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

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Bufo arenarum *young tadpoles were exposed to D-mannitol solutions from 0 to 271 mOsm under acute conditions for 10 days. The water balance condition was evaluated measuring the wet weight, dry weight, water content, as percentage of the body weight and related to dry body mass. Results were analyzed by a multivariate analysis of variance with multiple comparisons and multiple discriminant analysis. The first 24 h constituted a critical period in the acclimation of animals to the new media. Different degrees of compensatory response were observed particularly in 70 mOsm. The parameter with best discriminant power in response to environmental osmolarity alterations was the dry weight.*

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

INTRODUCTION

Most adult amphibians are considered typical freshwater vertebrates, practically intolerant to external saline concentrations higher than those found in pond water. However, a considerable number of both anuran and urodele species have been reported to be able to survive in somewhat brackish waters (Bentley, 1971; Duellman and Trueb, 1986).

The same capacity to tolerate higher external salinities than those of freshwater habitats was also reported in larvae of Anura and Urodela. In these cases the upper level of the osmotic tolerance range seemed to be slightly lower than in adults.

On the other hand, we have demonstrated that embryos and larvae of *Bufo arenarum* are able to survive in distilled water (Castañé *et al*, 1987; Ferrari and Salibián, 1987).

This wide range of salinity tolerated by adults and larvae of amphibians suggests the existence of mechanisms that potentially enable them to colonize different environments with dissimilar availability of water and ions or to overcome seasonal adverse ecological situations reflected in salinity changes in the environment.

The aim of this work was to study the water balance of *Bufo arenarum* young tadpoles exposed to non electrolytic acute osmotic stress; wet weight, dry weight and water content (as percentage of the body weight and related to dry mass) where the measured parameters and the responses were analyzed through an integrated multivariate analysis.

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A preliminary report of these results has been published elsewhere (Ferrari *et al*, 1988).

METHODS

Animals' breeding.

Ovulation of three females was induced by injection of an homologous hypophysis suspension into the coelomic cavity. Oocytes were fertilized *in vitro* with sperm of two males and the obtained embryos were maintained in 10% Holtfreter solution (Hamburger, 1969) until the larval stage was reached. Tadpoles were transferred to artificial pond water (APW) of the following composition (in mM): NaCl, 1.3; $CaCl_2$, 0.8; KCl, 0.1 and $NaHCO_3$, 0.2 (Alvarado and Johnson, 1966) and kept at 18-22°C. At this temperature range, growth rate was low so that larvae remained at the same stage throughout the experimental period.

Experimental design

We used tadpoles at the first larval stage (stage 26) (Echeverría and Fiorito de López, 1981). All tests were conducted with animals acclimated 48 h before the beginning of the experiment at constant temperature (20 ± 1°C) in a Lauda bath, and remaining under the same condition throughout the experiment. Animals stayed unfed during the experiments.

D-mannitol solutions of 70, 141, 176, 204, 247 and 271 mOsm in distilled water were prepared; osmotic pressures were checked with a Fiske osmometer; control series of distilled water (DW), APW and D-mannitol 5 mOsm (5 mOsm MAN) were run; the latter corresponded to the APW osmolarity.

Experiments were carried out in duplicate without previous osmolarity acclimation. At the beginning 160 tadpoles were placed in glass containers (320 tadpoles per assayed solution). A ratio of one larvae per 4 ml of solution was kept constant throughout the experiment. Solutions were renewed daily.

Sampling

Larvae were taken out from the solution by means of Pasteur's pipettes. At the end of the temperature acclimation period, a first sampling was made. It was considered representative of the initial condition (APW 5 mOsm). During the experimental period and for each assayed solution, daily samples were taken during the first week; a final one was taken on the 10th day. In order to lessen the weighing error due to the low weight of animals at this stage, each sampling unit was composed of 4 tadpoles. It is noteworthy to mention that at this stage the size and age of larvae are very homogeneous.

Each sampling included 8 units (4 in duplicate) corresponding to a total of 32 tadpoles for each solution.

Sampled animals were rinsed in distilled water for 1 min, after which they were put in aluminum paper cones and weighed in an analytical balance (± 0.01 mg). Each sample (made of 4 larvae) was drained out and the wet weight (**ww**) in mg was determined. After drying the sample at 100 °C for at least 12 h, the dry weight (**dw**) was determined and expressed in mg. The water content (**wc**) was estimated by the difference between wet and dry weights, and expressed as mg H_2O/mg dw, as well as % of body mass (**H**) (see Fig 1). The two expressions used to indicate water content are slightly different: **wc** is an absolute value refefred to the water content, that allows comparisons within a particular group of experimental animals, while **H** is a relative parameter that allows comparisons among different experimental groups.

Statistical analyses

All morphological variables were simultaneously considered through a multivariate

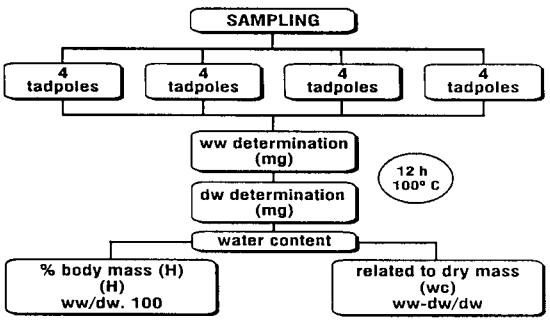

Fig 1. Flow diagram of the experimental design.

approach. The physiological responses to different solutions during the incubation period were evaluated by means of multivariate analysis of variance (MANOVA) (Morrison, 1976). The evaluation of the integrated responses of animals to environmental conditions was carried out using multiple discriminant analysis (MDA) (Dixon, 1981). MDA allow the graphic representation of both position and orientation of integrated responses of tadpoles, i.e. to compare the reaction to different incubation times with osmolarities as well as osmolarities to each other; it also allows to find out the variables with best discriminant ability to detect significant differences in the responses of animals incubated in different solutions.

RESULTS

The data from replicate experiments were considered together since in no case was there significant difference among them. The standard deviations for each of the considered parameters and each time were in all cases lower than 10 % of the means (Tables I to III).

A. 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 proportion of the total dispersion (as percentage) are reported in Table IV. For each one of the CV, the eigenvectors of the four measured physiological parameters are shown; the best discriminant capacity corresponded to the vector of higher absolute value. Figures 2 and 3 show the canonical discriminant analysis for each assayed solution.

A.1. Control solutions. As shown in figure 2, the global behaviour of the parameters along time in APW was relatively uniform, showing a marked inflection after 24 h mainly due to changes of **H** (Table IV).

The integrated responses of the variables in MAN-5 mOsm had a tendency to be similar to that found in APW; in this case, the situation at the end of the experimental period was equivalent to that observed during the beginning of the experiment. The most important discriminant parameter resulted to be dry weight (Table IV).

In DW there was a difference in time due to changes in dry weight, and a temporary

TABLE I

Means and standard deviations of wet weight (**ww**) for each assayed solution at each sampling time.

Means of eight pooled samples made of four larvae.

Solutions	0 h	24 h	48 h	72 h	96 h	144 h	168 h	240 h
Control:								
APW	42.36 ± 3.13	40.73 ± 4.08	36.81 ± 3.88	35.02 ± 3.42	31.18 ± 3.25	30.03 ± 4.84	29.81 ± 3.88	29.25 ± 2.99
DW	42.36 ± 3.13	37.07 ± 3.86	33.08 ± 2.70	29.34 ± 2.65	24.40 ± 2.32	25.47 ± 4.08	23.82 ± 1.53	20.11 ± 4.44
D-mannitol:								
5 mOsm	42.36 ± 3.13	36.40 ± 3.86	32.62 ± 5.33	25.10 ± 2.50	25.50 ± 3.06	25.88 ± 2.17	27.77 ± 5.67	28.92 ± 3.27
70 mOsm	42.36 ± 3.13	27.92 ± 2.10	25.22 ± 3.53	25.34 ± 3.36	26.27 ± 2.38	24.03 ± 2.26	28.01 ± 4.57	27.52 ± 3.97
141 mOsm	42.36 ± 3.13	21.60 ± 3.41	25.12 ± 2.67	22.92 ± 4.03	22.64 ± 1.77	21.46 ± 3.05	23.70 ± 1.96	22.33 ± 2.65
176 mOsm	42.36 ± 3.13	24.77 ± 2.78	22.82 ± 2.55	22.78 ± 1.85	20.08 ± 3.20	22.75 ± 2.29	21.69 ± 2.11	
204 mOsm	42.36 ± 3.13	24.02 ± 3.31	22.39 ± 2.01	20.62 ± 3.14	20.53 ± 1.86	18.65 ± 0.52		
247 mOsm	42.36 ± 3.13	2.87 ± 3.17						
271 mOsm	42.36 ± 3.13	22.35 ± 2.22						

APW, artificial pond water; DW, distilled water.

TABLE II

Means and standard deviations of dry weight (**dw**) for each assayed solution at each sampling time.
Means of eight pooled samples made of four larvae.

Solutions	0 h	24 h	48 h	72 h	96 h	144 h	168 h	240 h
Control:								
APW	1.95 ± 0.15	1.94 ± 0.18	1.61 ± 0.15	1.66 ± 0.34	1.52 ± 0.16	1.39 ± 0.19	1.41 ± 0.13	1.30 ± 0.11
DW	1.95 ± 0.15	1.73 ± 0.11	1.47 ± 0.15	1.53 ± 0.26	1.38 ± 0.09	1.26 ± 0.10	1.22 ± 0.09	0.95 ± 0.10
D-mannitol:								
5 mOsm	1.95 ± 0.15	1.75 ± 0.09	1.55 ± 0.19	1.35 ± 0.08	1.35 ± 0.10	1.28 ± 0.18	1.35 ± 0.25	1.26 ± 0.24
70 mOsm	1.95 ± 0.15	1.77 ± 0.09	1.54 ± 0.07	1.50 ± 0.10	1.62 ± 0.15	1.35 ± 0.18	1.47 ± 0.22	1.28 ± 0.21
141 mOsm	1.95 ± 0.15	1.89 ± 0.22	1.68 ± 0.33	1.83 ± 0.26	1.58 ± 0.12	1.51 ± 0.13	1.49 ± 0.08	1.26 ± 0.17
176 mOsm	1.95 ± 0.15	2.10 ± 0.16	1.72 ± 0.20	1.90 ± 0.20	1.63 ± 0.15	1.70 ± 0.13	1.51 ± 0.21	
204 mOsm	1.95 ± 0.15	2.09 ± 0.23	1.69 ± 0.17	1.94 ± 0.23	1.67 ± 0.10	1.61 ± 0.08		
247 mOsm	1.95 ± 0.15	2.29 ± 0.31						
271 mOsm	1.95 ± 0.15	2.31 ± 0.24						

TABLE III

Means values and standard deviations of water content (**wc**, related to dry mass) for each assayed solution and each time sampled.
Means of eight pooled samples made of four larvae.

Solutions	0 h	24 h	48 h	72 h	96 h	144 h	168 h	240 h
Control:								
APW	20.80 ± 1.69	20.02 ± 1.31	21.47 ± 0.73	20.13 ± 2.47	19.48 ± 1.26	20.77 ± 1.87	20.19 ± 1.83	21.53 ± 2.13
DW	20.80 ± 1.69	20.36 ± 1.31	21.71 ± 2.96	18.43 ± 2.63	16.65 ± 1.51	19.54 ± 2.07	18.61 ± 1.45	19.81 ± 2.85
D-mannitol:								
5 mOsm	20.80 ± 1.69	19.83 ± 1.74	20.29 ± 4.53	17.70 ± 2.06	17.84 ± 2.07	19.45 ± 1.71	19.62 ± 2.11	22.17 ± 1.98
70 mOsm	20.80 ± 1.69	14.77 ± 1.22	15.39 ± 1.95	15.94 ± 2.11	15.32 ± 1.60	16.97 ± 2.46	18.06 ± 1.78	20.79 ± 2.78
141 mOsm	20.80 ± 1.69	10.46 ± 1.67	14.28 ± 2.00	11.50 ± 0.77	13.37 ± 1.31	13.23 ± 1.47	14.80 ± 1.00	16.88 ± 2.00
176 mOsm	20.80 ± 1.69	10.79 ± 0.99	12.32 ± 1.00	11.01 ± 0.80	11.39 ± 2.14	12.37 ± 0.94	12.58 ± 1.27	
204 mOsm	20.80 ± 1.69	10.46 ± 1.43	12.32 ± 1.69	9.63 ± 1.24	11.29 ± 0.80	10.57 ± 0.38		
247 mOsm	20.80 ± 1.69	8.92 ± 0.73						
271 mOsm	20.80 ± 1.69	8.73 ± 1.40						

TABLE IV

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

	CONTROL SOLUTIONS				**D-MANNITOL SOLUTIONS (mOsm)**											
	APW		**DW**		**5**		**70**		**141**		**176**		**204**		**247**	**271**
	CV1	**CV2**	**CV1**	**CV2**	**CV1**	**CV2**	**CV1**	**CV2**	**CV1**	**CV2**	**CV1**	**CV2**	**CV1**	**CV2**	**CV1**	**CV2**
ww	0.21	0.24	0.46	0.18	0.01	1.24	-1.39	-0.24	-1.45	0.25	0.39	0.02	-1.10	-0.03	0.41	0.36
dw	1.24	-5.19	-1.35	-4.47	-6.55	-25.56	21.22	8.97	18.44	-0.72	2.13	5.03	10.70	4.86	-4.00	-2.21
wc	-0.78	-0.63	-0.33	-0.48	-0.35	-2.08	0.01	-0.09	0.19	-0.07	-0.61	-0.12	-0.48	0.34	0.37	0.49
H	3.22	-1.39	-0.22	-1.26	0.28	0.33	5.43	0.64	3.16	-1.39	0.99	-0.09	2.96	-0.88	-0.42	0.78
VAR	0.86	0.95	0.89	0.96	0.78	0.97	0.63	0.91	0.83	0.96	0.84	0.97	0.94	0.99	1.0	1.0

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

Table V

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

	INCUBATION TIMES													
	24 h		48 h		72 h		96 h		144 h		168 h		240 h	
	CV1	CV2	CV1	CV2	CV1	CV2	CV1	CV2	CV1	CV2	CV1	CV2	CV1	CV2
ww	-0.11	0.26	0.28	0.98	0.44	0.37	0.97	1.19	0.26	0.66	-0.04	0.19	-0.12	1.97
dw	0.81	-5.82	-2.52	-15.70	-5.24	1.56	-15.58	-12.56	-5.83	-17.68	-0.03	-1.50	9.01	41.77
wc	1.23	-1.27	-0.31	-0.18	-0.10	0.47	-0.16	-0.93	0.12	-0.95	-0.50	1.36	1.34	-3.04
H	-0.61	1.52	1.34	-4.20	0.08	-2.12	-1.60	-1.86	0.12	-0.76	3.72	-4.43	-4.17	4.59
VAR	0.91	0.96	0.90	0.96	0.82	0.97	0.82	0.94	0.86	0.94	0.88	0.95	0.88	0.98

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

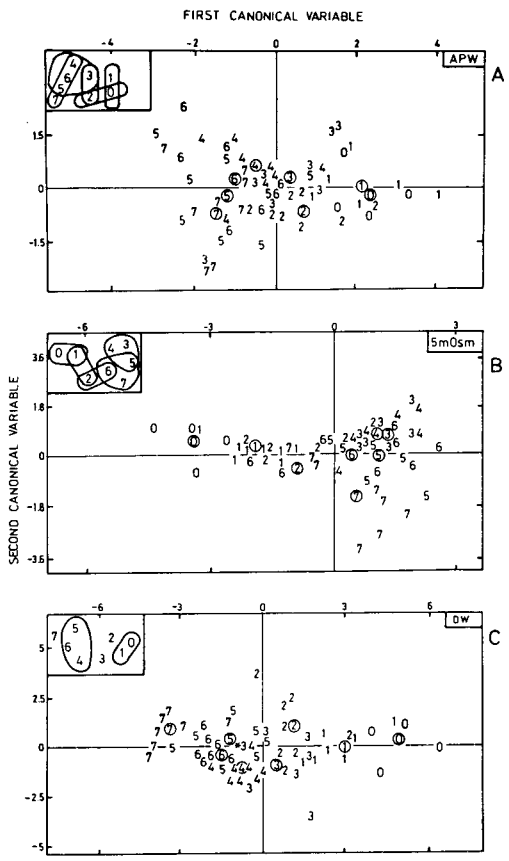

Fig 2. Multiple discriminant analyses for control solutions. **A**, artificial pond water (APW). **B**, D-mannitol 5 mOsm. **C**, distilled water (DW). Numbers, relative positions of animals (expressed as all physiological parameters measured, at each evaluation time.Encircled numbers, mean values at each time. Values at the upper left corners, means for each time. Numbers within circles do not differ statistically in the multiple comparisons tests. Evaluation times: **0**, 0 h; **1**, 24 h; **2**, 48 h; **3**, 72 h; **4**, 96 h; **5**, 144 h; **6**, 168 h; **7**, 240 h.

stabilization of the parameters between 24-48 and 96-168 h.

A.2. Experimental solutions. As shown in figure 3, in almost all cases the variable with the best discriminant power was the dry weight (Table IV). There was a clear cut tendency of the animals to re-establish the original conditions, with intermediate fluctuations, especially pronounced at 24 and 72 h.

In the 70-176 mOsm range, compensation after the seventh day was impossible; a similar behaviour was observed from the 6th day for 204 mOsm solution. It is noteworthy that from 24 h onwards, the tendency of the values was to unify with osmolarity increase.

B. Discriminant analysis of the time course of tadpoles' responses

In analyzing the simultaneous behaviour of the four studied variables 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 V. Figure 4 presents the canonical discriminant analysis for each time assayed.

After 24 h incubation, the water content (CV1) distinguished from controls, 70 mOsm

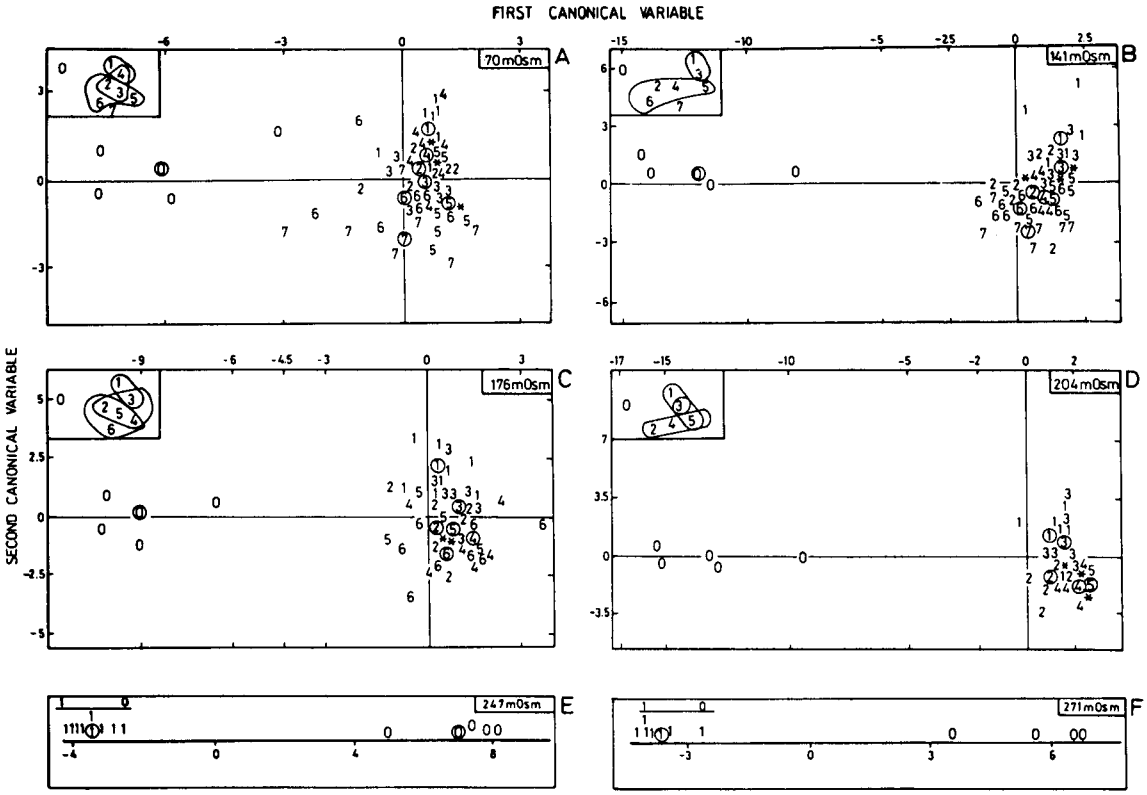

Fig 3. Multiple discriminant analyses for experimental D-mannitol solutions. **A**, 70 mOsm. **B**, 141 mOsm. **C**, 176 mOsm. **D**, 204 mOsm. **E**, 247 mOsm. **F**, 271 mOsm. Symbols and remaining details as in Fig 2.

and the remaining concentrations. Dry weight was the variable of highest discriminant incidence within concentrations higher than 70 mOsm. Dry weight (CV1) discriminated three well defined groups after 48 h of exposure: controls, intermediate (70 and 141 mOsm) and high (176 and 204 mOsm) osmolarities.

Twenty four hours later, APW was separated from the remaining two controls in their dry weight and humidity; 70 mOsm was not different from 5 mOsm MAN and the behaviour of all parameters together at 141 mOsm was similar to that observed in 176 mOsm.

After 96 h exposure APW was separated from the other controls. The integrated response of tadpoles at 141, 176 and 204 mOsm was similar; 70 mOsm was not different from 141 mOsm.

The responses at 144 and 168 h were similar in the control groups and 70 mOsm. The remaining solutions differed from those groups and from each other.

At 240 h, dry weight allowed to separate DW from the rest of solutions.

DISCUSSION

If we consider the organism as a system with several different interrelated variables, the correct approach to quantify differences between samples must be multivariate. The multiple discriminant analysis allows us to discriminate between various experimental groups and classify individuals where several variables have been quantified. The discriminant function represents the best way to integrate all measured variables linearly to obtain maximal difference among groups.

The water balance regulation mechanisms of young *Bufo arenarum* larvae were studied through alterations in wet weight, dry weight and water content as indicator parameters when transferred to, and incubated in solutions of a wide range of osmotic

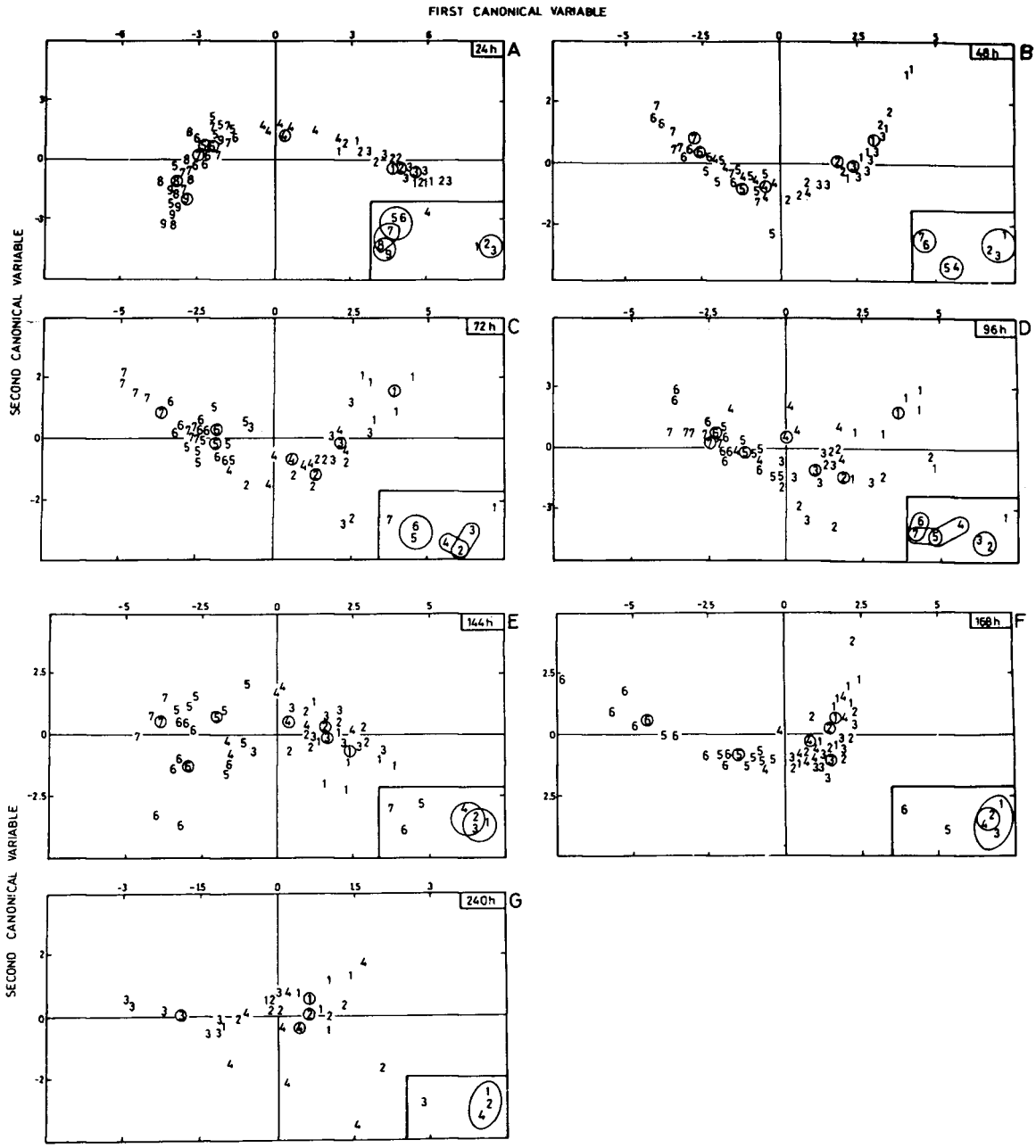

Fig 4. Multiple discriminant analyses for each assayed time. **A**, 24 h. **B**, 48 h. **C**, 72 h. **D**, 96 h. **E**, 144 h. **F**, 168 h. **G**, 240 h. Numbers, relative positions of solutions at each evaluation time. Encircled numbers: means for each solution. Values at the right bottom corners, means for each solution. Numbers within circles do not differ statistically in the multiple comparisons tests. **1**, APW. **2**, DW. **3**, D-mannitol 5 mOsm. **4**, 70 mOsm. **5**, 141 mOsm. **6**, 176 mOsm. **7**, 204 mOsm. **8**, 247 mOsm. **9**, 271 mOsm.

pressure. We also compared the effects of isoosmotic solutions made of ionic (APW) and non ionic (MAN) molecules. The experimental design allows us to gain an integrated evaluation of the adaptive mechanisms of larvae to those environmental changes.

Our results indicate that *Bufo arenarum* young tadpoles possess physiological mechanisms to overcome partially severe osmotic stress conditions. They survived for several days after a sudden osmotic change with osmolarities varying from 0 mOsm

(DW) to 204 mOsm (MAN solutions). This limit must be the upper limit of the osmoregulatory capacity of these animals.

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 the external osmolarity increased over the isoosmolarity level. On the contrary, the measured parameters in control larvae (APW and 5 mOsm-MAN) did not show significant changes.

In some cases, different degrees of compensatory responses were observed. With external osmolarities of 70 mOsm tadpoles reached control conditions at the end of the assayed time. The integrated compensatory response in these animals was gradual and completed after seven days.

In these experiments all measured parameters resulted similar after 24 hours incubation in 141, 176 and 204 mOsm. The values remained steady and in no case were compensation responses found; in addition, survival was shorter indicating that the osmoregulatory capacity of animals was not sufficient.

Continuous incubation of larvae in high osmotic pressure solutions must provoke a dehydration type response (Katz *et al*, 1984). When the external osmolarity was further increased to 247 mOsm, survival of the animals was reduced to less than 48 hours and the integrated response of the physiological variables was far from those measured at the beginning of the experiment.

External high osmolarity with mannitol decreases cellular volume, apical conductance and sodium transport in toad skin (Gazitua *et al*, 1988); in *Bufo viridis* skin, the acclimation in non electrolyte solutions decreases skin osmotic permeability (Katz, 1987). If we consider that tadpoles exposed to hyperosmolar non electrolytic media can not exchange ions with the external solution, they should lose water continuously; consequently, an adjustment of plasmatic osmotic pressure will be made through an increase in NaCl relative concentration, which will be higher with the osmolarity and with the increase of animal dehydration. In this way death may occur by hypernatremia (Balinsky, 1981; Schrock and Hanke, 1979; Schrock *et al*, 1980). This mechanism could explain the low survival in higher osmolarity solutions.

Another adaptive mechanism to survive in high osmolarity media is to accumulate organic osmolytes like urea and free aminoacids. There is no evidence that the *Bufo arenarum* young tadpoles can synthesize urea in hyperosmotic media; however, a transient adaptive response to non electrolytic hyperosmolarity by rapid increase of the nitrogen compounds concentration can not be discarded. So, if we consider the bibliographic references, we can expect the parameter with best discriminant power to be the **wc**, but our results indicate that it was the **dw**.

Mannitol is known to penetrate the skin via paracellular way (Fidelman and Watlington, 1987); the noted increase in dry weight would be considered a consequence of the influx of mannitol into the organism. When tadpoles are incubated in electrolytic solutions (NaCl) of higher osmolarities than those of APW control media, they also show the **dw** as the best discriminant parameter (Ferrari and Lombardo, 1990); thus, the penetration of mannitol could not be considered the only responsible for the **dw** increase.

We have also studied the effect of exposure of larvae at the same stage as those used in this study in NaCl solutions of the same osmolarity assayed with mannitol (Ferrari, Lombardo and Salibián, manuscript in preparation). In this case the dry weight also increased but to a lower level and the integrated response of the evaluated variables was similar. In these animals the differences found were attributed to the fact that the experimental media were comparable to that where *B. arenarum* inhabit, being their ionic exchange mechanisms functional.

Changes in water content can be produced by independent alterations of the dry and wet weight. Our results indicate that its regulation in controls from both ionic and non ionic solutions, was reached by simultaneous changes in dry and wet weight, but one of the alterations was dominant.

Finally, we believe that the way to get valuable quantitative information referred to these particular physiological responses is

the multivariate approach we followed (Ferrari and Lombardo, 1990). In our case it became evident that the parameter with best discriminant capacity was the dry weight, when considered both at different incubation times in a definite solution or at different solutions for each analyzed time. The differences of the integrated response of the evaluated parameters between controls and experimental tadpoles were remarkable and the similarity of the parameters behavior of the experimental tadpoles to the controls was associated with a similar survival rate.

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