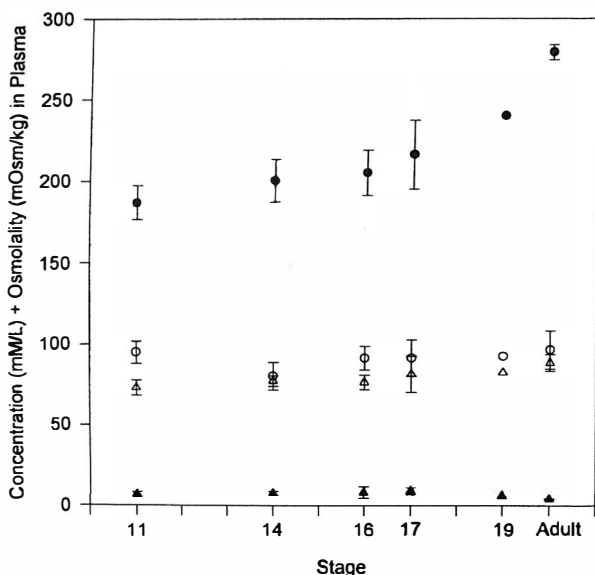


FIG 1. Total osmolality and concentrations of major electrolytes in plasma of *P. syriacus* tadpoles, stages 11-17 (mean $\pm$ SD). Filled circles: osmolality; open circles: sodium; filled triangles: potassium; open triangles: chloride. Stage 11,  $n=5$ ; stage 14,  $n=3$ ; stage 16,  $n=5$ ; stage 17,  $n=10$ ; stage 19,  $n=1$ ; adult,  $n=4$ .

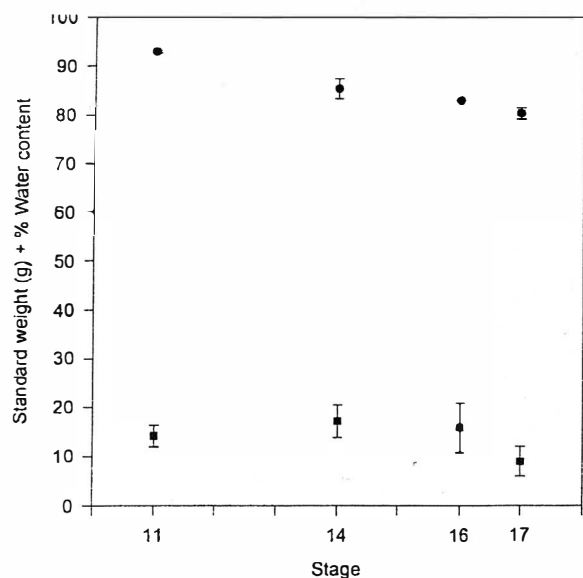


FIG 2. Percent water content and standard weight of *P. syriacus* tadpoles, stages 11-17 (mean $\pm$ SD). Circles: water content; squares: standard weight. Sample sizes (stages 11-17) as in Fig. 1.

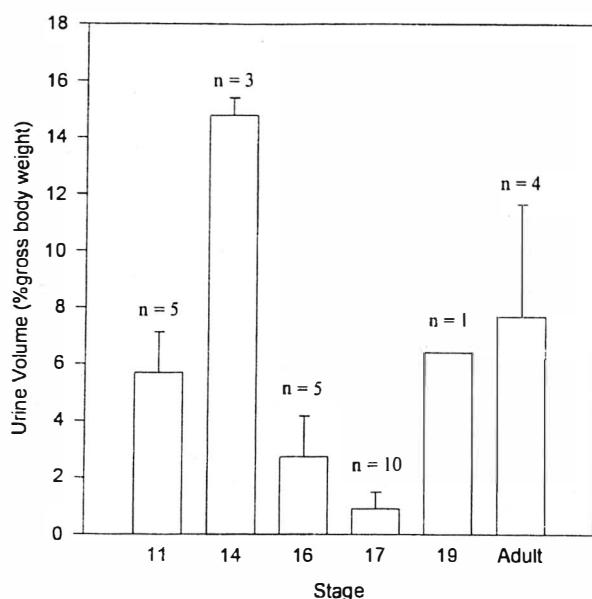


FIG 3. Urine volume expressed as percent total body weight, which itself reaches maximum at stage 14. The stages (Zabroda & Ilyenko, 1981) are not of equal duration; 11-16 together take several weeks, while 17 is completed in a few days.  $n$  = number of animals.

( $t=8.282$ ,  $df=36$ ,  $P<0.001$ ). The concentrations of sodium and chloride were increased (sodium:  $t=1.829$ ,  $df=33$ ,  $P<0.05$ ; chloride  $t=5.090$ ,  $df=37$ ,  $P<0.001$ ), and the presence of urea contributed to the increased total osmolality (Table 1). Apart from an increased concentration of urea, there was no significant difference in the composition of the blood of a single 8-year old animal and that of three newly metamorphosed individuals (Table 2). The urine was dilute, with the summed sodium and chloride concentrations about 17% of that in the plasma. Weights varied from 4.1 g in a 1-year old toad (6.2 g in a newly metamorphosed toadlet) to 46.0 g at 3 years; water content decreased from 81.6 to 75.2%. The data were, necessarily, collected from a small number of animals (according to permit issued by the Nature Conservation Board).

Ammonia ( $\text{Amm}_T = \text{NH}_3 + \text{NH}_4^+$ ) was found at concentrations between 5 and 15 mM in the plasma of all stage 11 tadpoles, but not in stages 12-14. Much higher concentrations ( $\sim 50$  mM) were present in a few stage 16 and several stage 17 animals, although none was present in others at the same apparent level of develop-

TABLE 2. Plasma osmolality and standard (bladder-empty) weight in *Pelobates syriacus* toadlets and toads.

Stage	Age	$n$	Osmolality (mOsm/kg)	Mass (g)
Postmetamorph	5 days	1	228	6.1
Postmetamorph	1 week	2	252	6.2
Postmetamorph	2 weeks	1	240	6.6
Juvenile	1 year	3	$293 \pm 12.0$	$4.3 \pm 0.55$
Adults	2 years	2	279	25.4
Adults	3-4 years	2	280	12.3
Adults	8 years	1	264	46.0

ment. The urine profile was similar but with slightly lower values to that of plasma.

Low levels of arginase activity (sp. act.  $\sim 15$   $\mu\text{moles urea mg}^{-1}$  protein  $\text{h}^{-1}$ ) were detected in the liver of some tadpoles just before the onset of metamorphic climax (stage 17). This remained unchanged at stage 19, but in juveniles and adults the specific activity of this enzyme was more than twice as high. The earliest appearance of any CPS activity ( $ca. 10$   $\mu\text{moles}^{-3}$  citrulline  $\text{mg}^{-1}$  protein  $\text{h}^{-1}$ ) was in a toadlet 1 week after metamorphosis. This was within the range of adult values ( $10-20$   $\mu\text{moles}^{-3}$  citrulline  $\text{mg}^{-1}$  protein  $\text{h}^{-1}$ ).

#### DISCUSSION

During development from stages 11-17, tadpole plasma osmotic pressure gradually increased from 185 to 215 mOsm/kg. The difference was significant but cannot be attributed to an increased sodium concentration (Fig. 1) as was found in *R. catesbeiana* (Just *et al.*, 1977). The total plasma osmolality and concentrations of major osmolytes in toads ranging in age from newly metamorphosed to 8 years, are in the range found previously in this species (Degani *et al.*, 1983; Shpun *et al.*, 1992; 1993), but the osmolality of the urine is higher largely because of an unexplained elevated chloride concentration. No previously published data could be found which gave changes in these fluids at metamorphic climax, but in *R. catesbeiana* plasma osmolality rose from 160 to 259 mOsm/l, and in *Scaphiopus hammondi* from 190-290 mOsm/l (Funkhouser, 1977). The increase in *P. syriacus* is of a similar magnitude.

The urine (but not plasma) of larval *Caudiverbera caudiverbera*, an anuran which is fully aquatic throughout its life-history, was collected at various stages of development (Zamorano *et al.*, 1988), but only the ex-

TABLE 1. Plasma and urine composition (Mean  $\pm$  SD) of *Pelobates syriacus* tadpoles (stages 11-17), toadlets and toads.

	Tadpoles				Toadlets and Toads			
	$n$	Plasma	$n$	Urine	$n$	Plasma	$n$	Urine
Osmolality (mOsm/kg)	26	$206 \pm 23.1$	25	$203 \pm 26.1$	12	$270 \pm 19.8$	7	$89 \pm 29.8$
Sodium (mM/l)	27	$88 \pm 10.8$	20	$88 \pm 10.3$	8	$96 \pm 11.1$	3	$12 \pm 1.7$
Potassium (mM/l)	27	$9.0 \pm 0.77$	20	$3.3 \pm 0.50$	8	$4.6 \pm 1.04$	3	$0.8 \pm 0.33$
Chloride (mM/l)	27	$76 \pm 9.1$	24	$78 \pm 11.6$	12	$94 \pm 12.4$	7	$22 \pm 6.6$
Ammonia (mM/l)	22	$17 \pm 20.8$	24	$10 \pm 17.1$	-	-	-	-
Urea (mM/l)		-		-	12	$32 \pm 25.3$	6	$26 \pm 12.8$

cretion rates of ammonia and urea were published. *C. caudiverbera* larvae gradually become ureotelic before the completion of metamorphosis. In *P. syriacus* the changeover is abrupt. Little or no urine appears as metamorphic climax approaches, and the fluid produced by post-metamorphic toadlets is the first to contain urea. This coincides with the first appearance of CPS activity in the liver and these two observations, together, suggest that the ornithine urea cycle becomes functional, in this species, only at metamorphosis.

Renal function also undergoes a rapid change during metamorphosis. The amphibian larval kidney, a pronephros, persists in anurans until metamorphosis (Fox, 1963; Michael & Yacob, 1974). Its primary function is to excrete water in order to balance the influx across the water-permeable integument. During the tadpoles' growth the mesonephros develops and gradually takes over. In *P. syriacus* tadpoles, urine is so similar in composition to that of the plasma that it must be presumed that plasma electrolytes are replaced adequately from diet as the bathing fluid is almost salt-free ( $\text{Na}^+$  5mM,  $\text{Cl}^-$  15mM). The pronephros degenerates and is replaced by a mesonephros which on completion of metamorphosis has assumed exclusive function (Pons *et al.*, 1982). Adult-type urine is produced within the first week of emergence on to dry land.

The long period of larval development of *P. syriacus*, although found in other members of this genus living in more temperate climes, is atypical of xeric inhabiting anurans. Rapid growth and a short larval period to ensure metamorphosis before ephemeral ponds dry out is much more usual (Warburg, 1988). The long developmental period, coupled with the females' "reluctance" to deposit eggs in shallow water, is habitat restrictive, in that it confines breeding to a limited number of ponds; consequently, it places the species at considerable risk from the cumulative effects of those human activities which involve changes in land usage. The sympatric anuran, *Bufo viridis*, is not subject to the same constraints. It breeds successfully even in temporary pools in the Negev desert (Dimentman & Margalit, 1981) and develops more rapidly than *P. syriacus*. Despite the same depredations, *B. viridis* remains fairly widely distributed from Galilee to the Negev.

*P. syriacus*, in Israel, is probably a relict population from the time when this region enjoyed a milder climate, at the end of the last glaciation period (about 10,000 years ago; Butzer, 1958). It owes its limited survival, in great part, to its longevity (15 years old from tadpole in our lab). The relationship between maximal larval size and metamorph size is therefore worthy of further exploration in this species. It has been suggested that larger metamorphs have greater locomotory capabilities or greater stamina (Newman & Dunham, 1994) either of which confers an advantage in the first, vital search for a suitable habitat. Once it leaves its pond, the toadlet must quickly find a patch of moist soil in which to burrow. The rapidity of the final stages leading to

metamorphosis may be an adaptation to climatic conditions but the trigger is not known.

In the laboratory, under controlled conditions of temperature and food availability, animals successfully metamorphose about two weeks before the summer solstice. This regularity suggests that while growth rate and development of tadpoles are strongly influenced by abiotic factors – particularly the size of ephemeral breeding ponds which are dependent on winter rainfall – the time of actual metamorphosis is possibly regulated by an internal clock. In the present climatic conditions of this region, this timing is not optimal for a successful search for a microhabitat of damp soil and may represent behaviour inherited from earlier times.

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#### REFERENCES

- Brown, G. W. Jr. & Cohen, P. P. (1959). Methods for the quantitative assay of urea cycle enzymes in liver. *J. Biol. Chem.* **234**, 1769-1174.
- Butzer, K. W. (1958). The Near East during the last glaciation: a palaeogeographical sketch. *Geographical J.* **124**, 367-369.
- Degani, G. & Nevo, E. (1986). Osmotic stress and osmoregulation of tadpoles and juveniles of *Pelobates syriacus*. *Comp. Biochem. Physiol.* **83A**, 365-370.
- Degani, G., Goldenberg, S. & Warburg, M. R. (1983). Changes in ion, urea concentrations and blood plasma osmolarity of *Pelobates syriacus* juveniles under varying conditions. *Comp. Biochem. Physiol.* **75A**, 619-623.
- Dimentman, C. & Margalit, J. (1981). Rainpools as breeding and disposal sites of mosquitoes and other aquatic insects in the Central Negev. *J. Arid Environ.* **4**, 123-129.
- Fox, H. (1963) The amphibian pronephros. *Quart. Rev. Biol.* **38**, 1-25.
- Funkhouser, A. (1977). Plasma osmolarity of *Rana catesbeiana* and *Scaphiopus hammondi* tadpoles. *Herpetologica* **33**, 272-274.
- Just, J. J., Sperka, R. & Strange, S. (1977). A quantitative analysis of plasma osmotic pressure during metamorphosis of the bullfrog, *Rana catesbeiana*. *Experientia* **33**, 1503-1505.
- Michael, M. L. & Yacob, A. Y. (1974). The development, growth and degeneration of the pronephric system in anuran amphibians of Iraq. *J. Zool. Lond.* **174**, 407-417.
- Newman, R. A. & Dunham, A. E. (1994). Size at metamorphosis and water loss in a desert anuran (*Scaphiopus couchii*). *Copeia* **1994**, 372-381.

- Pons, G., Guardabassi, A. & Pattano, P. (1982). The kidney of *Hyla arborea* (L.) (Amphibia Hylidae) in autumn, winter and spring: histological and ultrastructural observations. *Monitore Zool. Ital.* **16**, 261-281.
- Shpun, S., Hoffman, J. & Katz, U. (1992). Anuran amphibia which are not acclimable to high salt, tolerate high plasma urea. *Comp. Biochem. Physiol.* **103A**, 473-477.
- Shpun, S., Hoffman, J., Nevo, E. & Katz, U. (1993). Is the distribution of *Pelobates syriacus* related to its limited osmoregulatory capacity? *Comp. Biochem. Physiol.* **195A**, 135-139.
- Warburg, M. R. (1988). Adaptation of amphibians to life in xeric habitats. In *Ecophysiology of Desert Vertebrates*. Eds. P.K. Ghosh and I. Prakash. Scientific Publishers, Jodhpur.
- Zabroda, C. N. & Ilyenko, E. P. (1981). Peculiarities of reproduction and larval development of *Pelobates fuscus* (Laur.) from the Ukrainian woods. *Vestnik Zool.* **1981**, 66-71.
- Zamorano, B., Cortes, A. & Salibin, A. (1988). Ammonia and urea excretion in urine of larval *Caudiverbera caudiverbera* (L.) (Anura, Amphibia). *Comp. Biochem. Physiol.* **91A**, 153-155.

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