Quantitative evaluation of water balance in *Bufo arenarum* young tadpoles after acute exposure to concentrated NaCl solutions: a multivariate approach

LUCRECIA FERRARI*[1,2]

[1] Applied Ecophysiology Program, Biology Division, Basic Sciences Department, National University of Luján, Luján, Argentina.
[2] Scientific Research Commission, La Plata, Buenos Aires, Argentina.

In order to quantify the regulatory responses involved in water balance of amphibians immersed in high salinity media, wet weight, dry weight and water content were measured in Bufo arenarum young tadpoles exposed to NaCl solutions from 70 to 271 mOsm. Water balance was evaluated by a multivariate analysis of variance, with multiple comparisons and multiple discriminant analysis.

The first 24 h constituted a critical period in animals' acclimation to the new media. After the initial period, the effect of osmolarity on the tadpoles becomes independent of exposure times. A compensatory response was observed upon exposures to 70 mOsm external media. An osmoconforming behavior seems to appear at higher osmolarity values. The physiological parameter with best discriminant power in response to environmental osmolarity alterations was dry weight, both in ionic and in non-ionic solutions, but the behavior of hydric regulation variables is different according to the chemical nature of the media.

Key terms: Bufo arenarum *tadpoles, NaCl, multivariate analysis, water balance.*

INTRODUCTION

Due to its reproductive and developmental habits, the first stages in the life of most amphibians are exposed to an environment with changing salinity (Duelman & Trueb, 1983). Thus, the salinity of the medium is an important factor in the developmental ecology of the group (Padhye & Ghathe, 1992). As anuran tadpoles are unable to increase plasma osmolarity through the synthesis of organic osmolites, the compensatory response –when they are in a medium of higher than usual salinity– depends almost entirely upon increases in the concentration of plasmatic NaCl. Regulation of the water balance is possible, but when the plasma becomes hyperosmotic with regard to the environment, death by hypernatremia occurs (Balinsky, 1981). If the environment is not electrolytic, water balance should not be possible and tadpoles behave as limited osmoconformers (Katz, 1987).

Water balance in amphibians has mostly been studied from the perspective of feed-back mechanisms. Therefore, in the face of changes in the internal environment such as intracellular or extracellular dehydration, compensatory mechanisms begin to function that return that variable to its reference values (Shpun *et al*, 1992; Warburg & Rosenberg, 1990). Such studies have been undertaken analyzing different aspects, both *in vitro* and *in vivo*, at the level of

* **Correspondence to:** Dra Lucrecia Ferrari, Programa de Ecofisiología Aplicada, División Biología, Departamento de Ciencias Básicas, Universidad Nacional de Luján, Casilla de Correo 221, (6700)-Luján (B), Argentina. Fax: (54-323) 25795. E-Mail: prodea@unlu01.edu.ar

neuroendocrine, ethological and biochemical regulation and of adaptive strategies among other perspectives (Ardizzone & Lippe, 1985; Degani, 1985; Degani & Nervo, 1986; Gasser & Miller, 1986; Katz *et al*, 1984; Katz & Hoffman, 1989; Shoemaker *et al*, 1992). In every case the results were analyzed in an univariate manner, contrasting the effect of the treatment upon each of the variables under consideration with the responses of an untreated control. However, unless the organism is considered as an integral unit, in which every variable is interrelated and interdependent, the dimensionality of the variation among different treatments is overestimated. Therefore, the correct approach must be necessarily multivariate, considering the simultaneous covariation of each variable under study (Ferrari & Lombardo, 1990). In a previous study we have quantified the answer of the water balance in *Bufo arenarum* tadpoles exposed to non electrolytic osmotic stress through a multivariate approach (Ferrari *et al*, 1995). This work will attempt –under the same experimental design– to quantify the water balance in *Bufo arenarum* young tadpoles exposed to electrolytic acute osmotic stress.

METHODS

Animals' breeding methods, experimental design, sampling schedule and statistical analysis of the results were described in a previous paper (Ferrari *et al*, 1995). In the present experiments NaCl solutions of 70, 141, 204, 247 and 271 mOsm in distilled water were prepared. Control series of experiments in artificial pond water (APW 5 mOsm) were also run (Alvarado & Johnson, 1966). Osmotic pressures of solutions were checked with a Fiske osmometer.

Experiments were carried out in duplicates without previous osmolarity acclimation, during five days. One hundred sixty tadpoles of stage 26 (Gosner, 1960) were placed in glass containers (320 tadpoles per tested solution). The density of 4 g organism/l solution was kept constant throughout the experiment. As determined in previous tests, this density does not modify the survival rate as compared with the density recommended for bioassays by standard methods (APHA, 1992) of 1 g organism/l solution (Ferrari, 1995). Solutions were renewed daily.

Every 24 hours, larvae from each experimental solution were sampled and their wet weight (ww), dry weight (dw) and water content were determined. The water content (wc) was expressed as H_2O/mg dw and as % of body mass (H). These two expressions are slightly different: wc is an absolute value referred to the water content –that allows comparisons within a particular group of experimental animals–, while H is a relative parameter –that allows comparisons between different experimental groups.

The comparisons between integrated responses to different incubation times and osmolarities were carried out through a multivariate approach, under factorial design, using multiple analysis of variance (MANOVA) with multiple comparisons and multiple discriminant analysis (MDA) (Morrison, 1976; Dixon, 1981; Hair, 1995).

RESULTS

Data from replicate experiments were considered together since there was significant difference between them. The standard deviations for each one of the parameters considered at each time, were near to 10 % of the means in all cases studied (Tables I to III).

Table IV shows *F* values for the MANOVA tests applied to results obtained from tadpoles immersed in each given solution as a function of time, and for those exposed to different solutions at each given time. The results of the multiple comparisons are shown for each case in Figures I and II.

Results obtained with MANOVA show that for exposure to each solution as a function of time, the integrated behavior of the parameters under analysis varies, reaching highly significant differences ($p< 0.01$). The MANOVA for exposures to different osmolarities at each given time shows significant differences only at 24 and 48 h. As of 72 h (when tadpoles

Table I

Means and SD's of wet weight of tadpoles exposed to each assayed solution at each sampling time. Means of eight pooled samples made of four larvae each.

Solutions		0 h	24 h	48 h	72 h	96 h	120 h	144 h
APW		48.19 ± 3.42	40.45 ± 4.35	39.18 ± 2.86	36.58 ± 2.65	34.66 ± 4.12	31.24 ± 2.64	39.88 ± 4.48
NaCl	70 mOsm	48.19 ± 3.42	40.94 ± 3.51	37.14 ± 3.11	36.16 ± 3.51	32.82 ± 4.13	29.84 ± 2.92	37.41 ± 4.20
	141 mOsm	48.19 ± 3.42	43.03 ± 3.26	38.90 ± 2.76	36.60 ± 2.69	31.50 ± 2.67	30.90 ± 4.04	
	204 mOsm	48.19 ± 3.42	40.96 ± 2.47	44.84 ± 5.48	40.45 ± 2.25			
	247 mOsm	48.19 ± 3.42	39.69 ± 3.62	38.95 ± 3.82				
	271 mOsm	48.19 ± 3.42	34.07 ± 2.42					

APW, artificial pond water.

Table II

Means and SD's of dry weight of tadpoles exposed to each assayed solution at each sampling time. Means of eight pooled samples made of four larvae each.

Solutions		0 h	24 h	48 h	72 h	96 h	120 h	144 h
APW		1.47 ± 0.13	1.87 ± 0.22	1.84 ± 0.14	1.76 ± 0.11	1.82 ± 0.20	1.51 ± 0.21	1.61 ± 0.14
NaCl	70 mOsm	1.47 ± 0.13	2.08 ± 0.13	1.77 ± 0.18	1.68 ± 0.22	1.67 ± 0.15	1.45 ± 0.18	1.46 ± 0.14
	141 mOsm	1.47 ± 0.13	2.09 ± 0.22	1.80 ± 0.11	1.86 ± 0.19	1.64 ± 0.14	1.52 ± 0.18	
	204 mOsm	1.47 ± 0.13	2.16 ± 0.20	2.24 ± 0.21	2.01 ± 0.11	1.52 ± 0.18		
	247 mOsm	1.47 ± 0.13	2.14 ± 0.25	1.99 ± 0.14				
	271 mOsm	1.47 ± 0.13	1.89 ± 0.22					

APW, artificial pond water.

Table III

Means values and SD's of water content (related to dry mass) of tadpoles exposed to each assayed solution at each sampling time. Means of eight pooled samples made of four larvae each.

Solutions		0 h	24 h	48 h	72 h	96 h	120 h	144 h
APW		31.76 ± 2.15	20.71 ± 1.89	20.38 ± 2.69	19.81 ± 0.96	18.07 ± 2.17	20.11 ± 3.58	23.27 ± 2.19
NaCl	70 mOsm	31.76 ± 2.15	18.67 ± 1.08	20.16 ± 1.40	20.87 ± 1.64	18.66 ± 2.01	19.94 ± 3.64	24.37 ± 1.99
	141 mOsm	31.76 ± 2.15	19.67 ± 1.65	20.69 ± 1.23	18.80 ± 1.50	18.44 ± 1.71	19.40 ± 2.21	
	204 mOsm	31.76 ± 2.15	17.73 ± 0.90	18.90 ± 2.04	18.43 ± 0.92			
	247 mOsm	31.76 ± 2.15	17.63 ± 1.24	18.49 ± 1.36				
	271 mOsm	31.76 ± 2.15	17.11 ± 1.25					

APW, artificial pond water.

Table IV

Multiple analysis of variance (MANOVA) of results from exposures to each given solution for different intervals, and to different solutions at each given time.

Solution / Time		*d.f.*	F
APW		24.144	11.38 **
NaCl	70 mOsm	24.151	8.38 **
	141 mOsm	29.124	14.54 **
	204 mOsm	12.45	9.28 **
	247 mOsm	8.30	15.79 **
	271 mOsm	4.9	66.17 **
Interval:	24 h	20.127	3.28 **
	48 h	16.86	2.47 *
	72 h	9.58	2.61 ns
	96 h	8.36	0.91 ns
	120 h	8.36	0.34 ns
	144 h	4.7	1.13 ns

d.f., degrees of freedom; **F**, ratio F from Fisher test; **, $p < 0.01$; *, $p < 0.05$; **ns**, no statistical significance

survive exposed to 204, 141 and 70 mOsm), there are no significant statistical differences between the experimental solutions and the control.

Discriminant analysis of tadpoles' responses to exposure to different solutions.

The values of eigenvectors for the first and second canonical variables (CV) and their cumulative proportions of the total dispersions (as percentages) are reported in Table V. For each CV, the eigenvectors of the four physiological parameters measured are shown; the best discriminant capacity corresponded to the vector of higher absolute value. Figure 1 shows the canonical discriminant analysis for each solution assayed and the diagrammatic representation of the multiple comparisons results.

For exposures to each solution, there are highly significant differences between time 0 and 24 h of exposure. For immersions in APW (Fig 1A), we can observe two well defined periods as a function of time: from 24 to 72 h and from 48 to 144 h, within which there are no statistically significant differences; however, a trend to return to initial conditions is evidenced. Differences are mainly explained by the dry weight.

Results obtained from exposures to experimental solutions (Figs 1B, C, D, E and F) present a behavior very close to the control group and there is a trend towards grouping for all times, which becomes more marked at higher osmolarities. The principal discriminant parameter resulted to be dry weight (Table V).

From the integrated analysis of the variables, it could be concluded that –after the initial 24 h period– the effect brought by osmolarity upon tadpoles is independent from time.

Discriminant analysis of the time course of tadpoles' responses.

Figure 2 shows the canonical discriminant analysis for each considered time and the diagrammatic representation of multiple comparison results. In analyzing the

Table V

Canonical discriminant analysis: evaluation of the hydric balance by means of an integrated response of *Bufo arenarum* young tadpoles incubated in different solutions.

	APW		70 mOsm		141 mOsm		204 mOsm		247 mOsm		271 mOsm
	CV1	CV2	CV1	CV2	CV1	CV2	CV1	CV2	CV1	CV2	CV
ww	0.87	-0.06	0.84	-0.44	0.46	-0.12	0.11	0.11	-0.05	0.06	0.08
dw	17.08	7.58	-17.71	16.25	-8.09	9.20	-3.10	4.30	1.27	1.27	2.32
wc	0.60	-30.30	0.41	0.67	1.25	-0.19	0.80	0.35	0.66	1.05	0.37
H	-8.09	3.46	-6.40	1.25	-7.02	3.38	-2.24	-1.16	0.39	-6.30	1.83
VAR	0.85	0.95	0.96	0.96	0.85	0.99	0.98	0.99	0.99	1.00	1.00

APW, artificial pond water; **ww**, wet weight; **dw**, dry weight; **wc** and **H**, water content; **CV1** and **CV2**, eigenvectors for first and second canonical variables; **VAR**, cumulative proportion of total dispersion.

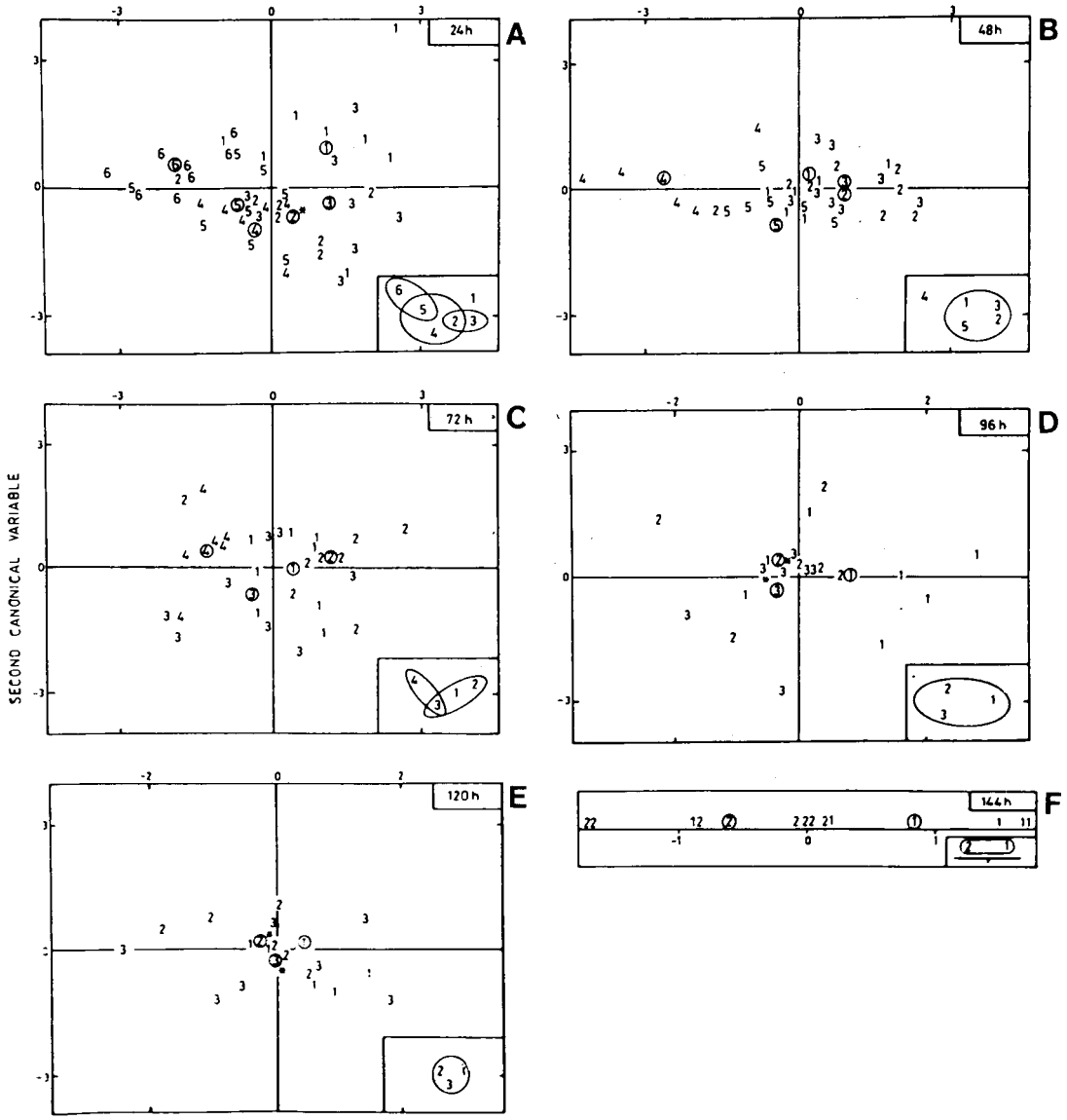

Fig 1. Multiple discriminant analyses for results obtained with exposures to control (**A**; APW, artificial pond water) and NaCl solutions: 70 mOsm (**B**); 141 mOsm (**C**); 204 mOsm (**D**); 247 mOsm (**E**); and 271 mOsm (**F**). Numbers, relative positions of animals (expressed as all physiological parameters measured, at each evaluation time). Encircled numbers, mean values at each time. Values at right bottom corners, means for each time. Numbers within circles do not differ statistically in multiple comparisons tests. Evaluation times: **0**, 0 h; **1**, 24 h; **2**, 48 h; **3**, 72 h; **4**, 96 h; **5**, 120 h; **6**, 144 h.

simultaneous behavior of the four variables studied by using MANOVA and multiple comparisons, groups are defined as concentration sets that show absence of significant statistical differences.

The values of eigenvectors for CV1 and CV2, as well as the cumulative proportion of the total dispersion, are given in Table VI. Figure 2 illustrates the canonical discriminant analysis for each time assayed. At 24 h of exposure (Fig 2A), exposures to experimental solutions show a clear separation from that to control solution. In accordance with the results of the multiple comparison analysis, the statistical significance of these differences increases with osmolarity. There is a grouping between neighboring osmolarities; thus we have two groups, 70-141 mOsm and 204-247 mOsm. The relationship between both groups is furnished by the lack of statistical significance between exposures to 70, 204

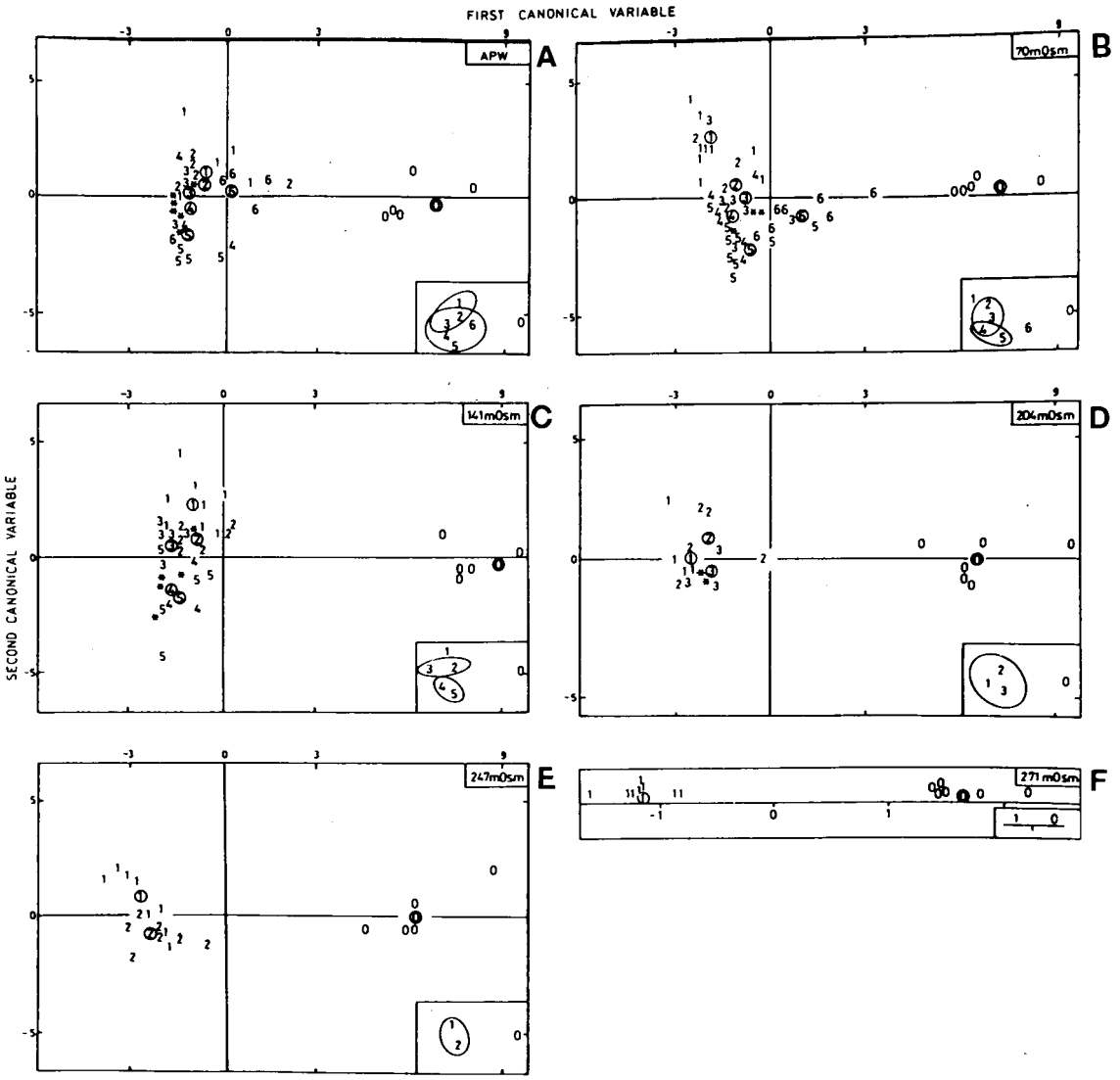

Fig 2. Multiple discriminant analyses for results obtained with exposures at different intervals: 24 h (**A**); 48 h (**B**); 72 h (**C**); 96 h (**D**); 120 h (**E**); and 144 h (**F**). Numbers, relative positions of solutions at each evaluation time. Encircled numbers, means for each solution. Values at right bottom corners, means for each solution. Numbers within circles do not differ statistically in multiple comparisons tests. Solution: **1**, APW; **2**, 70 mOsm; **3**, 141 mOsm; **4**, 204 mOsm; **5**, 247 mOsm; **6**, 271 mOsm.

and 247 mOsm; the behavior of tadpoles immersed in 141 and 204 mOsm NaCl solutions differed by a significance value of 95%.

We can then conclude that there are no significant differences between exposures to NaCl 70 and 141 mOsm, at none of the sampled times. As of 48 h of exposure, there is a grouping of results obtained with exposures to almost all experimental solutions with those to control solution. Nevertheless, it is important to notice that as the compensatory behavior of the parameters under study in the experimental solutions approach the control one– death of the tadpoles occurs. This takes place at 48 h for tadpoles immersed in 247 mOsm NaCl solution, at 72 h for those exposed to 204 mOsm solution and at 120 h for those immersed in 141 mOsm solution (Fig 2). In every case, differences can be accounted for by the dry weight (Table VI).

Table VI

Canonical discriminant analysis: evaluation of the hydric balance by means of an integrated response of *Bufo arenarum* young tadpoles to all the assayed solutions, for each time considered.

	24 h		48 h		72 h		96 h		120 h		144 h
	CV1	CV2	CV1	CV2	CV1	CV2	CV1	CV2	CV1	CV2	CV
ww	0.35	-1.08	0.11	0.29	-0.47	0.40	1.29	2.56	-2.75	-1.65	-1.01
dw	-3.42	16.45	-9.36	-4.48	-4.73	5.40	32.08	-49.23	59.01	29.09	32.42
wc	-0.51	2.72	-1.54	1.37	0.13	-0.57	0.78	-3.16	1.15	1.30	4.94
H	2.72	-2.25	5.90	-6.51	0.48	6.20	5.92	-4.39	12.31	3.77	-22.30
VAR	0.66	0.89	0.87	0.96	0.74	0.92	0.83	1.00	0.72	1.00	1.00

ww, wet weight; **dw**, dry weight; **wc** and **H**, water content. **CV1** and **CV2**, eigenvectors for first and second canonical variables; **VAR**, cumulative proportion of total dispersion.

DISCUSSION

The integrated response of water balance regulation mechanisms of young *Bufo arenarum* larvae was studied through changes in wet weight, dry weight and water content induced by the transference to and incubation in NaCl solutions from 70 to 271 mOsm of osmotic pressure. Under our experimental conditions, the first 24 hours were clearly a critical period in the adaptation of animals to the new media. The observed differences were more evident as external osmolarity increased.

The initial responses to NaCl exposures were similar to those found in exposures to non-electrolytic solutions of the same osmolarity. *Bufo arenarum* young tadpoles possess physiological mechanisms to partially overcome severe osmotic stress conditions (Ferrari *et al*, 1995). The results of this study indicate that the initial response to osmotic stress is independent of the nature of the incubation media. The main parameter of the discriminating function is dry weight, which increases at higher osmolarities, though in the same magnitude and with a different behavior than those of exposures to the same osmolarities of non-electrolytical (mannitol) solutions. When tadpoles are incubated in NaCl solutions, the dry weight presents a two-phase behavior of decrease as a function of time until reaching 141 mOsm, and increase at higher osmolar values (Table II). The integrated behavior of the variables indicates a trend to stabilize along time, which is more noticeable at higher osmolarities in which they come on a level with the control. It is interesting to see that as this takes place, survival is impossible (Fig. 2). At 70 mOsm, the integrated behavior of the variables drifts slightly away from the control. This would indicate an osmoconforming behavior in NaCl solutions of osmolarities higher than 70 mOsm, in a different way than that observed in a non-electrolytic solutions (Ferrari *et al*, 1995).

Under similar experimental conditions, the behavior of hydric regulatory variables is very different according to the chemical nature of the media: whereas for high osmolarities with mannitol, the wet weight and water content at each sampling time always stay far from those of control conditions (Ferrari *et al*, 1995), upon exposures to NaCl solutions within the same osmolarity range, these variables remain close to those of control conditions (Tables I and III). This suggests the unleashing of different hydric balance regulation mechanisms in accordance with the nature of the environment.

In general, the osmolarity of the environment for most amphibians is found between 10 and 15 mOsm. When tadpoles are exposed to external osmotic pressures higher than the internal ones (approximately 200 mOsm), they are incapable of

osmoregulation (Burggren & Just, 1992). This agrees with our finding that tadpoles' mortality increases when the osmolarity of the incubation media is higher than 200 mOsm, in spite of the fact that the behavior of the physiological variables is close to control in the case of NaCl enriched media and far from it in the case of mannitol enriched media.

The principal osmoregulatory activity of tadpoles is carried out through their skin. Ion interchange can be either passive due to the electrochemical gradient or active (Na^+K^+-ATPase dependent). In young anuran tadpoles the level of this enzyme is very low (Kawada *et al*, 1969); thus, it can be considered that the only sodium transport mechanism is passive (Warburg & Rosenberg, 1990). This evidence suggests that tadpoles' death in ionic solutions with osmolarities higher than 200 mOsm probably occurs due to the passive entrance of Na^+ to the tadpole's internal *milieu*, which becomes isoosmotic with the external medium. This would also accompany the continuous loss of water. Therefore, death would be caused by hypernatremia. Then, 200 mOsm must be the upper limit of the osmoconforming capacity of these animals, independently of the chemical nature of the solutes of the external medium.

Changes in water content can be produced by independent alterations of the dry and wet weights. Our results indicate that osmotic regulation in tadpoles immersed in both ionic and non-ionic solutions may be reached by simultaneous changes in dry and wet weights, but dry weight being dominant.

The results shown in this work, together with those reported previously (Ferrari *et al*, 1995), allow us to reach the following conclusions: *i-* the initial response (0 to 24 h) to osmotic stress is similar for exposures to NaCl and mannitol solutions; *ii-* independently from the chemical nature of the incubation media, 200 mOsm may be considered the maximum tolerable limit, for immersions in ionic or non-ionic media; *iii-* in the response to osmotic stress without acclimation, the dry weight is the parameter with best discriminant power for immersions both in ionic and non ionic media; nevertheless, the magnitude of change in dry weight is smaller in NaCl solutions than in mannitol solutions; *iv-* from an integrated point of view, the response of the parameters evaluated differs according to the chemical composition of the solution, suggesting that different compensating mechanisms are unleashed, probably linked to the presence of Na^+ in the media.

ACKNOWLEDGMENTS

We wish to thank Dr Alfredo Salibián for directing the present work, Prof Rubén Lombardo for statistical advice and Eng Roberto Yoshihara for technical help.

This work was supported by grants from CONICET, CIC and the National University of Luján (UNLu).

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