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CRITICAL ELECTROLYTE CONCENTRATION OF SPERMATOZOAL CHROMATIN CONTAINING HISTONE H1 VARIANTS

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ABSTRACT

The critical electrolyte concentrations (CEC) of sperm chromatin from animal species known or suspected to contain histone H1 variants were compared by examining the affinity of their DNA-protein complexes for toluidine blue in the presence of Mg^{2+}. Bullfrog, sea urchin, bee and bumblebee spermatozoa were studied. The CEC for *Rana catesbeiana* and two sea urchin species were similar to that of histone H5-containing chromatin from chicken erythrocytes, thus confirming the biochemical and structural similarities of these DNA-protein complexes. The CEC for bees and the bumblebee, *Bombus atratus*, showed no particular phylogenetic relationship. We concluded that the CEC of histone H1-containing sperm cell chromatin is a useful indicator of variability in DNA-protein complexes but is of little phylogenetic value.

INTRODUCTION

Spermatozoa have been grouped into many dissimilar classes on the basis of the protein elements of their DNA-protein complexes. One of these categories of sperm nuclear proteins includes somatic-like histones which belong to Bloch's (1969) "*Rana*" type or "type 4" class. The nuclear basic protein of *Rana* spermatozoa contains sperm-specific, very lysine-rich histone H1 variants (Kasinsky *et al*., 1985; Itoh *et al*., 1997). DNA-protein spermatozoal complexes in other animals such as sea urchins and honey bees also contain histone H1 variants (Subirana and Palau, 1968; Verma, 1972; Mello and Vidal, 1973; Strickland *et al*., 1976, 1980; Poccia *et al*., 1987; Puigdomenech *et al*., 1987; Mello and Falco, 1996).

The availability and proximity of free DNA phosphates to each other in histone H1-containing nucleoprotein complexes allows the binding of cationic dyes such as toluidine blue (TB), resulting in a basophilic reaction. When histone H1 variants complex with DNA, different spectral patterns of basophilia are expected to be found cytochemically, especially under TB and inorganic cation (Mg^{2+}, for instance) competitive conditions (Vidal and Mello, 1989).

The critical electrolyte concentration (CEC) for nucleic acids in nucleoprotein complexes has been defined as the inorganic ion concentration at which the metachromasy due to nucleic acid TB staining (violet color) is abolished, with the appearance of a green color (Vidal and Mello, 1989). The CEC of different DNA-protein complexes occurs at different Mg^{2+} concentrations (Vidal and Mello, 1989; Amaral and Mello, 1989; Mello and Falco, 1996; Taboga *et al*., 1996; Monteiro and Mello, 1998).

For spermatozoa, the CEC assay is particularly relevant when comparing the DNA-protein complexes of individual cells and when histone electrophoresis and other biochemical tests are not possible because of the small amount of sperm sample and/or the rarity of the species investigated.

In this study we compared the CEC of DNA-protein complexes of spermatozoa from animal species in which different histone H1 variants are known or suspected to occur.

MATERIAL AND METHODS

Bullfrog, sea urchin, bee and bumblebee spermatozoa were studied. The species and specimen source are enlisted in Table I. At least three specimens of each species were used.

The testes of sexually mature bullfrogs, *Rana catesbeiana*, were fixed in 1.75% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 30 min. The fixed organs were embedded in paraffin and 10-μm thick sections were prepared. Sperm smears were obtained from the other species and fixed for 15 min in the same solution as for *R. catesbeiana*. Sea urchin gametes were collected following the intracoelomic injection of 2 ml of 0.5 M KCl.

The sections and smears were treated with a 0.025% TB solution at pH 4.0 in the absence or presence of 0.02-0.20 M $MgCl_2$. The preparations were then rapidly (5 s) rinsed in distilled water, air-dried, cleared in xylene, and mounted in Canada balsam.

The richness in arginine residues of spermatozoal nuclei was assessed by staining some preparations with fast green with or without previous deamination (Bloch and Hew, 1960).

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Table I - Species and specimen source investigated for sperm nucleoprotein cytochemistry.

Animal group	Species	Specimen source
Bullfrog	*Rana catesbeiana* (Amphibia, Anura)	Ranario Aquavale (Jundiaí, SP)
Sea urchin	*Lytechinus variegatus, Arbacia lixula* (Echinodermata, Echinoidea)	Canal de São Sebastião (North seaside of the São Paulo State)
Bee	*Apis mellifera ligustica, A.m. adansonii* (Hymenoptera, Apoidea)	Dept. of Genetics, FMRP/USP (Ribeirão Preto, SP)
	Plebeia minima, Plebeia droryana, Friesella schrottkyi (Hymenoptera, Apoidea)	Dept. of General Biology, Fed. Univ. Viçosa (Viçosa, MG)
	Eufriesia violacea, Eulaema nigrita (Hymenoptera, Apoidea)	Serra do Japi (Jundiaí, SP)
Bumblebee	*Bombus atratus* (Hymenoptera, Apoidea)	Dept. of Biology, FFCLRP/USP (Ribeirão Preto, SP)

RESULTS

Sperm cell nuclei from all species examined stained violet (metachromasy) with TB in the absence of Mg^{2+} (Figure 1a, c, e, f). When the staining solution was supplemented with Mg^{2+} ions, the color of the stained nuclei changed to green once reached the DNA-protein CEC (Figure 1b, d). Although most sea urchin nuclei stained green when the CEC was reached, some nuclei still stained metachromatically (Figure 1d).

The lowest CEC for the studied hymenopteran was found in honeybee subspecies (*Apis mellifera ligustica* and *A.m. adansonii*) and the highest in a stingless bee (*Plebeia minima*) and an orchid bee (*Eufriesia violacea*) (Table II).

All the species responded positively to the alkaline fast green test but were negative when this test was preceded by deamination.

DISCUSSION

The metachromatic staining after treatment with the TB solution at pH 4.0 and the negative response to the fast green staining after deamination are characteristic indications of the presence of lysine-rich histone H1 types (Bloch and Hew, 1960; Mello, 1997).

Rana catesbeiana sperm cells contain nucleosomes with no regular spacing and sperm-specific histone H1 variants essential for chromatin condensation in the sperm nuclei (Itoh *et al*., 1997). These histones, which migrate faster than somatic histone H1 in AUT-PAGE, share extensive structural similarity with the chicken erythrocyte histone H5 (Itoh *et al*., 1997).

The sperm chromatin of sea urchin such as *Arbacia lixula* contains nucleosomes with DNA linkers that are longer than those of the somatic counterpart (Spadafora *et al*., 1976). Sea urchin spermatozoa also contain a sperm-specific histone H1 (Poccia, 1986) that is similar in many aspects to chicken erythrocyte H5 (Strickland *et al*., 1976) but differs in helicity (Puigdomenech *et al*., 1987), and may be responsible for the special compaction of the chromatin (Poccia, 1995).

The presence of histone H1 has been indicated cytochemically in *Apis mellifera* (Verma, 1972; Mello and Vidal, 1973; Mello and Falco, 1996). The electrophoretic mobility of this histone differs from that of somatic histone H1 of the same species, and from H1 standards from calf thymus and rat liver, as well as histones H1 of sea urchin spermatozoa (Falco, J.R.P. and Mello, M.L.S., unpublished observations). The spermatozoal nuclear proteins of bees other than *Apis mellifera* and of bumblebee have not been determined either by electrophoresis or cytochemistry. However, the results above indicate that histone H1 proteins also occur in the sperm cells of phylogenetically distant Apoidea species (Michener, 1990).

The CEC for DNA-protein complexes has been considered to be a function of the availability of free DNA phosphates for the competitive binding of TB and Mg^{2+} (Vidal and Mello, 1989), but may be influenced by the degree of chromatin condensation, which determines the proportion of anionic binding sites per chromatin area

Table II - CEC values for sperm chromatin DNA-protein complexes.

Species	CEC (M)
Rana catesbeiana	0.10
Lytechinus variegatus	0.12
Arbacia lixula	0.12
Apis mellifera ligustica	0.08
A.m. adansonii	0.08
Plebeia minima	0.15
P. droryana	0.12
Friesella schrottkyi	0.12
Eufriesia violacea	0.15
Eulaema nigrita	0.12
Bombus atratus	0.12

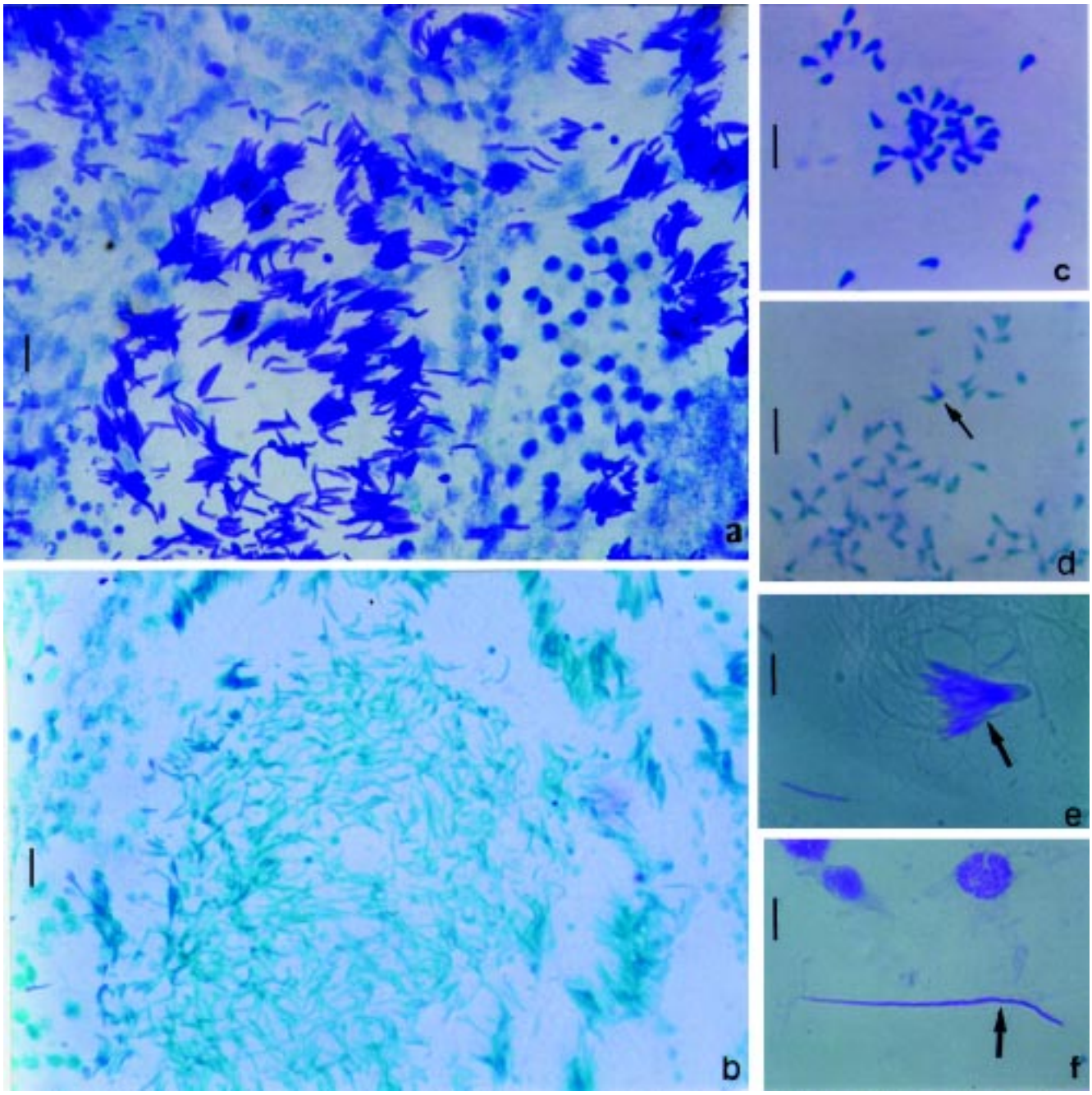

Figure 1 a-f - TB staining in the absence (a, c, e, f) or presence of Mg^{2+} ions (b, 0.10 M; d, 0.12 M) in sperm cell nuclei. a,b: *Rana catesbeiana*; c,d: *Lytechinus variegatus*; e: *Bombus atratus*; f: *Eulaema nigrita*. d, Metachromatically stained nucleus (arrow) of *L. variegatus* at the CEC. Most of the nuclei are stained green. e,f, A group of spermatozoal heads and an individual sperm cell nucleus are indicated (arrow). Bar: 10 µm.

(Amaral and Mello, 1989; Mello and Falco, 1996). Differences in the CEC indicate corresponding differences in the DNA-protein complexes, as seen inclusive among the bees in this study. However, the possible correlation with phylogenetic position (Michener, 1990) was not observed for the hymenopteran studied here.

The CEC for *Rana catesbeiana* sperm chromatin was close to that of the chromatin from chicken erythrocytes under the same conditions of fixation (Falco *et al*., 1999). The CEC for the spermatozoal chromatin of the two sea urchin species examined was similar to that of *Rana catesbeiana* and identical to that of chicken erythrocyte chromatin (Falco *et al*., 1999). The cytochemical data thus confirm the biochemical and structural similarities in the DNA-protein complex and chromatin supraorganization among these species.

The observation that some sea urchin sperm cell nuclei exhibited metachromasy when the CEC had been

reached in most of the cell nuclei suggests the occurrence of abnormalities in the DNA-protein complexes similar to those reported for bull spermatozoa (Mello, 1982; Beletti and Mello, 1996). The abnormalities in the sea urchin sperm cells may reflect the effects of environmental pollution caused by the oil spillage in the Canal de São Sebastião on the north-eastern shore of São Paulo State (Schaeffer-Novelli, 1990). This damage to the DNA-protein complexes of the spermatozoa may have resulted in more loosely packed chromatin with an increased susceptibility to protein removal by the $MgCl_2$ solution in a manner similar to that for somatic chromatin treated with Mg^{2+} concentrations much higher than the CEC (Amaral and Mello, 1989; Vidal and Mello, 1989).

We conclude that the CEC of histone H1-containing sperm cell chromatin is a useful indicator of the influence of histone H1 variants on the organization of DNA-protein complexes influenced by histone H1 variants, but is of little phylogenetic value. The biological significance of spermatozoal histone diversity as a phylogenetic attribute is itself still unclear (Bloch, 1969; Oliva and Dixon, 1991).

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RESUMO

Valores de concentração crítica de eletrólitos (CEC) da cromatina de espermatozóides de espécies conhecidas ou suspeitas de apresentarem variantes da histona H1 foram comparados entre si. O objetivo foi estabelecer semelhanças ou diferenças nos complexos DNA-proteína de espermatozóides dessas espécies em nível citoquímico. A afinidade por moléculas de azul de toluidina em condições de competição com íons Mg^{2+} foi investigada nos espermatozóides do sapo boi e de ouriços do mar, abelhas e mamangava. Uma íntima relação entre os valores de CEC de *Rana catesbeiana* e de duas espécies de ouriço do mar com os da cromatina de eritrócitos de frango, que contém a histona H5, foi vista estar de acordo com certas semelhanças bioquímicas e estruturais entre seus complexos DNA-proteína. Quanto aos dados para abelhas e para a mamangava *Bombus atratus*, não se pôde associar a variabilidade em valores de CEC com a posição das espécies na respectiva árvore filogenética. Conclui-se, portanto, que a CEC de cromatina de espermatozóides que contêm histona H1 é um indicador útil da influência de variantes de H1 na organização de complexos DNA-proteína, mas é de pouco valor em estudos filogenéticos.

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