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Role of cations as components of jelly coats in *Bufo arenarum* fertilization

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Summary

The role of monovalent (Na^+, K^+) and divalent (Ca^{2+}, Mg^{2+}) cations in *Bufo arenarum* fertilization was analysed. Our results showed that the highest fertilization percentages were obtained when strings of uterine oocytes (UO) were inseminated. Under these conditions, full jelly (FJ), which represents the jelly coats surrounding the oocytes at the time of deposition, contained 68.5 ± 7.0 mM Na^+, 27.4 ± 2.4 mM K^+, 6.3 ± 0.9 mM Ca^{2+} and 6.9 ± 0.9 mM Mg^{2+}. When the strings of oocytes were washed in deionized water, these cations diffused into the liquid medium surrounding them. There was a marked similarity between the loss of Ca^{2+} in the jelly and the decrease in the fertilizability of the UO. Furthermore, the use of chelating agents of divalent cations showed the importance of the Ca^{2+} contained in the jelly. When Ca^{2+} was sequestered from the jelly coats by the addition of the chelating agents to the insemination medium as well as by pretreatment of the UO strings, a decrease in fertilization percentages occurred, this effect being dose dependent and more marked with EGTA. These results demonstrate that the Ca^{2+} in the jelly plays a role in fertilization. Nevertheless, taking into account that during the washing of the jelly other jelly coat components were diffused and considering that the addition of Ca^{2+} to the insemination medium reverted significantly, but only partially, the loss of fertilizability of jellied UO (washed), the participation of other components in the fertilization mechanism is suggested.

Keywords: Amphibian, Calcium, Cations, Fertilization, Jelly

Introduction

In anuran amphibians, when ovulation takes place, oocytes are released into the pleuroperitoneal cavity and then enter the oviduct, where they come into contact with its secretions. After traversing the most cephalic portion of the oviduct, the pars recta (PR), oocytes continue their transit through the pars convoluta (PC). There the envelope that surrounds them, the vitelline envelope (VE), comes into contact with a gelatinous secretion that becomes deposited and is sequentially organized into several jelly coats around the gametes forming long strings to be finally stored in the ovisac or uterus. These oocytes, called uterine oocytes (UO), are later deposited on the water during amplexus and it is in this medium that they encounter the sperm and fertilization occurs (Fernández & Ramos, 2003).

The importance of these envelopes in fertilization has been confirmed for all amphibian anuran species studied so far, as jellyless oocytes are not fertilizable under physiological conditions (Fernández & Ramos, 2003).

The ultrastructural organization of each of the jelly coats has been studied only in *Xenopus laevis* (Bonnell & Chandler, 1996). Basically, these envelopes revealed an organization characterized by its high degree of complexity based on a stable fibrillar matrix, the structural component, made up of glycoproteins of high molecular weight to which globular proteins of low molecular weight become bound. Some of them are strongly associated with the stable matrix and so are not diffusible, while others, approximately 30% of the total protein content of the jelly coats, are free and can diffuse at different rates to the surrounding medium. It has been determined that approximately

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10% of the free proteins diffuse after the first 5 min, while the remaining 20% are released during the next 4 h (Bonnell *et al.*, 1996).

In *Bufo arenarum*, Arranz & Cabada (2000) isolated and characterized two highly glycosylated proteins, one of them of high molecular weight (300 kDa) that would represent the major structural component in the jelly; the other protein, of low molecular weight (100–120 kDa), was a diffusible molecule. There are data concerning the presence of other diffusible molecules in the jelly: hydrophobic substances (Díaz Fondevila *et al.*, 1991) and cations, especially Na^+, K^+, Ca^{2+} and Mg^{2+} (Ishihara *et al.*, 1984).

Thus, when oocyte strings are immersed in water or hypotonic saline solution, a variety of compounds with the ability to diffuse (glycoproteins, lipids and salts) pass into the liquid medium making up the so-called diffusible factor (DF).

Studies carried out in *Bufo arenarum* and *Bufo japonicus* demonstrated that the loss of diffusible components makes the oocyte refractory to fertilization. When these components are restored to the insemination medium, the ability to fertilize is regained (Barbieri & Villecco, 1966; Katagiri, 1973). Additional data obtained for *Bufo arenarum* provided evidence that the loss of this ability was reversed more efficiently in UO that had been deprived of the diffusible jelly components than in UO that had been deprived of their jelly coats when they were inseminated in the presence of the DF (Barbieri & Raisman, 1969; Barbieri & Del Pino, 1975). This finding means that, for this species and under these experimental conditions, the presence of the structural matrix is also essential in the process (Barbieri & Del Pino, 1975).

With respect to the nature of the molecules involved in this process, the data obtained from the different amphibian species analysed show great dispersion. Thus, in *Bufo arenarum*, there is evidence for the participation of a dialyzable heat stable molecule with an essential amino group (Barbieri & Villecco, 1966; Barbieri *et al.*, 1977), of a substance of lipidic nature (Díaz Fontdevila *et al.*, 1991) and of a highly glycosylated glycoprotein of low molecular weight (Arranz & Cabada, 2000). In other species the importance of other molecules has been pointed out, e.g., a non-dialyzable heat-labile compound in *Rana pipiens* (Elinson, 1971), a molecule of basic nature and low molecular weight in *Bufo japonicus* (Katagiri, 1973), and small proteins (<50 kDa) in the three jelly coats in *Xenopus laevis* (Olson & Chandler, 1999).

In contrast with these alternatives, experiments carried out with *Bufo japonicus* demonstrated that UO that had been deprived of their jelly coats could be fertilized in the absence of organic compounds, both structural and diffusible, if the insemination medium is a balanced saline solution of ionic composition similar to that of jelly (Ishihara *et al.*, 1984). Based on these data, these authors suggested that the glycoproteins present in the jelly coats would have, as their sole function, binding of the divalent cations Ca^{2+} and/or Mg^{2+}, which would be essential for the induction of the acrosomic reaction (AR) (Ishihara *et al.*, 1984). However, the role attributed to these cations has not been analysed in any other amphibian species.

With respect to the origin of the cation and the process involved in its secretion, previous ultracytochemical studies in *Bufo arenarum* demonstrated for the first time in amphibians that, in the PC, both epithelial and glandular secretory cells exhibit prominent Ca^{2+} deposits and Ca-ATPase activity. This cation is stored in the cellular structures that, through different secretion mechanisms that also involve apocrine, merocrine and holocrine processes, release their products toward the oviductal lumen to form the jelly coats (Medina *et al.*, 2000).

Taking these data into account, the present study was carried out to examine the participation of cations (Ca^{2+}, Mg^{2+}, K^+ and Na^+) present in the jelly coats in the process of fertilization of *Bufo arenarum*.

Materials and methods

Animals

Sexually mature *Bufo arenarum* females and males were captured during their breeding period in the area of San Miguel de Tucumán, Argentina. Animals were used immediately after capture or stored for brief periods under appropriate conditions of humidity and temperature until use.

Gametes

Sperm suspensions were prepared by dilaceration of testes in Ringer's solution, pH 7.4, with or without 10 mM Tris–HCl and then filtered to eliminate tissue debris. The filtrate was centrifuged at 100 *g* for 5 min in order to remove the red cells. The supernatant was centrifuged again at 650 *g* for 10 min and the pellet, which contained sperm, was suspended in Ringer's solution, pH 7.4, with or without 10 mM Tris–HCl. For each experiment sperm concentration was determined by means of a Neubauer chamber and suspensions were maintained in a cold bath (4°C) during use. Preliminary experiments allowed us to determine that the fertilizing capacity of the sperm did not show differences when gametes were resuspended in the presence or absence of Tris–HCl.

The UO were obtained using the technique of *in vivo* ovulation. For this purpose, adult females previously maintained at 20–22°C for 24 h were induced to ovulate

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by injecting a suspension of homologous hypophysis (1 hypophysis/animal) (Houssay *et al.*, 1929) preserved according to Pisanó (1956) into the dorsal lymphatic sac. After 10 ± 2 h at 25 ± 1°C, oocytes, surrounded by their jelly envelopes forming long strings, were removed from the ovisac.

As an experimental alternative, we used oocytes that had been obtained by spontaneous ovulation, i.e. without hormone induction. Animals captured during the ovulatory period were maintained at 20–22°C for up to 48 h until the beginning of oviposition.

Preparations of egg-jelly materials

Full jelly (FJ)

Strings of jellied oocytes, carefully removed from the ovisac, were hydrated for 2–3 min in deionized water (1:3 w/v) and later irradiated with ultraviolet (UV) light until complete dissolution of the jelly coats. The solubilized full jelly (FJ) was obtained by decanting the oocytes.

Washed jelly (WJ)

Strings of UO with their jelly coats were immersed in deionized water (1:20 w/v) and shaken gently at room temperature to allow diffusion (washing or extraction) of jelly coat components into the liquid medium. At different time intervals (1–60 min) after immersion, the washed strings were transferred to Petri dishes and irradiated with UV light in the presence of deionized water (1:3 w/v) until the complete dissolution of the jelly coats. The solubilized material obtained was separated by decantation of the oocytes.

Diffusible factor (DF)

This term alludes to the liquid medium (deionized water) that contained the components that diffused from the jelly during the washing periods mentioned above. All the preparations obtained from the jelly were used immediately or fractioned and kept at −20°C until use.

In order to eliminate those samples that might be contaminated with components of intracellular origin the following controls were carried out: (1) stereoscopic microscope observation of the jelly and of the morphology of the UO. The samples were discarded if the oocytes were not intact or the jelly was contaminated with red blood cells; and (2) dosage of the lacticodehydrogenase enzyme. The activity of this enzyme in the preparations of the oviductal secretion (FJ, WJ and DF) indicates cell death or damage to the plasma membrane. The presence of enzymatic activity was determined using Noll's technique (1984).

Determination of cation concentration in jelly materials

Calcium analyses were performed using a Jarrell Ash model 590 atomic absorption photometer. Cation standards (Riedel de Haën) gave a linear standard curve for Ca^{2+} at 4227 Å from 0.1 to 3 ppm. Additional analyses to estimate total calcium content was carried out with a commercial kit supplied by Wiener Laboratory. Determinations were performed according to the manufacturer's instructions. The results obtained with both methods of Ca^{2+} determination showed a high correlation ($r = 0.95$). Thus, the data presented in this study are those obtained by the atomic absorption photometer.

The determination of total magnesium was performed by atomic absorption spectrophotometry using a spectrophotometer Hitachi Z 5000. Cation standards (Riedel de Haën) gave a linear standard curve for Mg^{2+} at 2852 Å from 0 to 2 ppm.

The determination of monovalent cations was carried out using a flame photometer (Metrolab Model 305). Sensitivity to Na^+ and K^+ was 3 ppm. The range of concentrations for Na^+ was 0–200 mEq/l and for K^+ 0–10 mEq/l. In all determinations deionized water was used as a blank target. Cation concentrations in the different preparations obtained from the jelly coats were determined as previously described by Ishihara *et al.* (1984) and expressed as moles/l.

Bioassays

In vitro *fertilization*

Uterine oocytes surrounded by their jelly coats were fertilized with a sperm suspension obtained as described above. Gametes were kept in contact for 20–30 min in the insemination medium indicated for each case and, after this time, the oocytes were transferred to 10% Ringer's solution (10% R) and kept at room temperature for their embryonic development. Results were expressed as percentage of fertilized oocytes that reached the morula or blastula stage in relation to total inseminated oocytes.

Relationship between the ability to fertilize, washing time of jelly and sperm concentration

In order to assess the participation of the cations present in the jelly and determine the importance of their concentration at the time of fertilization, strings of jellied oocytes were washed in deionized water (1:20 w/v). At different times, ranging from 1 to 60 min, three lots of samples were collected; two of these were transferred to Petri dishes in which fertilization was carried out in 10% R or 10 mM NaCl. Unwashed UO strings were used as controls and insemination was carried out in the same media.

Solubilized jelly obtained from the third lot of samples was used to estimate the concentration of

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monovalent (Na^+, K^+) and divalent (Ca^{2+}, Mg^{2+}) cations in order to analyse the correlation between these values and the fertilization percentages.

In another series of experiments we determined the optimum sperm concentration for our working conditions. For this purpose samples of jellied oocytes (unwashed) and jellied oocytes washed in deionized water (1/20 w/v) for 1 min were inseminated with different sperm concentrations (1×10^4–1×10^8 cell/ml) using 10% R or 10 mM NaCl as insemination medium.

Relationship between the ability to fertilize and cation type
In this series of experiments we analysed the contribution to fertilization of certain cations present in the jelly. Strings of UO were immersed in deionized water (1:20 w/v) for 1 or 2 min and then placed in Petri dishes. The oocytes were inseminated with a suspension containing 1×10^6 cell/ml in the presence of $CaCl_2$, $MgCl_2$ or KCl at increasing concentrations (1–10 mM). When necessary, total cation concentration in the insemination medium was adjusted to 10 mM by addition of adequate amounts of NaCl. Jellied oocytes that were: (i) unwashed; or (ii) washed for 1 or 2 min and inseminated in the presence of only 10 mM NaCl were used as controls.

Analysis of the effect of divalent cation chelating agents
The participation of the divalent cations Ca^{2+} and Mg^{2+} in fertilization was also analysed by studying the effect of EGTA or EDTA on the process by means of two experimental models:

Addition of chelating agents to the fertilization medium
Strings of UO were washed in deionized water (1/20 w/v) for 1–5 min. Then, oocytes were inseminated with a sperm suspension containing 1×10^6 cell/ml in the presence of EGTA or EDTA at increasing concentrations (0.2–3.2 mM). Insemination was carried out in either 10 mM NaCl or 10% R.

As controls we used: (1) strings of UO without washing and fertilized in the presence or absence of EGTA or EDTA in the conditions described above; and (2) strings of UO washed and inseminated in the absence of the chelating agents in the conditions described above.

Pretreatment of gametes with the chelating agents
Aliquots of the sperm suspensions were treated for 1 or 3 min at 4 or 22°C with two concentrations of EDTA or EGTA (0.2 and 0.8 mM). Sperm that had been treated with Ringer's solution were used as controls. After the incubations the sperm suspensions were washed twice by centrifugation at 650 *g* for 5 min at 4°C, and the sediment was resuspended at the volume of 10% R necessary to obtain a concentration of 1×10^6 cell/ml. These sperm were used to fertilize UO according to the *in vitro* fertilization technique mentioned above.

In another series of experiments UO strings were incubated with an EDTA or EGTA solution (0.2 and 0.8 mM) for 1 or 3 min at 4 or 22°C. Then the samples were transferred to Petri dishes and inseminated with sperm at a concentration of 1×10^6 cell/ml. Strings of UO that had been washed with deionized water for the same time periods and at the same temperatures were used as controls.

Table 1 Concentration of monovalent and divalent cations in the full jelly.

Cations	Concentration (mM)
Ca^{2+}	6.3 ± 0.9
Mg^{2+}	6.9 ± 0.9
Na^+	68.5 ± 7.0
K^+	27.4 ± 2.4

Strings of uterine oocytes collected from the ovisac were immersed for 2–3 min in deionized water (1:3 w/v) and later irradiated with UV light until the complete dissolution of the jelly coats. The resulting total solubilized jelly was obtained by decanting the oocytes. Cation concentration was determined by atomic absorption spectrophotometry and flame photometry. Results are expressed as the mean ± SD of determinations carried out in different animals ($n = 11$).

Statistical analyses

Statistical analysis was performed using the Kruskal–Wallis non-parametric test followed by the multiple comparisons method. Spearman's rank correlation test was used for all variables. All statistical comparisons were made above the 95% confidence level ($p < 0.05$).

Results

The presence and concentration of monovalent (Na^+, K^+) and divalent (Ca^{2+}, Mg^{2+}) cations was determined in samples of full jelly (FJ), washed jelly (WJ) at different time periods and diffusible factor (DF).

The results presented in Table 1 showed that FJ, which represents the jelly coats at the time of deposition, has high Na^+ concentrations, medium K^+ levels, and lowered Ca^{2+} and Mg^{2+} concentrations.

When jellied oocytes were immersed in deionized water, the initial concentration of cations present in the FJ decreased with the washing time. During the first minutes a rapid diffusion was observed with a decrease of about 30% in the levels of Ca^{2+}, Mg^{2+} and Na^+ (Fig. 1*a*–*c*), while the K^+ concentration showed no variation (Fig. 1*d*).

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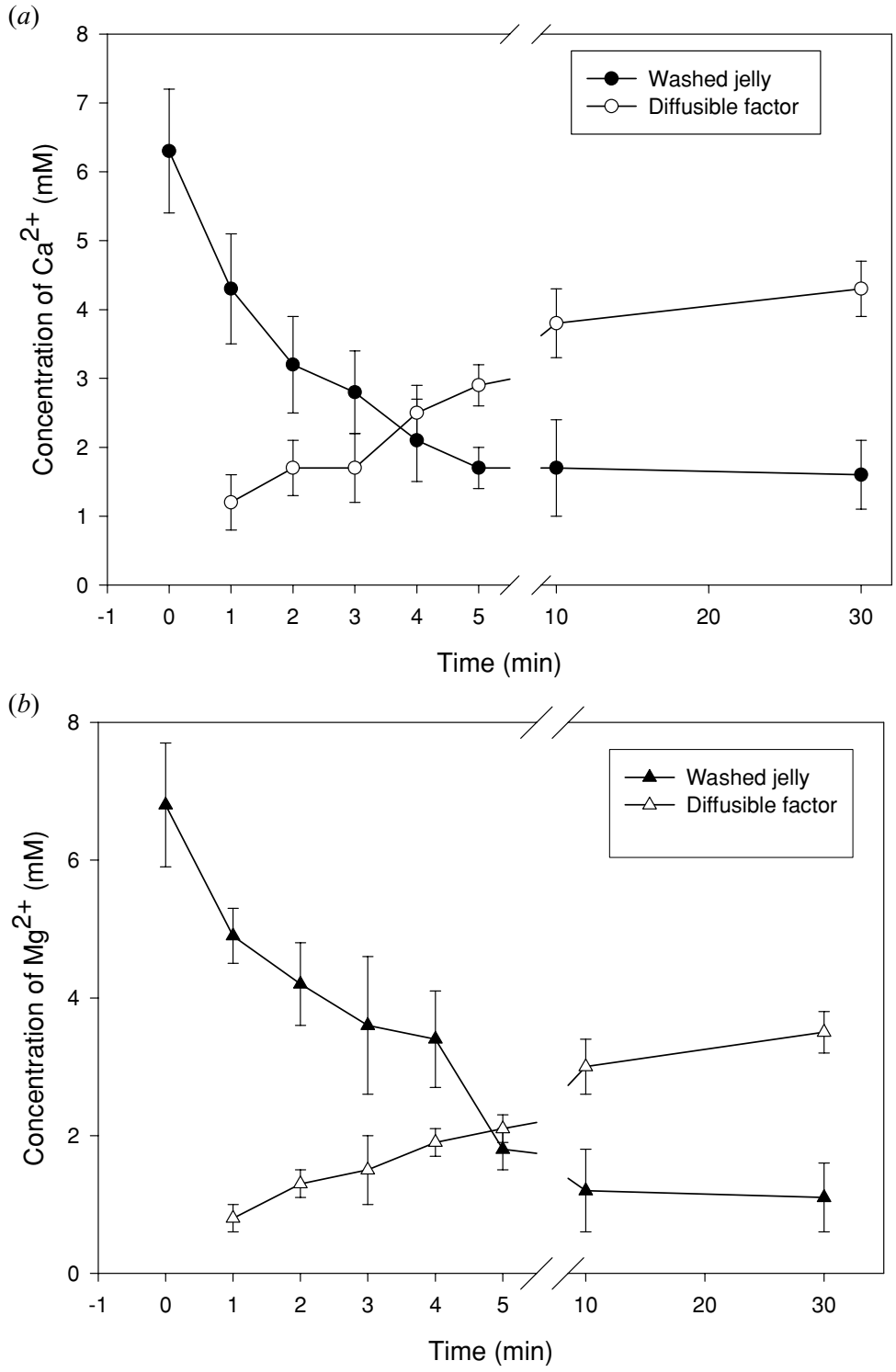

Figure 1 For legend see next page.

The diffusion of 50% of the total content of the cations present in the jelly coats was observed after washing for 2 min for Ca^{2+}, 3 min for Na^{+} and 4 min for Mg^{2+} and K^{+}. It is important to notice that after 5 min the concentration of all the cations reached basal values that remained without variations for 30 min.

As shown in the above figures, the loss of these cations in the WJ was associated with the increase in the DF. This observation was verified by the statistical analysis, which revealed a significant inverse correlation between the levels of each cation in the two media studied (Table 2).

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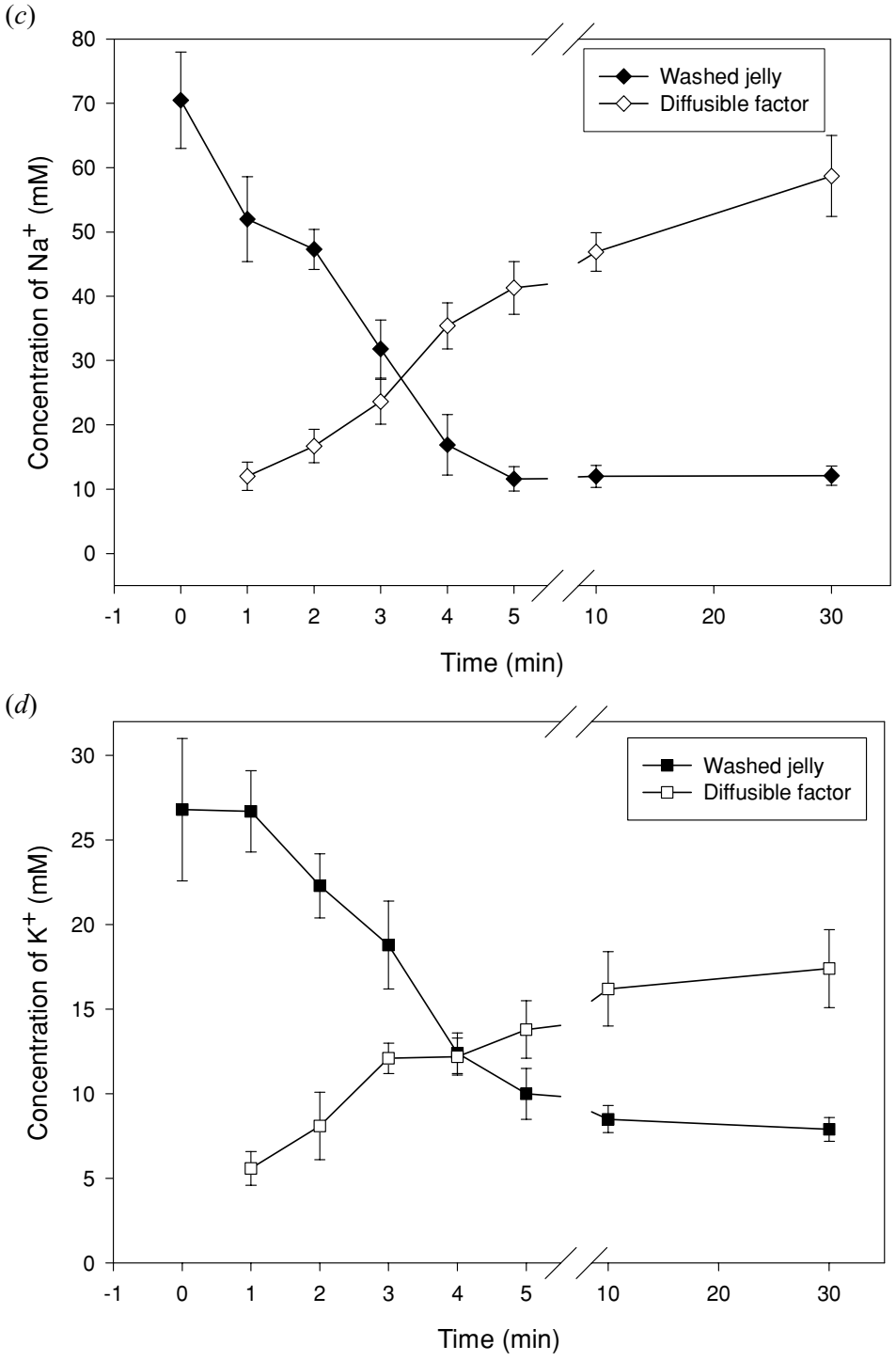

Figure 1 Quantitative analysis of the cations present in the diffusible factor and jelly coats washed in deionized water at different time periods. (*a*) Ca^{2+}; (*b*) Mg^{2+}; (*c*) Na^{+} and (*d*) K^{+}. Data represent the mean ± SD of experiments carried out in different animals ($n = 10$).

For fertilization assays, preliminary experiments were made in order to determine the optimum concentration of sperm suspensions in our experimental conditions. For that purpose, control jellied oocytes (unwashed) and jellied oocytes washed with deionized water for 1 min were inseminated with sperm suspensions at increasing concentrations (1×10^4–1×10^8 cell/ml). Insemination was carried out in 10 mM NaCl or 10% R.

Figure 2 shows that when control jellied oocytes were inseminated with a sperm suspension equal to or higher than 1×10^6 cell/ml the highest fertilization

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Table 2 Correlation between cation concentrations in two compartments: jelly coats and diffusible factor.

	Cations			
Value	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}
r	−0.80	−0.73	−0.91	−0.81
p	<0.0001	< 0.0001	< 0.0001	< 0.0001

r, Spearman's simple correlation coefficient.

percentages were obtained, this parameter decreased progressively with concentrations below 1×10^6 cell/ml.

Insemination of jellied oocytes washed for 1 min showed, with sperm concentrations of 1×10^7 and 1×10^8 cell/ml, fertilization percentages similar to those of the controls. With concentrations of 1×10^6 cell/ml, the ability to fertilize started to decrease markedly from 1×10^5 cell/ml, no fertilization was observed for samples at a concentration of 1×10^4 cell/ml.

On the basis of these data we selected a concentration of 1×10^6 cell/ml for the fertilization experiments, which enabled us to obtain fairly good fertilization percentages and to detect variations in this parameter under different experimental conditions.

Figure 2 shows that the results obtained with strings of control UO and with strings of jellied UO (washed) presented no significant difference when fertilization was carried out in the presence of 10 mM NaCl or 10% R. This finding allowed us to use as an alternative the 10 mM NaCl solution as an insemination medium for the determination of the effect of cations on fertilization.

In order to analyse the oocyte's ability to fertilize after the loss of diffusible components, samples of jellied oocytes were washed in deionized water or 10% R at different time periods between 1 and 60 min and then inseminated.

Figure 3 shows that the oocyte's ability to fertilize decreased as a function of time and dependent upon the wash medium. After washing for 1 min with deionized water there was a significant decrease of about 30% in the fertilization percentage with respect to the unwashed control jellied oocytes ($p < 0.001$). This percentage continued to decrease constantly until, after 5 min, complete inhibition was reached. When oocytes were washed with 10% R, the decrease in fertilization rate was less marked, total inhibition being observed only after 60 min.

Due to the above results that indicated the presence of monovalent and divalent cations in the FJ and that their concentration decreased as a function of washing time with a parallel decrease in the fertilization percentages, we considered it important to study the contribution of the cations in this process.

For this purpose, samples of strings of UO washed in deionized water for 1 and 2 min were inseminated

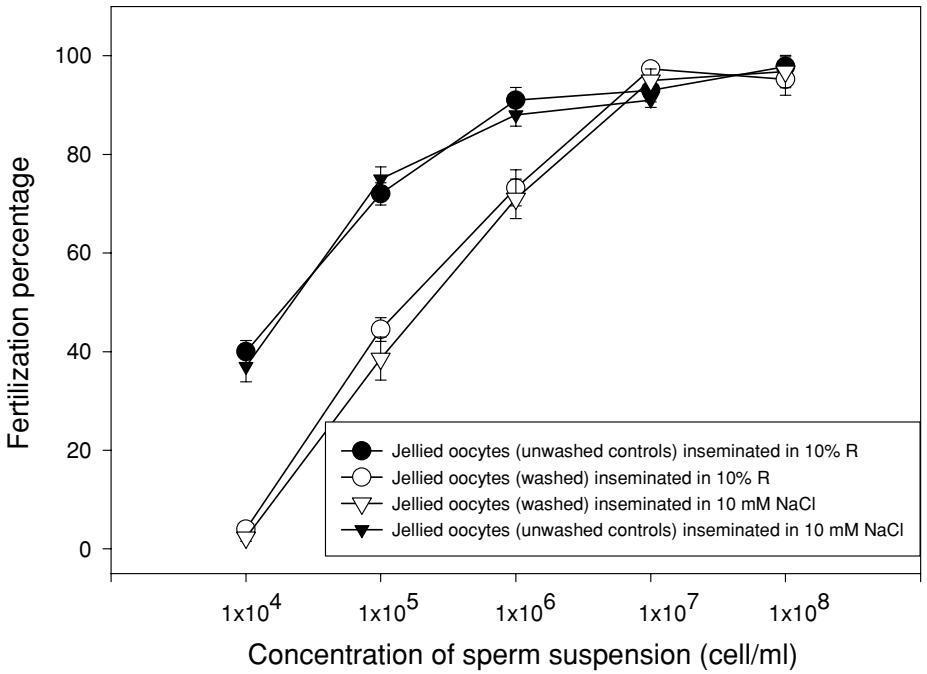

Figure 2 Analysis of the ability to fertilize of jellied uterine oocytes (unwashed controls) and jellied uterine oocytes washed for 1 min in deionized water and inseminated in 10% R or 10 mM NaCl, as a function of different sperm concentrations. Data represent the mean ± SD of experiments carried out in different animals ($n = 12$).

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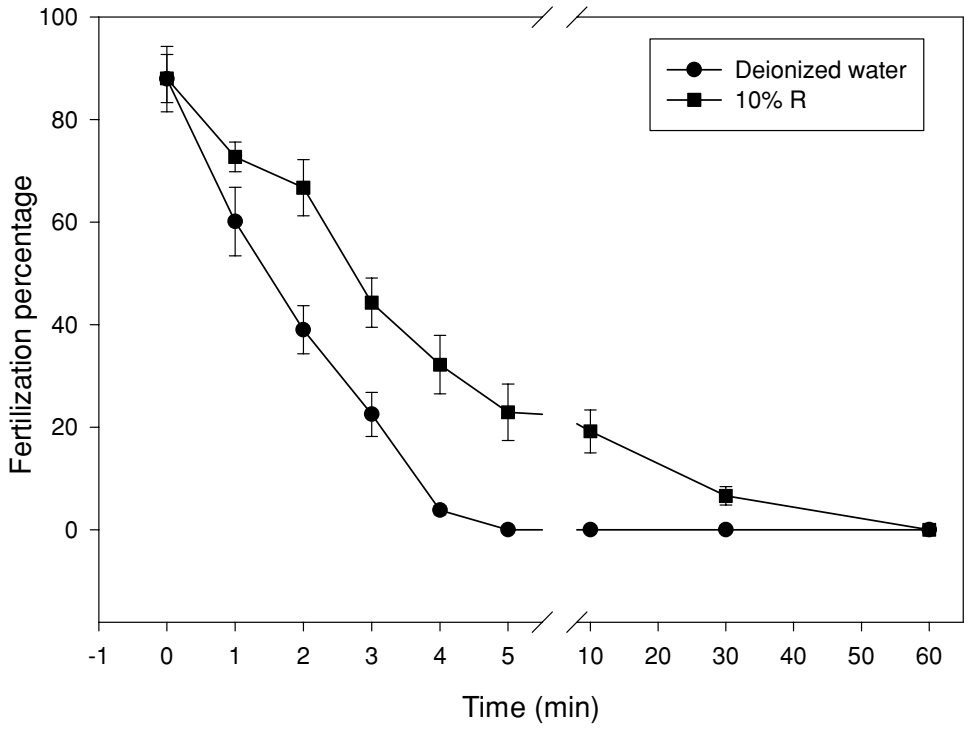

Figure 3 Fertilizability of jellied uterine oocytes washed for different time periods in deionized water or 10% R and inseminated in 10 mM NaCl. Data represent the mean ± SD of experiments carried out in different animals ($n = 12$).

in the presence of different $CaCl_2$, $MgCl_2$ or KCl concentrations. The final concentration of the cations in the insemination medium was adjusted to 10 mM by addition of the appropriate amounts of NaCl, previously determined to be biologically inactive. In this respect it should be pointed out that our controls had allowed us to determine that this concentration of cations in the fertilization medium did not affect sperm motility.

Figure 4*a* shows that the addition of Ca^{2+} to the insemination medium of the jellied oocytes that had been washed for 1 min induced a significant increase in the fertilization percentages with respect to the control (jellied oocytes washed for 1 min and inseminated in 10 mM NaCl), when the Ca^{2+} concentration added was around 2 mM ($p < 0.01$) or 3 mM ($p < 0.05$).

When the washing period of the strings of UO was increased to 2 min (Fig. 4*b*), although an increase in fertilization percentages was shown by the addition of Ca^{2+} at a broad range of concentrations (1–10 mM), the most significant increase occurred with 2 mM ($p < 0.01$) and 3 mM ($p < 0.001$).

Figure 4 also shows that the ability to fertilize of the oocytes whose jelly coats were washed for 1 or 2 min was only partially reverted by the addition of Ca^{2+}; fertilization percentages were always significantly lower than those obtained with the unwashed controls ($p < 0.0001$).

The addition of Mg^{2+} to the insemination medium (Fig. 4) induced only a slight increase in the fertilization percentages of the jellied oocytes washed for 1 or 2 min with respect to the control (jellied oocytes washed and inseminated in 10 mM NaCl). Statistical analysis showed that this increase was not significant.

Similar experiments were carried out to analyse the effect of K^+. The results, summarized in Table 3, showed that at none of the K^+ concentrations used could differences in the fertilization percentages be observed with respect to the control (UO washed in deionized water for 1 min).

The importance of the divalent cations in the fertilization process was also analysed through the use of chelating agents. Strings of control UO (unwashed) and strings of UO washed for different time periods (1–5 min) in deionized water were fertilized in the presence of different EGTA or EDTA concentrations (0.2–3.2 mM).

Results showed that the presence of divalent cation chelating agents in the insemination medium (Fig. 5) inhibited fertilization in a dose-dependent manner, EGTA being more effective than EDTA. It was also observed that, in both cases, inhibition was greater in unwashed jellied oocytes than in washed ones.

In order to elucidate whether the inhibitory effect on fertilization obtained with the chelating agents was due to the direct action on the oocytes or on the sperm, the following experiments were carried out. Aliquots of sperm suspensions were incubated in Ringer's solution

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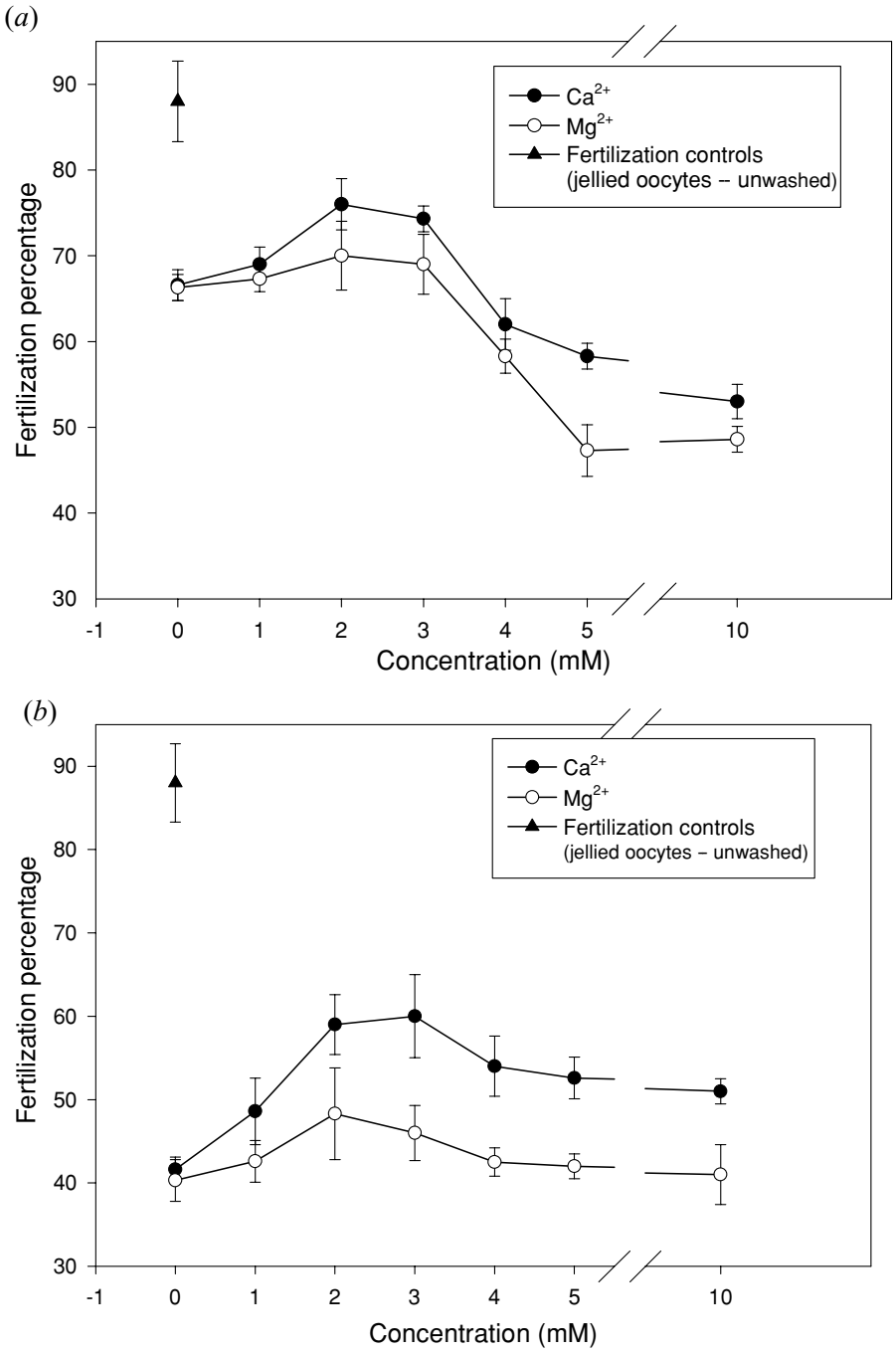

Figure 4 Fertilizability of jellied uterine oocytes washed in deionized water for 1 min (*a*) or 2 min (*b*) as a function of the addition of different concentrations of Ca^{2+} or Mg^{2+} to the fertilization medium. Data represent the mean ± SD of experiments carried out in different animals ($n = 8$).

(control) or 0.2 or 0.8 mM EGTA or EDTA (treated) for 1 or 3 min at 4 or 22°C. Then, sperm were centrifuged, washed twice in Ringer's solution to eliminate the chelating agent and resuspended in 10% R. Afterwards, sperm aliquots were taken to inseminate jellied UO (unwashed).

The results (Table 4) showed that there were no differences between the fertilization percentages obtained with control sperm and with treated sperm at any of the time periods and temperatures assayed.

Strings of UO were washed in an EGTA or EDTA solution (0.2 or 0.8 mM) for 1 or 3 min at 4 or 22°C. For the controls we used jellied oocytes washed in deionized water for the same time periods. The results summarized in Table 4 indicated that the ability to fertilize of the UO that had been treated

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Table 3 Effect of potassium on the fertilization of strings of uterine oocytes washed for 1 min.

K^+ (mM)	Fertilization (%)
0	66.0 ± 2.7
1	63.5 ± 1.5
2	68.3 ± 3.5
3	67.4 ± 3.1
5	68.5 ± 2.2
10	65.7 ± 3.7

Strings of uterine oocytes were washed in deionized water (1:20 w/v) for 1 min and then placed in Petri dishes. Insemination was carried out with a suspension of 1×10^6 cell/ml in the presence of KCl at increasing concentrations (1–10 mM). In each insemination, total cation concentration in the medium was adjusted to 10 mM by addition of the adequate amounts of NaCl. Results are expressed as the mean ± SD of data obtained with different animals ($n = 8$).

with chelating agents decreased in a dose- and time-dependent manner at the two temperatures assayed, this effect being more evident with EGTA.

Discussion

The results obtained in the present work demonstrated that, as found for *Bufo japonicus* (Ishihara *et al.*, 1984), the jelly coats surrounding the *Bufo arenarum* oocytes at the time of deposition contained monovalent (Na^+ and K^+) and divalent (Ca^{2+} and Mg^{2+}) cations.

When strings of oocytes collected directly from the ovisac or uterus were immersed in deionized water, these cations diffused rapidly from the jelly to the surrounding medium, and the diffusion rate was greater for Ca^{2+} than for Na^+, Mg^{2+} or K^+. The decrease in the concentration of cations in the washed jelly showed a close correlation with the increase in the liquid medium (DF). This finding would indicate that the oocyte has no significant participation in the exchange between these two compartments.

According to our results, there was a marked parallel between the loss of the Ca^{2+} present in the jelly and the decrease in the oocyte's ability to fertilize. The ability to be fertilized was significantly reverted by restitution of the cation, when it was added to the insemination medium in a large enough amount to reach the concentration present in the jelly coats at the time of oviposition (6.3 ± 0.9 mM). It is important to point out that the addition of Ca^{2+} at concentrations higher than that above caused a decrease in fertilizability. To date, the mechanisms and/or processes that enable a satisfactory explanation of this fact are still unknown.

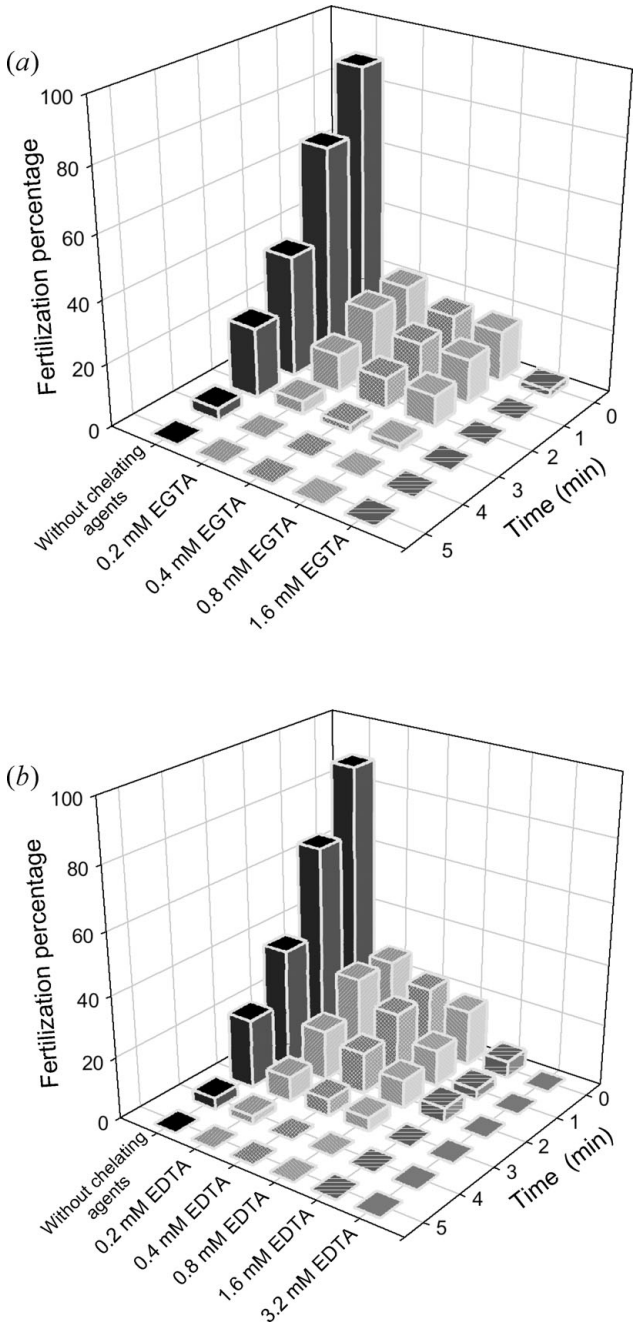

Figure 5 Fertilizability of jellied uterine oocytes (unwashed) and jellied uterine oocytes washed in deionized water for different time periods as a function of the addition of EGTA (*a*) or EDTA (*b*) to the fertilization medium. Data represent the mean ± SD of experiments carried out in different animals ($n = 7$).

However, it is possible to suggest that high Ca^{2+} concentrations in the insemination medium might induce a premature AR before sperm arrival near the VE.

Moreover, the experiments in which divalent cation chelating agents were used showed the importance of the presence of the Ca^{2+} in the jelly at the time of fertilization. Sperm did not show a decrease in their

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Table 4 Effect of chelating agents on gamete fertilization after 1 and 3 min incubation.

Treatment		Fertilization (%) 1 min		Fertilization (%) 3 min	
		Sperm	Egg	Sperm	Egg
Control	Ringer's solution	92.1 ± 4.5	–	93.5 ± 6.1	–
	Deionized water	–	68.7 ± 6.0	–	22.7 ± 1.9
EDTA	0.2 mM	95.3 ± 4.5	24.7 ± 3.6	92.1 ± 6.6	1.5 ± 0.8
	0.8 mM	89.1 ± 9.1	10.0 ± 3.0	89.0 ± 8.5	0.4 ± 0.4
EGTA	0.2 mM	88.2 ± 4.3	17.4 ± 4.3	88.5 ± 3.5	1.1 ± 0.8
	0.8 mM	87.1 ± 3.8	6.8 ± 1.1	91.1 ± 7.3	0.6 ± 0.8

Aliquots of sperm suspensions or lots of jellied uterine oocytes were incubated as indicated in the text. The results of experiments carried out at 4°C are expressed as the mean of fertilization percentage ± SD of data obtained from different animals ($n = 7$).

fertilizing capacity when they were preincubated in the presence of EDTA or EGTA, which indicated that these molecules have no direct effect on the male gamete. However, when the cation was sequestered from the jelly coats by the addition of the chelating agents to the insemination medium or by pretreatment of the strings of UO, a decrease in the fertilization percentages was observed. This differential and dose-dependent effect was more evident with EGTA. These data presented together with the fact that this inhibition could be reverted by the addition of Ca^{2+} at concentrations higher than those of the chelating agent (results not shown), strongly suggest that this cation plays an important role in the process of gamete interaction.

It is important to emphasize that the addition to the insemination medium of the other cations present in the jelly, even at a broad concentration range, was either not very effective (Mg^{2+}) or had no ability (Na^{+} and K^{+}) to revert the loss of fertilizability of the UO present in the previously washed strings.

In this sense our results differ from those obtained for *Bufo japonicus* (Ishihara *et al.*, 1984), a species in which it was demonstrated that both Ca^{2+} and Mg^{2+} were necessary for normal fertilization. While in *Bufo arenarum* the effectiveness of Ca^{2+} required the presence of the jelly coats, the fact that in *Bufo japonicus* the oocytes deprived of the jelly coats could be fertilized in a medium that contained only an adequate concentration of the divalent cations would indicate that, in this species, no organic compound from the jelly is involved in gamete interaction.

Another difference to be considered lies in the range of Ca^{2+} concentrations required for fertilization. In *Bufo japonicus*, the cation concentrations present in the jelly immediately after deposition (around 130 mM), 8.5 and 6.0 mM of which correspond to Ca^{2+} and Mg^{2+} respectively. Under these conditions, sperm exhibited a low fertilizing capacity (Ishihara *et al.*, 1984). Jelly coats had to be washed for 2–3 min to dilute the total cation content present at 20–25 mM. At this time the adequate Ca^{2+} or Mg^{2+} concentration (2–5 mM) needed to ensure high fertilization percentages was reached. This factor means that the fertilizing ability of the sperm in this species depends on the balance between the decrease in salinity and the minimum level of divalent cations essential for gamete fusion.

In *Bufo arenarum*, in contrast, the cation concentration present in the jelly coats at the time of deposition is approximately 110 mM, Ca^{2+} being between 5.5 and 7 mM, the optimum concentration range for the attainment of the highest fertilization percentages. This situation would explain in this species the loss of the oocyte's ability to fertilize when the jelly is washed, even for very short periods.

As pointed out above, highest fertilization percentages were obtained when strings of unwashed oocytes were inseminated. Under these conditions, the FJ had a Ca^{2+} concentration of 6.3 ± 0.9 mM. After washing for 5 min in deionized water, complete inhibition of fertilization was observed in the oocytes together with the decrease in Ca^{2+} concentration to basal level (1.7 ± 0.5 mM), which under our experimental conditions and for the time period assayed remained unchanged.

These data indicate that 27–30% of the total Ca^{2+} contained in the jelly coat was not involved in the fertilization process and, as it did not diffuse to the medium, it would be strongly related to the structural component of these coats. In contrast, the remaining 70–73% of the cation content, which was free or weakly bound to organic compounds and so could diffuse freely from the different jelly coats to the DF, would be the fraction with an active participation in gamete interaction. This correlation became evident when we demonstrated that the decrease in approximately 50% of the diffusible Ca^{2+} present in the jelly coats determined the loss of approximately 30–35% of oocyte fertilization rate. The importance of the participation of the cation in the process was shown in the experiments that demonstrated that the loss of fertilization was 100% when all free Ca^{2+} was diffused.

However, as together with this cation other jelly coat components diffuse to the medium and considering

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that the addition of Ca^{2+} to the insemination medium did not totally revert the loss of fertilizability in strings of UO washed in deionized water, we cannot rule out the possible coparticipation of organic compounds in the fertilization mechanism. These compounds would favour Ca^{2+}storage in the jelly coats or participate together with the cation in AR induction.

In this respect Shimoda *et al.* (1994) demonstrated that in the jelly of *Bufo japonicus* there is a high molecular weight glycoprotein that has the capacity to bind to Ca^{2+} thus maintaining the cation concentration necessary to insure gamete interaction. A glycoprotein with similar characteristics, with domains for binding two Ca^{2+} molecules, has been identified in *Lepidobatrachus laevis* (Peavy *et al.*, 2003). Although all its functions have not been determined, it has been suggested that this macromolecule would participate in the maintenance of the jelly structure to enable sperm transit. As an alternative, it has been postulated that this glycoprotein could be a Ca^{2+}storage site for AR induction.

In our laboratory, experiments are underway to determine the direct participation of the glycoproteins present in the jelly coats in the process of fertilization of *Bufo arenarum* and the corresponding mechanisms of action of these molecules.

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References

Arranz, S.E. & Cabada, M.O. (2000). Diffusible highly glycosylated protein from *Bufo arenarum* egg-jelly coat: biological activity. *Mol. Reprod. Dev.* **56**, 392–400.

Barbieri, F.D., Del Pino, E.J. & Armador, M.R. (1977). Properties and isolation of the diffusible factor involved in *Bufo arenarum* fertilization. *Acta Physiol. Lat. Am*. **27**, 293–302.

Barbieri, F.D. & Del Pino, E.M. (1975). Jelly coats and diffusible factor in anuran fertilization. *Arch. Biol. (Brussels)* **86**, 311–21.

Barbieri, F.D. & Raisman, J.S. (1969). Non-gametic factors involved in the fertilization of *Bufo arenarum* oocytes. *Embryologia* **10**, 363–72.

Barbieri, F.D. & Villecco, E.I. (1966). A fertilizin-like substance in the egg jelly of the toad, *Bufo arenarum*. *Arch. Zool.* **51**, 227–37.

Bonnell, B.S. & Chandler, D.E. (1996). Egg jelly layers of *Xenopus* leavis are unique in ultrastructure and sugar distribution. *Mol. Reprod. Dev.* **44**, 212–20.

Bonnell, B.S., Reinhart, D. & Chandler, D.E. (1996). *Xenopus laevis* egg jelly coats consist of small diffusible proteins bound to a complex system of structurally stable networks composed of high molecular weight glycoconjugates. *Dev. Biol*. **174**, 32–42.

Díaz Fondevila, M.F., Bloj, B. & Cabada, M.O. (1991). Effect of egg water from *Bufo arenarum* on the fertilizing capacity of homologous spermatozoa. *J. Exp. Zool*. **257**, 408–14.

Elinson, R.P. (1971). Fertilization of partially jellied and jellyless oocyte of the frog *Rana pipiens*. *J. Exp. Zool.* **176**, 415–28.

Fernández, S.N. & Ramos, I. (2003). Endocrinology of reproduction. In *Reproductive Biology and Phylogeny of Anura*, vol. 4 (ed. B.G.M. Jamieson), pp. 73–117. Enfield, New Hampshire, USA: Science Publishers, Inc.

Houssay, B.F., Giusti, L. & Lascano González, J.M. (1929). Implantation d'hypophyse et stimulation des glandes et des fonctions sexuelles du crapaud. *CR Séanc. Soc. Biol.* **102**, 864–66.

Ishihara, K., Hosono, J., Kanatani, H. & Katagiri, C. (1984). Toad egg-jelly as a source of divalent cations essential for fertilization. *Dev. Biol.* **105**, 435–42.

Katagiri, C. (1973). Chemical analysis of toad egg jelly in relation to its "sperm-capacitating" activity. *Dev. Growth Differ.* **15**, 81–92.

Medina, M.F., Winik, B., Romero, I.R. de, Crespo, C. & Fernández, S. (2000). Subcellular localization of Ca-ATPase and calcium in *Bufo arenarum* oviduct. *Acta Histochem. Cytochem*. **33**, 49–58.

Noll, F. (1984). L^+ Lactate. In *Methods of Enzyme Analysis* (ed. J. Bergmeyer & M. Grassl), pp. 582–88. Weinheim: Verlag Chemie.

Olson, J.H. & Chandler, D.E. (1999). *Xenopus laevis* egg jelly contains small proteins that are essential to fertilization. *Dev. Biol*. **210**, 401–10.

Peavy, T.R., Hernandez, C. & Carroll, E.J. Jr (2003). Jeltraxin, a frog egg jelly glycoprotein, has calcium-dependent lectin properties and is related to human serum pentraxins CRP and SAP. *Biochemistry* **42**, 12761–69.

Pisanó, A. (1956). Método para mantener la hipófisis de anfibio fisiológicamente *in vitro*. *Arch. Bioq. Quim. Farm. Tucumán*. **7**, 387–92.

Shimoda, Y., Kitajima, K., Inoue, S. & Inoue, Y. (1994). Isolation, structural determination, and calcium-binding properties of the major glycoprotein present in *Bufo japonicus* egg jelly. *Eur. J. Biochem*. **223**, 223–31.