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OSMOTIC REGULATION IN THE GREEN TOAD (*BUFO VIRIDIS*)

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INTRODUCTION

The green toad (*Bufo viridis*) of Europe and the Middle East and the crab-eating frog (*Rana cancrivora*) of Thailand are the only two euryhaline amphibians known. Adult green toads have been reported from aquatic environments of salinities as high as 20‰ in northern Europe (Gislén & Kauri, 1959); Rumanian green toads can tolerate artificial sea-water salinities of 22–29‰, depending upon place of origin and season (Stoicovici & Pora, 1951). Adult crab-eating frogs will tolerate artificial sea-water salinities at least as high as 28‰; tadpoles tolerate natural sea-water salinities up to 39‰ (Gordon, Schmidt-Nielsen & Kelly, 1961).

Gordon *et al.* (1961) have described the major features of the physiological mechanism upon which the euryhalinity of *R. cancrivora* rests. Stoicovici & Pora (1951) and Pora & Stoicovici (1955) have described part of the mechanism employed by *B. viridis*. The present paper describes the results of some further observations on *B. viridis*.

MATERIALS AND METHODS

Adult *B. viridis* of 30–60 g. weight were obtained from the area north and east of Belgrade, Yugoslavia, during July 1961. Two similar sized *B. viridis* were also obtained from an area near Naples, Italy, after an extensive search. All toads were maintained at room temperature (fluctuating diurnally from about 23° to 29° C.) in plastic pans containing desired dilutions of Bay of Naples sea water (100% sea water = 38‰ salinity = 1100 m-osmoles/l. osmotic concentration). Experiments were carried out over a period of about 6 weeks. The toads were not fed. No attempt was made to separate the sexes.

Unless noted otherwise, the following series of observations were made on groups of five or six toads, variously acclimatized to different salinities. Except as noted, methods and precision were as described by Gordon *et al.* (1961).

(1) Survival and change in body weight following transfers from fresh water to various dilutions of sea water, or from one dilution of sea water to another. The two Italian toads were used for survival study only.

(2) On plasma and urine samples: Δ, Cl, Na, K and, on some plasma samples, urea. Samples were stored frozen at −20° C. Chloride was determined in 10 μl. aliquots using the method of Schales & Schales (1941). Urea was measured in 10 μl. aliquots using the colorimetric method of Friedman (1953).

(3) Electrical potentials and short-circuit currents across isolated pieces of belly skin. A Hewlett–Packard Model 412A electronic voltmeter was used for both potential

and current measurements. A 3 V. dry battery and precision variable resistor were used for short-circuiting the skins. Precision of potential measurements was ± 1 mV.; of current measurements ±0·01 μA./mm.² (113 mm.² skin area across opening of cell).

(4) On duplicate 150–250 mg. samples of muscle dissected from the thighs:

(*a*) *Total solids.* Samples were weighed before and after drying for 24 hr. at 105° C. Precision ± 2 g./kg. wet weight.

(*b*) *Sodium.* Dried muscle samples were thoroughly digested in small volumes of concentrated nitric acid and 30% hydrogen peroxide, first for several hours at room temperature then to complete digestion in a boiling-water bath. Samples were then evaporated to dryness over several days in a 60° C. oven and finally redissolved in known volumes of glass-distilled water. Sodium analysis was by flame photometer on final diluates. Precision ± 2 m-equiv./kg. wet weight.

(*c*) *Potassium.* By flame photometer on final diluates. Precision ± 2 m-equiv./kg. wet weight.

(5) Preference of the animals themselves for concentration of their external medium (Gordon *et al.*, 1961 for procedure).

RESULTS

Survival and weight changes

Data on survival and percentage changes in body weight of Yugoslavian toads transferred from fresh water to various concentrations of external medium, and from one sea-water concentration to another, are presented in Fig. 1.

Virtually no mortality occurred over periods of 2 weeks following transfers from fresh water to concentrations up to 40% sea-water (15‰ salinity). Direct transfers from fresh water to 50% sea water (19‰ salinity) and higher concentrations were uniformly fatal within 6–24 hr.

Acclimatization to 40% sea water for a week increased tolerance to 50% sea water somewhat. Heavy mortality only began to occur among groups of such acclimatized toads 3 days after transfer to the higher concentration. Insufficient numbers of animals were available to permit determining whether any of these toads could survive for longer periods in 50% sea water.

The two toads collected near Naples tolerated much higher salinities. Both animals survived with no apparent ill effects 4 days in 40% sea-water, then 4 days in 50% sea water. After 4 days more in 60% sea water (23‰ salinity), the smaller of the two animals died. The larger, still in apparent good condition, was then transferred to 70% sea water. It died between 12 and 24 hr. later.

Compared with controls maintained in fresh (Naples tap) water, weight changes following tolerable transfers from fresh water were almost completely eliminated within 24–48 hr. The small increase in weight in toads transferred to 20% sea water may have been the result of drinking, or of the temporary accumulation of urine in the bladder. The sharp decrease in weight of toads transferred to 40% sea water was probably due to osmotic removal of water. The rapid recovery from this loss may have been due to drinking. Toads transferred to 50% sea water and higher concentrations showed no sign of recovery from even larger weight losses during their short periods of survival.

Second transfers of toads after a week's acclimatization, from 20 to 40% sea water and from 40 to 50% sea water, produced decreases in weight which were only partly compensated after 2 days. Osmotic losses of water across the skin were probably again the cause of these changes.

All these results from Yugoslavian toads are similar to those obtained by Stoicovici & Pora (1951) in comparable short-term experiments with a Rumanian freshwater population of *B. viridis*. The greater salinity tolerance of the two Italian toads indicates that their population may be similar in its abilities to the Rumanian brackish water population (Stoicovici & Pora, 1951).

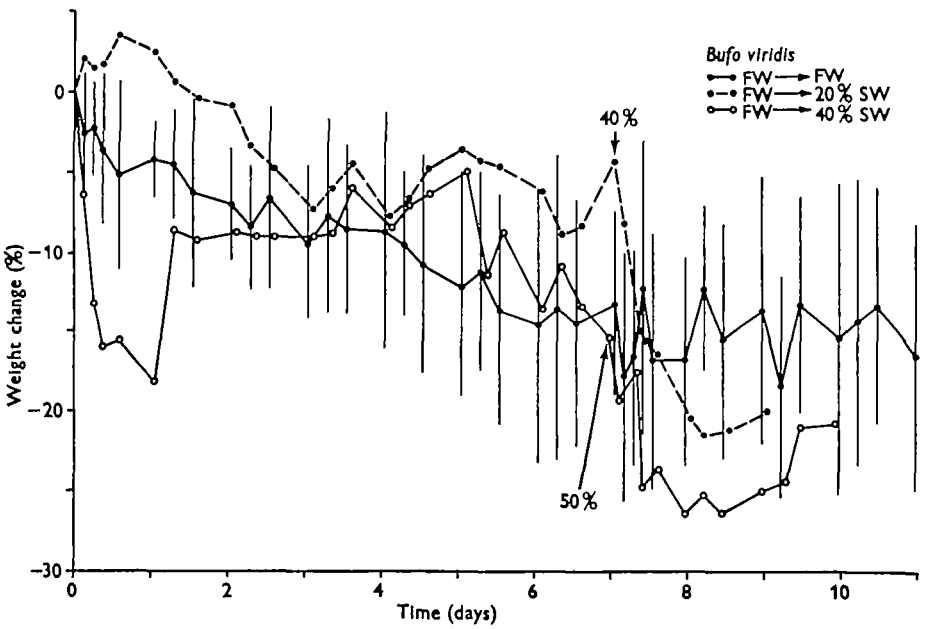

Fig. 1. Mean percentage weight changes in groups of *B. viridis* acclimatized to fresh water, then transferred to fresh water (controls), 20 and 40% sea water. After the passage of 1 week, at the points indicated by arrows, the toads in 20% sea water were transferred to 40%, the toads in 40% were transferred to 50%. Vertical lines on freshwater points indicate ±2 S.E.'s of the means. S.E.'s of other points comparable, but omitted for clarity

Plasma and urine concentrations

Results of analyses for Δ, Cl, Na, K and, for some plasma samples, urea of plasma and urine in variously acclimatized *B. viridis* are summarized in Table 1 and Fig. 2. The toads in 50% sea water had previously been acclimatized to 40% sea water for a week.

The plasma of *B. viridis* is hypertonic to the external medium over the range of tolerable salinities, but the amount of hypertonicity decreases as the concentration rises. The increase in concentration above freshwater levels appears due primarily to increased NaCl. Plasma osmotic concentration increased by 300 m-osmoles/l. between toads in fresh water and toads in 50% sea water. The sum (Na + Cl) in these two groups differed by 253 mM., or 84% of the total difference. The difference between plasma urea levels in these two groups made up another 5–10% of the total.

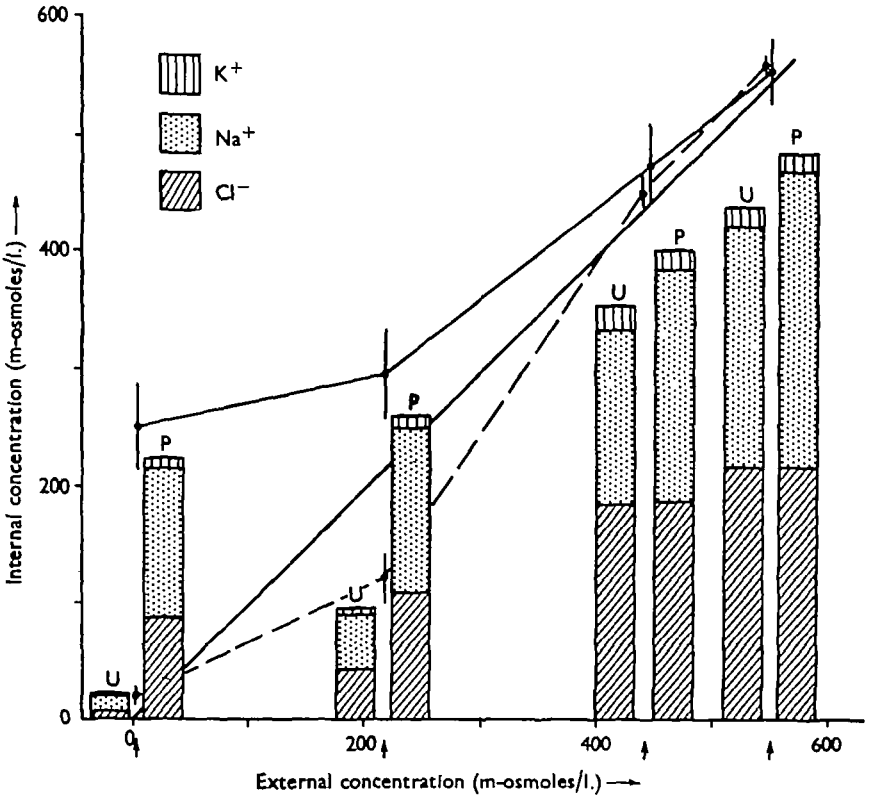

Fig. 2. Plasma (P) and urine (U) osmotic concentration, Cl, Na and K in variously acclimatized *B. viridis*. Sampling times, numbers of analyses, and precision as indicated in Table 1. Upper continuous line, plasma osmotic concentration; middle line, equality between internal and external concentrations; lower dashed line urine osmotic concentration. Vertical lines on each point, ±2 S.E.'s of means. Right-hand bar in each pair, plasma concentrations; left-hand bar, urine concentrations. Four groups are toads in fresh water and 20, 40 and 50% sea water. Arrows along abscissa mark actual acclimatization concentrations.

Table 1. *Plasma and urine concentrations in* Bufo viridis

State of acclimatization	Concentrations [$\bar{X}\pm$S.E. (N)] Δ (m-osmoles/l.)	Cl (m-equiv./l.)	Na (m-equiv./l.)	K (m-equiv./l.)	Urea (m-moles/l.)
Plasma					
Freshwater, 3+ days	247±18 (5)	86±3 (5)	129±2 (5)	9±1 (5)	35±10 (2)
20% sea water, 5 days	295±18 (5)	109±4 (5)	141±4 (5)	8±1 (5)	—
40% sea water, 6 days	472±16 (5)	188±7 (5)	199±3 (5)	13±2 (5)	—
50% sea water, 2 days	549±15 (5)	219±7 (5)	249±8 (5)	14±1 (5)	60± 5 (3)
Urine					
Freshwater, 3+ days	22±4 (5)	7±1 (5)	16±5 (5)	1±0·02 (5)	—
20% sea water, 5 days	122±10 (5)	42±6 (5)	49±5 (5)	5±1 (5)	—
40% sea water, 6 days	457±8 (5)	182±4 (5)	150±6 (5)	21±6 (5)	—
50% sea water, 2 days	563±1 (5)	219±4 (5)	200±6 (5)	19±4 (5)	—

The urine of *B. viridis* is very hypotonic to the blood in more dilute media, but becomes isotonic in the higher concentrations. These increases in urine concentration are again primarily due to NaCl, but not to quite so great an extent as for the blood changes. Urine osmotic concentration increased by 540 m-osmoles/l. between fresh water and 50% sea water. The sum (Na+Cl) in these two groups increased by 400 mM., or 74% of the total difference. Urinary Cl was identical with plasma Cl levels in toads in 40 and 50% sea water. Urinary Na was 50 mM. lower than plasma levels in the same two groups.

No measurements were made of rates of urine production, but large volumes (up to several ml.) were readily obtained even from toads in 50% sea water.

The only analyses for osmotically significant substances carried out by Stoicovici & Pora (1951) were a number of plasma Cl determinations and a very few urine Cl measurements. They found no differences between their freshwater and brackish water populations in this regard. Where direct comparisons are possible, the present data are virtually identical with the earlier observations. All populations of *B. viridis* thus seem to use the same ionic regulatory mechanisms.

Electrical potentials and short-circuit currents across the skin

Table 2 summarizes the results of measurements of electrical potentials and short-circuit currents across isolated pieces of abdominal skin from variously acclimatized *B. viridis*. All measurements were made within 5 min. of removal of the skins. Ringer

Table 2. *Electrical properties of the skin of* Bufo viridis

State of acclimatization	Electrical property [$\bar{X} \pm$ S.E. (N)]	
	Potential (mV.) (inside positive)	Short-circuit current density (μA./mm.^{2})
Fresh water, 3+ weeks	20±1 (5)	0·38±0·08 (5)
20% sea water, 2+ weeks	13±6 (5)	0·09±0·05 (5)
40% sea water, 2 weeks	10±2 (5)	0·06±0·03 (5)
50% sea water, 2 days	3±1 (4)	0·02±0·01 (4)

solutions of 100, 120, 140 and 140% of the concentration used by Adrian (1956) were used, respectively, on both sides of the skins of toads from fresh water, 20, 40 and 50% sea water.

Independent of the concentration of the external medium, skins developed lasting potentials such that their serosal surfaces were positive. The magnitude of this potential decreased with increase in external concentration. This behaviour would be consistent with an inwardly directed sodium pump of the type usual in freshwater amphibia, decreasing in rate of pumping with increase in external salt concentration.

Short-circuit current measurements on the skins were also consistent with this interpretation. Similar inverse relationships between skin short-circuit currents and external salt concentrations have been described in two freshwater frogs (*Rana esculenta*, *R. tigerina*) by Maetz (1959) and Gordon *et al.* (1961).

A few measurements of skin short-circuit currents were made on variously acclimatized *B. viridis* not starved for as long as the toads used for the observations listed in Table 2. These observations indicate that the currents developed by skins from

relatively unstarved toads in a given medium can be as much as 3 to 5 times larger than the currents developed by skins from starved toads. The decrease in current with increase in external salt concentration existed in the less starved toads as well.

Water content and ionic concentrations in the muscles

The results of measurements of the amounts of solids, Na and K in skeletal muscles of variously acclimatized *B. viridis* are presented in Table 3. These data substantiate and extend the data on muscle water, mineral and organic content presented by Stoicovici & Pora (1951).

No satisfactory estimates of extracellular volumes in the muscles of *B. viridis* are available (muscle samples were too small to permit sufficiently precise Cl analyses). Even in the absence of this information, however, the data in Table 3 indicate disproportionalities between changes in blood concentrations and changes in muscle concentrations.

The osmotic concentration of the blood increased by 191% between toads in fresh water and toads in 40% sea water (Table 1). The total solid content of the muscles increased by only 167% between the same two media. Plasma Na increased by 154%

Table 3. *Muscle concentrations in* Bufo viridis

State of acclimatization	Concentration [$\bar{X} \pm$ S.E. (N)] Total solids (g./kg. wet wt.)	Na (m-equiv./kg. wet wt.)	K (m-equiv./kg. wet wt.)
Fresh water, 3+ weeks	165 ± 12 (5)	27 ± 1 (4)	72 ± 4 (4)
20% sea water, 2+ weeks	190 ± 6 (5)	36 ± 2 (4)	71 ± 2 (4)
40% sea water, 2 weeks	275 ± 2 (5)	59 ± 2 (4)	89 ± 5 (4)

between fresh water and 40% sea water, while muscle Na increased by 218%. Plasma K in the same two groups of toads increased by 145%, while muscle K increased by 124%.

Unless quite large changes in extracellular volume in the muscles occurred, these discrepant ratios indicate shifts in the distribution of water and salts which must have been due to active adjustments in intracellular concentrations of osmotically active substances. If such adjustments had not occurred and the muscle fibres had behaved simply like osmometers, the degree of dehydration to which intracellular constituents were exposed would have been greater.

Environmental salinity preference

The results of experiments in which groups of six *B. viridis* in various states of salinity acclimatization were given the opportunity to choose their own environmental salinity are summarized in Table 4. The results of the first 4 hr. of observations, taken by themselves, show the same pattern.

It is apparent that the Yugoslavian freshwater population of *B. viridis* studied, while able to survive in environmental salinities almost twice as high as those tolerable by more ordinary amphibia, prefers media of less than half its own maximum tolerable

salinity. Actually, excepting only the toads in 50% sea water, they really preferred sitting on moist sand to being in any aquatic environment.

Table 4. *Environmental salinity preference by* Bufo viridis

(Occurrences in each medium in 12 hr. (% of all observations).)

	State of acclimatization			
Medium	Fresh water, 5+ days	20% sea water, 6 days	40% sea water, 7 days	50% sea water, 2 days
Fresh water	20	8	17	54
20% sea water	12	18	27	10
40% sea water	6	4	0	0
60% sea water	1	1	1	0
80% sea water	1	1	0	0
100% sea water	1	0	0	0
Sand	59	68	55	36
No. observations	180	180	180	122

DISCUSSION

The osmoregulatory mechanism used by *Bufo viridis* shows both a significant similarity to, and several differences from, the mechanism used by *Rana cancrivora*. The similarity is the fact that neither species seems to have modified significantly the general water permeability and ion transport properties which appear to be characteristic of all amphibian skins. As a result of this situation the basis of high salinity tolerance in both forms has had to be a tolerance of high osmotic concentrations in the body fluids. The need for some supply of free water to permit excretion of metabolic wastes by kidneys apparently incapable of producing urine hypertonic to the blood has made the actual requirement a tolerance of body-fluid concentrations hypertonic to the external medium.

One of the major differences between the two species is the kind of tissue tolerance developed. *B. viridis* tolerates salt in much the same way as do various euryhaline crustaceans (Shaw, 1959) and polychaete worms (Smith, 1959). *R. cancrivora* tolerates some increase in blood salt levels, but relies mostly on the accumulation of urea, somewhat after the fashion of elasmobranch fishes.

Another difference is in the pattern of response of the animals' kidneys to elevated environmental salinities. *B. viridis* appears able to maintain urinary osmotic and salt concentrations significantly below plasma levels only in salinities below about 10‰. Above 10‰ the urine becomes isotonic with the plasma and the ability of the kidney to control urinary salt concentrations declines considerably—it apparently becomes nil with respect to chloride. *R. cancrivora*, on the other hand, produces urine significantly more dilute than the plasma even when in the highest tolerable salinities. *Cancrivora* also maintains urinary monovalent ion content at levels below 10% of plasma content. Differences in renal tubular reabsorptive capacities are presumably the basis for this contrast, combined with the much greater salt loads presented to the kidneys of *B. viridis*.

The ease with which large volumes of urine were obtained from *B. viridis* even when in 50% sea water argues for significant rates of urine production in higher salinity media. Combined with high urinary salt concentrations this would mean that green

toads in saline environments suffer severe continuing losses of the salt they need to maintain internal hypertonicity. This salt loss could be made up by drinking external medium (no obvious drinking was observed) and/or by active uptake and passive diffusion across the skin. Concentration gradients exist which could cause passive entrance of chloride, for instance, even for toads isotonic with their medium in 50% sea water. Environmental Cl in 50% sea water was 298 mM., plasma Cl was 219 mM. If passive diffusion is a significant part of the salt intake mechanism, differences in specific permeability of the skin to different ions could be part of the regulatory process controlling ionic ratios in the blood.

A feature of the present work is the quantitative agreement which exists at many points where comparisons are possible with the earlier work on a Rumanian freshwater population of *B. viridis*. This is especially true with regard to the patterns of changes in blood and urine salt concentrations. The agreement in this connexion includes also the brackish water population studied by Stoicovici & Pora (1951). This uniformity in overall osmotic and ionic regulatory patterns probably implies that the differences in salinity tolerance of the different populations are due to differences in tissue tolerance to elevated internal salinities. The toads which can withstand the highest external salinities are those which can tolerate the highest internal salinities. This is probably also the basis for the seasonal changes in salinity tolerance which occur within single populations.

The fact that the Yugoslavian toads did not do so well, in terms of maximum tolerable salinity, as either Rumanian group of toads is not surprising when one considers that both Rumanian groups came from areas relatively close to salt environments. The Yugoslav toads have probably not been less than several hundred miles from sea water in many thousands of years. What is very surprising, however, is that they showed any particular abilities in this regard.

B. viridis is probably a very old species of toad, if breadth of distribution is any measure of species age. It occurs from western Europe to central Asia in a broad area bounded by the latitudes of southern Scandinavia on the north and North Africa on the south (Kauri, 1948). Amphibians are not noted for their dispersal abilities, hence it seems probable that areas more or less toward the middle of this distribution have been populated by resident groups of green toads for a long time. We presently know nothing of how much gene flow there is between adjacent populations of this form, or of mutation rates in genic loci controlling characteristics such as osmoregulatory mechanisms. However, it seems quite unusual that the mechanisms permitting toleration of salinities almost twice as high as those fatal to virtually all other amphibia should still exist in freshwater populations. It is possible that greater salinity tolerance may be correlated with increased tolerance for desiccation (*B. viridis* usually lives in quite dry areas), but I know of no demonstration of such a correlation in any amphibian.

SUMMARY

1. The osmotic and ionic regulatory abilities of adults of a freshwater population of the green toad (*Bufo viridis*) have been studied. These toads tolerated environmental salinities as high as 19‰ at temperatures near 25° C. Two individuals of another population of this species tolerated salinities as high as 23‰.

2. Changes in body weight of toads transferred to different environmental salinities indicate that the skin of this form is permeable to water. Rapid return to control levels of body weight indicate that drinking of external medium may be an important part of the initial adjustment to high salinities.

3. Above salinities of about 8‰ plasma Δ rises with increasing environmental Δ. Marked hypertonicity of the plasma is maintained in low salinities, but isotonicity with the medium is approached in higher salinities. Increases in plasma concentration above freshwater levels are due primarily to increased NaCl concentration (about 84%), partly to increased concentrations of urea (5–10%) and other osmotically active substances.

4. Urinary Δ is much lower than plasma Δ in dilute media, but becomes identical with plasma Δ above salinities of about 15‰. Increases in urine concentration above freshwater levels are also due primarily to NaCl increase (74%). Considerable quantities of salt are lost via the urine. The kidneys seem to lose much of their ability to regulate urinary salt concentrations in high-salinity media.

5. Measurements of electrical potential and short-circuit current indicate that active uptake of inorganic ions by the skin continues in concentrated media, but at reduced rates.

6. Changes in muscle water, Na and K contents indicate the occurrence of some redistribution of water and salts between various body-fluid compartments as part of the salinity adaptation process.

7. In preference experiments, *B. viridis* chooses the land over any aquatic environment. Among aquatic environments it prefers those with salinities below 8‰.

8. When combined with some earlier data by other workers who studied other populations of *B. viridis*, the present data indicate great uniformity of ionic and osmotic regulatory abilities among populations of this species. The marked differences between salinity tolerances of different populations are indicated to be due to differences in tissue tolerance of high body-fluid salinities.

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