

## OSMOTIC REGULATION IN THE TADPOLES OF THE CRAB-EATING FROG (*RANA CANCRIVORA*)

BY MALCOLM S. GORDON AND VANCE A. TUCKER\*

*Department of Zoology, University of California, Los Angeles*

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### INTRODUCTION

The major features of the osmoregulatory mechanisms which form the basis for the unusually great salinity tolerance of the adults of the southeast Asian crab-eating frog (*Rana cancrivora*) have been described by Gordon, Schmidt-Nielsen & Kelly (1961) and by Schmidt-Nielsen & Lee (1962). Adults of this species respond to high external salinities with an increase in internal osmotic concentration, largely due to accumulation of urea.

Gordon *et al.* also found that the tadpoles of this frog appeared to have an even greater tolerance for high salinities than the adults, confirming the original observations of Pearse (1911). Since studies on other amphibians have led to a presumption that pre-metamorphic anuran larvae in general lack enzymic machinery for the production of significant amounts of urea (Bennett & Frieden, 1962, for review), the question of how *R. cancrivora* tadpoles adjust to high salinities is raised.

The present paper describes the unexpected pattern of osmotic regulation in the tadpoles of the crab-eating frog, and investigates the change from the larval to the adult pattern at metamorphosis. Ecological information on the role of salinity in the life history of the species is also presented. For comparative purposes, and to supplement the very meagre information in the literature on osmoregulation in the larvae of freshwater frogs, data are also presented for the tadpoles of the freshwater frog *R. limnocharis*, a species very similar morphologically to *R. cancrivora*.

### MATERIALS AND METHODS

An expedition was made to Thailand, lasting from June to the end of August 1963. This working period coincided with the first months of the rainy season and, therefore, of the breeding season for *R. cancrivora*. The description of the breeding biology and development of this species given by Alcalá (1962) for the Philippines holds in general for Thailand as well.

Tadpoles in various developmental stages were abundant in brackish ponds near high-tide marks in the mangrove swamps along the north shore of the Gulf of Thailand. Collection sites ranged from the mouth of the Chao Phya River due south of Bangkok to the village of Ang Hin, about 60 miles to the south-east. Adult frogs, both calling males and ripe females, were numerous around many of these ponds at night. However, neither egg-laying nor egg masses were observed.

The salinity of the ponds was measured with an Industrial Instrument Co. Model

\* Present address: Department of Zoology, Duke University, Durham, North Carolina.

RS-5 induction salinometer precise to  $\pm 0.2\%$ . Salinometer results were checked from time to time by chloride titration of water samples brought to the laboratory. Pond temperatures were determined to  $\pm 0.1^\circ\text{C}$ . with a thermistor built into the salinometer.

Tadpoles were transported in natural pond water to the laboratory at Chulalongkorn University in Bangkok. There they were maintained in their own pond water or acclimatized to fresh water, and to 20, 40, 60, 80, or 100% sea water. Acclimatization was carried out in steps by changing the salinity 20% every 2-3 days. The temperature of the room used for animal maintenance approximated to that of the outside air, ranging from  $31-33^\circ\text{C}$ . in mid-afternoon to  $27-28^\circ\text{C}$ . in the hours before dawn.

All sea water used was obtained directly from the Gulf of Thailand. The maximum salinity obtainable was  $32\%$  (= 100% sea water, =  $\Delta$ ,  $-1.72^\circ\text{C}$ ., = 930 m-osmoles/l.). It should be noted that this salinity is 3% lower than the laboratory sea water concentration called 100% l.s.w. by Gordon *et al.* (1961).

Fresh water (chloride concentration 0.04 mm/l.) used for dilutions was obtained from a canal adjacent to the laboratory and inhabited by a large fauna and flora. It was filtered through glass wool before use.

Tadpoles kept in the laboratory for more than a few days were fed on algae taken from a nearby canal. They were not fed during salinity acclimatization. Movements of food materials along the gut were apparently slow. Only the first one or two loops of intestine were emptied of solid contents in tadpoles unfed for 8-10 days.

Survival of tadpoles in the laboratory was only moderately good. A great part of the mortality observed seemed due to cannibalism, which occurred even in the presence of abundant algal foods.

*R. limnocharis* tadpoles were collected from a small freshwater ditch near the laboratory. Maintenance conditions were as for *R. cancrivora*.

Developmental stages discussed in this paper are based upon those established for *R. pipiens* by Taylor & Kollros (1946).

Samples of blood, generally mixed with small quantities of lymph and pericardial fluid, were collected directly from the heart. After careful blotting of the skin the pericardial cavity was opened, leaving the gill and peritoneal cavities undamaged. Most of the pericardial fluid was blotted up, the heart was snipped with scissors, and the blood was collected in lightly heparinized glass capillary tubes. Plasma, separated by centrifugation, was stored frozen at  $-20^\circ\text{C}$ .

Cloacal fluid was obtained by inserting fine polyethylene catheters (PE 10) into the cloacal opening. The surrounding skin was first carefully blotted dry. The samples were immediately transferred to the bottom of a clean polyethylene dish filled with heavy paraffin oil. All samples containing solid matter or blood cells were discarded.

Fluid from the first turn of the intestine (hereafter called the foregut), as close to the oesophagus as possible, was obtained by dissecting open the gut cavity, displacing the rest of the intestine, blotting the area dry, raising the desired section of intestine with jeweller's forceps, and then puncturing the intestinal wall with a clean, dry 27-gauge hypodermic needle. The samples taken into the needle by capillarity were then handled in the same way as the samples of cloacal fluid. All samples containing blood cells were discarded.

Freezing-point measurements were made on samples of both cloacal and foregut

fluids within a few hours after the samples were taken. The samples were too small to permit of any other analyses.

Freezing-point depression ( $\Delta$ ), chloride (Cl), sodium (Na) and potassium (K) were determined with the methods and precision described by Gordon (1962), except that sample volumes for ion analyses were 1  $\mu$ l. Comparisons between as nearly pure samples of lymph, pericardial fluid and blood plasma as could be obtained demonstrated no significant differences in composition between these fluid compartments.

The effects of different salinities on development of fertilized eggs were investigated. Eggs stripped from ripe female *R. cancrivora* were fertilized with sperm contained in *breis* made from excised ripe testes. Groups of adult frogs acclimatized to 20 and 60% sea water were used. Eggs and sperm from the 20% sea-water frogs were mixed in 20% sea water. Eggs and sperm from some 60% sea-water frogs were mixed in 20% sea water, others in 60% sea water. After mixing for 10 min., groups of 50–100 eggs were placed in Petri dishes containing 50 ml. each of water ranging from fresh water to 100% sea water in 20% increments. The dishes were then observed at intervals.

## RESULTS

### *Field observations*

Salinities in the ponds from which *R. cancrivora* tadpoles were obtained ranged from 6 to 24% (19–75% sea water). Salinities were affected by many influences, especially the time elapsed since the last heavy rain. Pond water temperatures varied from 26 to 35° C.

Tadpoles ranging in development from stages III–XIX were obtainable throughout our stay in Thailand, and stages XX–XXV were obtainable by the end of June. The majority of individuals in a particular pond were, however, in about the same stage of development. The size of tadpoles in the pre-metamorphic stages XIV–XVII varied considerably from pond to pond. There was a correlation between high pond salinity and large tadpole size.

### *Osmoregulatory mechanisms*

*Rana cancrivora* tadpoles are good osmoregulators. Plasma  $\Delta$  increased by only 250 m-osmoles/l. in the face of increases in environmental  $\Delta$  of over 900 m-osmoles/l. (Fig. 1). The Na, Cl, and K content of the plasma accounted for 90–100% of the total osmotic concentration in all salinities, leaving little if any 'space' for urea. Table 1 summarizes the data for  $\Delta$ , Cl, Na and K of plasma samples taken from pre-metamorphic tadpoles (stages XIV–XVII) acclimatized to various salinities from fresh water to 100% sea water. Tadpoles survived moderately well and were active in all salinities used.

Cloacal fluid from tadpoles in fresh water had an osmotic concentration less than half that of the plasma (Table 1). This fluid was easily obtained in relatively large volumes. However, only small volumes were obtainable from the cloacas of tadpoles in 60 and 100% sea water. Differences between the osmotic concentrations of these samples and plasma concentrations were not statistically significant.

Foregut fluid from tadpoles in fresh water had only one-ninth the osmotic concentration of the plasma (Table 1). These tadpoles were, therefore, probably swallowing some medium. Tadpoles in 60% sea water had foregut fluid significantly more

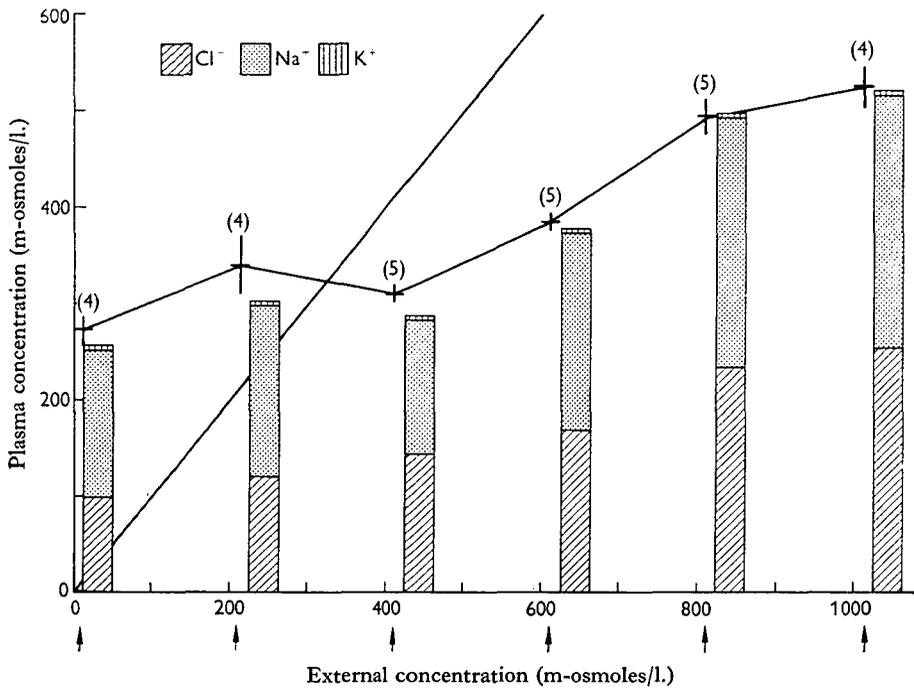


Fig. 1. Plasma osmotic concentration, Cl, Na and K in variously acclimatized tadpoles of *R. cancrivora* in developmental stages XIV–XVII. All samples taken after at least 48 hr. acclimatization to each environment. Diagonal solid line, line of equality between internal and external concentrations. Solid line joining crosses (—+—), plasma osmotic concentration. Horizontal bars of crosses indicate means for indicated numbers of observations; vertical bars  $\pm 2$  S.E. of mean. Six groups are tadpoles in fresh water and in 20, 40, 60, 80 and 100% sea water. Arrows along abscissa mark actual acclimatization concentrations.

Table 1. *Composition of body fluids in tadpoles (stages XIV–XVII) of Rana cancrivora*

State of acclimatization (%)	Concentrations [ $\bar{x} \pm$ S.E. (N)]			
	$\Delta$ (m-osmoles/l.)	Cl (m-equiv./l.)	Na (m-equiv./l.)	K (m-equiv./l.)
SW		Plasma		
o (FW)	272 $\pm$ 14 (4)	99 $\pm$ 3 (5)	153 $\pm$ 6 (4)	5.0 $\pm$ 0.9 (4)
20	339 $\pm$ 30 (4)	120 $\pm$ 3 (5)	179 $\pm$ 4 (3)	6.3 $\pm$ 0.2 (3)
40	309 $\pm$ 9 (5)	142 $\pm$ 3 (5)	141 $\pm$ 4 (4)	4.2 $\pm$ 0.6 (5)
60	385 $\pm$ 9 (5)	169 $\pm$ 8 (5)	207 $\pm$ 12 (5)	6.2 $\pm$ 0.2 (5)
80	494 $\pm$ 18 (5)	233 $\pm$ 6 (5)	263 $\pm$ 21 (4)	8.1 $\pm$ 0.6 (5)
100	523 $\pm$ 22 (4)	253 $\pm$ 15 (5)	265 $\pm$ 23 (3)	6.4 $\pm$ 0.6 (4)
		Foregut fluid		
o (FW)	30 $\pm$ 3 (4)	—	—	—
60	445 $\pm$ 12 (5)	—	—	—
80	568 $\pm$ 35 (5)	—	—	—
		Cloacal fluid		
o (FW)	124 $\pm$ 47 (5)	—	—	—
60	406 $\pm$ 14 (5)	—	—	—
100	662 $\pm$ 48 (4)	—	—	—

concentrated than the plasma (at the 1% level by 't' test), while samples from animals in 80% sea water were not significantly more concentrated than plasma. These data are equivocal, but suggest that drinking of hyperosmotic media may occur.

Several attempts were made to demonstrate drinking by tadpoles in 60% sea water by deeply staining their medium with chlorphenol red. No dye was visually detectable in the guts of six animals, either in daylight or under an ultraviolet lamp, after periods of more than 60 hr. in the dye solutions.

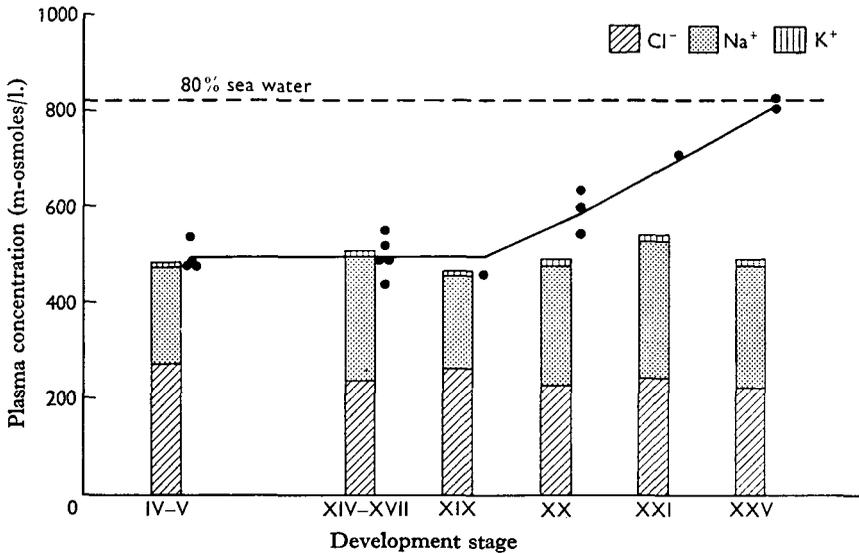


Fig. 2. Plasma osmotic concentration, Cl, Na and K in tadpoles of *R. cancrivora* uniformly acclimatized to 80% sea water at developmental stages IV–XXV. Solid circles (●) represent individual observations of plasma  $\Delta$ ; the line joining these groups passes through the means for the groups. Horizontal dashed line is osmotic concentration of 80% sea water. Other symbols as for Fig. 1.

Table 2. Composition of plasma of tadpoles of *Rana cancrivora* acclimatized to 80% sea water at various stages of development

Stage of development	Concentrations [ $\bar{x} \pm$ s.e. (N) or single values]			
	$\Delta$ (m-osmoles/l.)	Cl (m-equiv./l.)	Na (m-equiv./l.)	K (m-equiv./l.)
IV–V	492 $\pm$ 11 (4)	269 $\pm$ 4 (4)	197; 206	6.0; 7.1
XIV–XVII	494 $\pm$ 18 (5)	233 $\pm$ 6 (5)	263 $\pm$ 21 (4)	8.1 $\pm$ 0.6 (5)
XIX	456	259	193	5.8
XX	583 $\pm$ 23 (3)	225 $\pm$ 14 (3)	252 $\pm$ 10 (3)	8.2; 8.2
XXI	700	240	284	9.0
XXV	805; 820	197; 245	282; 222	10.2

Fig. 2 and Table 2 summarize plasma  $\Delta$ , Cl, Na and K in tadpoles ranging in developmental stage from IV to XXV inclusive when all stages were uniformly acclimatized to 80% sea water. No statistically significant variations in the plasma ionic concentrations were found. However, starting with stage XX and continuing until metamorphosis was complete at stage XXV, plasma osmotic concentration

increased progressively until the plasma was isosmotic with the environment. The plasma composition in stage XXV is only slightly different from that found in adult frogs acclimatized to the same salinity. It was unfortunately not possible to obtain sufficient plasma from stage XXV froglets to permit determination of urea concentrations. However, it seems likely that the difference between total osmotic concentration and osmotic concentration attributable to inorganic salts is due to urea.

Tadpoles of *Rana limnocharis* (stage XIV) survived in media up to 30% sea water, but died within 5 hr. after being placed in 40% sea water. They regulated both osmotic and chloride concentrations of plasma quite well over the range of tolerable salinities (Fig. 3). In this respect they resembled the adults of most freshwater frogs. The upper limit for salinity tolerance was apparently reached when plasma and medium became isosmotic.

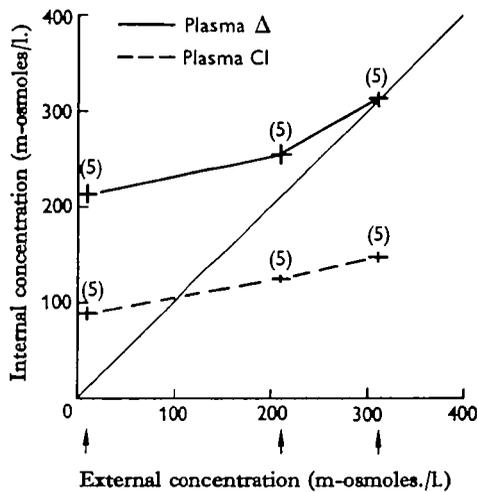


Fig. 3. Plasma osmotic concentration and Cl in variously acclimatized tadpoles of *R. limnocharis* in developmental stage XIV. Symbols as for Fig. 1, except that lower dashed line joins means of groups of Cl measurements. Three groups are tadpoles in fresh water and in 20 and 30% sea water.

#### *Laboratory observations on embryology and metamorphosis*

Development of artificially fertilized eggs in the laboratory was limited to the first few cleavages. Even this small amount of development, however, occurred only in eggs from adults in 20% sea water, fertilized in 20% sea water, then placed in either fresh water or 20% sea water. All other groups of eggs rapidly became distorted or shrank noticeably.

Tadpoles kept in the laboratory metamorphosed during the first week of captivity or not at all. Metamorphosis occurred most frequently in fresh water and 20% sea water. Groups of 10 pre-metamorphic tadpoles were acclimatized to fresh water, and to 20, 40, 60, 80 and 100% sea water. Only a few metamorphoses occurred, and these were restricted to tadpoles in fresh water and 20% sea water.

## DISCUSSION

The osmoregulatory ability of *R. cancrivora* tadpoles is unique among amphibians and is similar to that shown by euryhaline teleost fishes such as the rainbow trout (Gordon, 1963). It is unlikely that the integument is completely impermeable to water. The large osmotic gradients maintained across the body surfaces imply continuous flooding in hyposmotic media and dehydration in hyperosmotic environments. By analogy with euryhaline teleosts, one would expect production of large quantities of urine hyposmotic to the blood in the former situation, and small quantities of urine isosmotic with the blood in the latter. The foregut contents might be dilute in hyposmotic media due to adventitious ingestion of water with food, and hyperosmotic in hyperosmotic media due to ingestion of water to compensate for osmotic dehydration.

Tadpoles of *R. cancrivora* generally fulfil these expectations. The evidence for drinking of hyperosmotic media is suggestive but inconclusive. However, in the absence of direct active transport of water, absorption of a hyperosmotic medium depends on isolating a small volume of it. The digestive tract is the most likely region of the body where isolation could occur.

If hyperosmotic media are drunk, then the fluid ingested must be desalted in some way. The analyses of cloacal fluid indicate that neither the kidneys nor the gut are used for salt excretion. The gills of teleosts and the integument of amphibians have long been studied as sites of active salt transport. Either of these organs could be sites of salt excretion in *R. cancrivora* tadpoles. The correlation between the change-over to the adult osmoconforming pattern with the loss of the gills during metamorphosis is at least suggestive. Adolph (1927*a, b*) similarly interprets the development of volume-regulating ability in the tadpoles of the freshwater *R. pipiens* and *R. catesbeiana*. Krogh, Schmidt-Nielsen & Zeuthen (1938) considered the gills to be the principal site of active ion uptake in tadpoles of *R. temporaria* in early developmental stages.

Histological examination of fixed and stained (haematoxylin and eosin) gills taken from tadpoles acclimatized to fresh water, and to 60 and 80% sea water, demonstrated no signs of 'acidophil cells' similar to those often found in the gills of teleost fishes.

The apparent requirement for dilute media during early embryonic development and at metamorphosis must await further studies for its explanation. One might speculate, however, that the lack of gill development in the former period, and the loss of gills during the latter period, are involved.

The osmoregulation of *R. cancrivora* tadpoles is in striking contrast to the osmoconformity shown by the adults of the same species (Gordon *et al.* 1961; Schmidt-Nielsen & Lee, 1962). The tadpoles appear to have traded the metabolic demands associated with the extensive urea synthesis required by the adults (Gordon & Tucker, in preparation) for the metabolic demands associated with active transport of inorganic ions.

The difference in the mechanisms of salinity tolerance between tadpoles and adults suggests that a shift from ammonotelism to ureotelism occurs in this species, as in freshwater anurans, at metamorphosis (Bennett & Frieden, 1962, for review). At metamorphosis thyroid hormones influence the development of urea-cycle enzymes in the liver of the freshwater frogs *R. catesbeiana* and *R. clamitans* (Brown, 1962;

Brown, Brown & Cohen, 1959; Forster, Schmidt-Nielsen & Goldstein, 1963; Paik & Cohen, 1960). Similar changes probably occur during metamorphosis of *R. cancrivora*. However, *R. cancrivora* does not appear to develop tubular secretion of urea (Schmidt-Nielsen & Lee, 1962) as occurs in *R. clamitans* (Forster *et al.* 1963). It is interesting to note that in *R. cancrivora* the apparent change in nitrogen metabolism *causes* a change from a situation in which water is in short supply (dilute body fluids in concentrated media) to one in which an osmotic supply of water is available (body fluids of adults are always somewhat hyperosmotic to the medium). The usual presumption has been that nitrogen metabolism changes as a *result* of changes in availability of water.

Our observations in the laboratory indicate that the initial stages of embryonic development and metamorphosis are interfered with by salinities greater than 20% sea water. These observations, together with field data, suggest that the torrential summer rains of Thailand play an important role in the developmental biology of this frog. Spawning may occur only during or soon after heavy rains when the salinity of the spawning pools is low. We spent several nights collecting dozens of frogs with ripe gonads at the edges of the spawning pools but never observed amplexus. Spawning could have been restricted to periods of heavy rainfall, when we did not collect. The general synchrony of developmental stages in a single pond suggests that spawning could have been synchronized by a period of heavy rain.

Tadpoles in the laboratory usually did not metamorphose if the medium was more concentrated than 20% sea water. In the field the largest immediately pre-metamorphic tadpoles were found in the saltiest ponds. These observations suggest that metamorphosis may be delayed as long as the pond salinity is high.

These requirements for dilute media, and the freshwater nature of all other ranids, make it seem probable that *R. cancrivora* has invaded the marine environment from fresh water in relatively recent times. The high temperatures of its spawning ponds would permit rapid embryonic development and metamorphosis when torrential monsoon thunderstorms temporarily dilute the ponds. Its otherwise great salinity tolerance permits this frog and its tadpoles to enter a rich environment closed to all other amphibians.

#### SUMMARY

1. Salinity tolerance, osmoregulatory mechanisms and some effects of salinity on embryonic development and metamorphosis have been studied in the tadpoles of the euryhaline crab-eating frog (*Rana cancrivora*).

2. Tadpoles in developmental stages III–XIX survive well in all salinities from fresh water to 32‰ sea water. They are good osmoregulators over this entire range, plasma osmotic concentration changing by only 250 m-osmoles/l. in the face of environmental concentration changes of 900 m-osmoles/l. Na, Cl, and K account for over 90% of plasma osmotic concentration in all external concentrations.

3. The osmoregulatory mechanisms used by the tadpoles appear to be similar to those used by euryhaline teleost fishes. In hyperosmotic media they probably drink, then excrete salts by an extrarenal pathway.

4. A metamorphosis in osmoregulatory mechanisms occurs simultaneously with morphological metamorphosis. The osmoconforming behaviour of adult frogs is

fully developed in the tadpoles by developmental stage XXV. As in other anuran species there probably is no significant urea production before metamorphosis.

5. Both early embryonic development and the completion of metamorphosis beyond stage XIX apparently require low salinities.

6. The pattern of osmoregulation shown by tadpoles of the freshwater *R. limncharis* is identical with osmoregulatory patterns found in adult freshwater anurans.

7. Evolutionary and ecological implications of the data are discussed.

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#### REFERENCES

- ADOLPH, E. F. (1927*a*). Changes of body volume in several species of larval amphibia in relation to the osmotic pressure of the environment. *J. Exp. Zool.* **47**, 163-78.
- ADOLPH, E. F. (1927*b*). Changes in the physiological regulation of body volume in *Rana pipiens* during ontogeny and metamorphosis. *J. Exp. Zool.* **47**, 179-95.
- ALCALA, A. C. (1962). Breeding behaviour and early development of frogs of Negros, Philippine Islands. *Copeia*, no. 4, 679-726.
- BENNETT, T. P. & FRIEDEN, E. (1962). Metamorphosis and biochemical adaptation in amphibia. In M. Florkin and H. S. Mason (eds.), *Comparative Biochemistry* 4B, 483-556.
- BROWN, G. W., JR. (1962). Comparative biochemistry of urea synthesis. IV. (<sup>14</sup>C) urea synthesis by liver slices of the metamorphosing tadpole. *Biochim. Biophys. Acta*, **60**, 185-6.
- BROWN, G. W., JR., BROWN, W. R. & COHEN, P. P. (1959). Comparative biochemistry of urea synthesis. II. Levels of urea cycle enzymes in metamorphosing *Rana catesbeiana* tadpoles. *J. Biol. Chem.* **234**, 1775-80.
- FORSTER, R. P., SCHMIDT-NIELSEN, B. & GOLDSTEIN, L. (1963). Relation of renal tubular transport of urea to its biosynthesis in metamorphosing tadpoles. *J. Cell. Comp. Physiol.* **61**, 239-44.
- GORDON, M. S. (1962). Osmotic regulation in the green toad (*Bufo viridis*). *J. Exp. Biol.* **39**, 261-70.
- GORDON, M. S. (1963). Chloride exchanges in rainbow trout (*Salmo gairdneri*) adapted to different salinities. *Biol. Bull., Woods Hole*, **124**, 45-54.
- GORDON, M. S., SCHMIDT-NIELSEN, K. & KELLY, H. M. (1961). Osmotic regulation in the crab-eating frog (*Rana cancrivora*). *J. Exp. Biol.* **38**, 659-78.
- KROGH, A., SCHMIDT-NIELSEN, K. & ZEUTHEN, E. (1938). The osmotic behaviour of frog's eggs and young tadpoles. *Z. vergl. Physiol.* **26**, 230-8.
- PAIK, W. K. & COHEN, P. P. (1960). Biochemical studies on amphibian metamorphosis. I. The effect of thyroxin on protein synthesis in the tadpole. *J. Gen. Physiol.* **43**, 683-96.
- PEARSE, A. S. (1911). Concerning the development of frog tadpoles in sea water. *Philippine J. Sci.* **6**, 219-20.
- SCHMIDT-NIELSEN, K. & LEE, P. (1962). Kidney function in the crab-eating frog (*Rana cancrivora*). *J. Exp. Biol.* **39**, 167-77.
- TAYLOR, A. C. & KOLLROS, J. J. (1946). Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.* **94**, 7-23.