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SESSION IV

Testicular Activity and Sperm Glycoproteins in Giant Red Shrimp *Aristaeomorpha foliacea*

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Abstract

The reproduction of male giant red shrimp *Aristaeomorpha foliacea*, collected from the late winter to the summer in the north-western Ionian Sea (Mediterranean Sea), was investigated using histological and histochemistry methods. Seasonal changes in the spermiogenesis and the glycoprotein pattern were found and sperm glycoproteins matured as gametes moved from the testis to the terminal ampulla. In serial sections stained with hematoxylin and eosin the testicular activity appeared to be discontinuous. In late winter the testes had no meiotic activity and the seminiferous epithelium consisted of interkinetic spermatogonia and spermatozoa. In spring, spermiogenetic activity was high and the seminiferous epithelium mainly consisted of spermatocytes and spermatozoa while in summer, the testes were again inactive since both spermatocytes and spermatozoa were lacking. The use of twelve different lectins indicated that the intratesticular spermatozoa from late winter to summer contain surface binding sites for SNA, MAA, Con A and KOH-sialidase (si)-WGA. In March and July they also exhibited nuclear and cytoplasmic reactivity for SNA and Con A. In the hemispermatophore the spermatozoa displayed a more complex lectin-binding pattern because they also reacted with PNA, DBA, HPA, GSA II. The staining with DBA, KOH-si-DBA, and GSA II showed differences between the spermatozoa from late winter-spring hemispermatophores and summer hemispermatophores: the former showed a nuclear affinity whereas the latter displayed surface and/or cytoplasm staining. No reaction was observed with SBA, GSA I-B$_4$, UEA I, and LTA.

Key words: histology, Mediterranean, reproduction, shrimp

Introduction

The giant red shrimp *Aristaeomorpha foliacea* (Risso, 1827), generally occurring at depths between 300–700 m, is widely distributed in the eastern and western Atlantic, Indian Ocean and western Pacific, in the waters of Japan, Australia, New Zealand and in the Mediterranean Sea (Holthius, 1980). This species plays an important role in the overall biomass of the muddy bottoms of the Mediterranean Sea and represents an important commercial resource among crustaceans since it is much appreciated by the consumer.

The general biology, distribution and population dynamics of *A. foliacea* have been studied (D'Onghia *et al*., 1998 and references cited therein) but, as in many crustacean species, the male reproductive tract has received little attention. Only histological observations of spermatophore formation (Tunesi, 1987) and, more recently, the ultrastructural aspects of spermatozoa (Medina, 1995) have been described, whereas studies on both the testicular activity and the glycoconjugate composition of spermatozoa are lacking.

Glycoconjugates are a fundamental component of eukaryotic cells, and although their biological or physiological functions are known in many cases, the biological roles of their oligosaccharides remain mostly undefined (Lis and Sharon, 1993; Varki, 1993). Lectins are useful probes for intracellular localization of sugar residues and characterization of distinct cellular populations as well as cell-to-cell interactions and variation of biological activity (Spicer and Schulte, 1992; Danguy *et al*., 1994). Since *A. foliacea* spermatozoa lack an acrosome (Medina, 1995), the sperm surface as well as the cytoplasmic content seems to be of critical importance in the process of fertilization.

The aim of this study was to investigate the testicular activity as well as the glycoconjugate pattern in testicular and hemispermatophore spermatozoa from late winter to summer using a series of lectins with the attainment of sperm physiological maturation in understanding the reproductive dynamics of this commercially important species.

Materials and Methods

A total of 15 males of *Aristaeomorpha foliacea* (carapace length >32 mm), which were considered sexually mature for the presence of the hemispermatophores in the terminal ampullae and joined petasma (Sardà and Demestre, 1989), were collected in late winter (March) ($n = 2$), spring (April) ($n = 8$) and summer (July) ($n = 5$) by commercial bottom-trawl gear in the north-western Ionian Sea (Mediterranean Sea) (Fig. 1). The testes and the vasa deferentes were removed immediately after capture, fixed in Bouin's solution, dehydrated in an ascending ethanol series, and embedded in paraffin wax. Sections 5 µm thick were cut and, after dewaxing, were stained with Mayer's hematoxylin and eosin for histological observations or processed for lectin histochemical studies.

Lectin histochemistry

The lectins used are listed in Table 1. Dewaxed and rehydrated tissue sections were immersed in 3% H_2O_2 for 10 min to suppress the endogenous peroxidase activity, rinsed in 0.05 M Tris-HCl buffered saline (TBS) pH 7.4 and incubated in lectin solution at appropriate dilutions (10–25 µg ml^{-1}) for 1 h at room temperature (RT). After 3 rinses in TBS, peroxidase activity was visualized by incubation in a solution containing 0.05% 3,3'-diaminobenzidine (DAB) and 0.003% H_2O_2 in 0.05 M TBS (pH 7.6) for 10 min at RT before dehydration and mounting. Tissue sections incubated in biotinylated lectins (SNA, MAA and GSA I–B_4) were rinsed 3 times with 0.05 M phosphate-buffered saline (PBS) and then incubated in streptavidin/peroxidase complex (Vector Lab. Inc., Burlingame, CA) for 30 min at RT. After washing in PBS, peroxidase was developed in a DAB-H_2O_2 solution.

Controls for lectin staining included: (1) substitution of the substrate medium with buffer without lectin; (2) incubation with each lectin in the presence of its hapten sugar (0.2 M).

Sialidase digestion

Before staining with SNA, MAA, PNA, DBA and WGA, some sections were incubated at 37°C for 16 h in 0.1 Uml^{-1} of sialidase (Type V, from *Clostridium perfringens*, from Sigma, St. Louis, MO) dissolved in 0.1 M sodium acetate buffer, pH 5.5, containing 10 mM $CaCl_2$. Prior to the neuraminidase treatment, a saponification technique was performed to render the enzyme digestion effective, with 0.5% KOH in 70% ethanol for 15 min at RT (Reid *et al.*, 1978). As controls of the enzyme digestion procedure, certain sections were incubated in the enzyme-free buffer solution under the same experimental conditions.

Results

Histology

The testes of *A. foliacea* are a pair of convoluted tubules containing germinal cells, involved in the production of sperm, and somatic (accessory) cells. Spermatogonia are found along one margin, whereas the bulk is occupied by developing germ cells and/or spermatozoa (Fig. 2a, c, d, e, f).

Testes showed a different spermatogenetic activity from March to July. In March the testes display interkinetic spermatogonia (Ø 12.08 ± 0.56 µm) and spermatozoa (Ø 3.14 ± 0.17 µm) (Fig. 2c). The spermatozoa were slightly elliptic in shape and were characterized by a strongly hematoxylinophil nucleus surrounded by an unstained peripheral band of cytoplasm. In April the testes contained less spermatogonia, while they were packed with developing spermatocytes and spermatozoa (Fig. 2d). In July the testes were found in the following three conditions: 1) spermatogonia and primary spermatocytes (Fig. 2e), 2) spermatogonia and secondary spermatocytes (Ø 5.34 ± 0.24) (Fig. 2f), 3) spermatogonia and spermatozoa.

The hemispermatophores are hardened structures constituted of four layers, the first of them surrounds the spermatozoa that are enmeshed in an extracellular matrix (Fig. 2b).

Lectin histochemistry

Since the cytoplasm of spermatozoa consists of a thin perinuclear band it was very difficult to

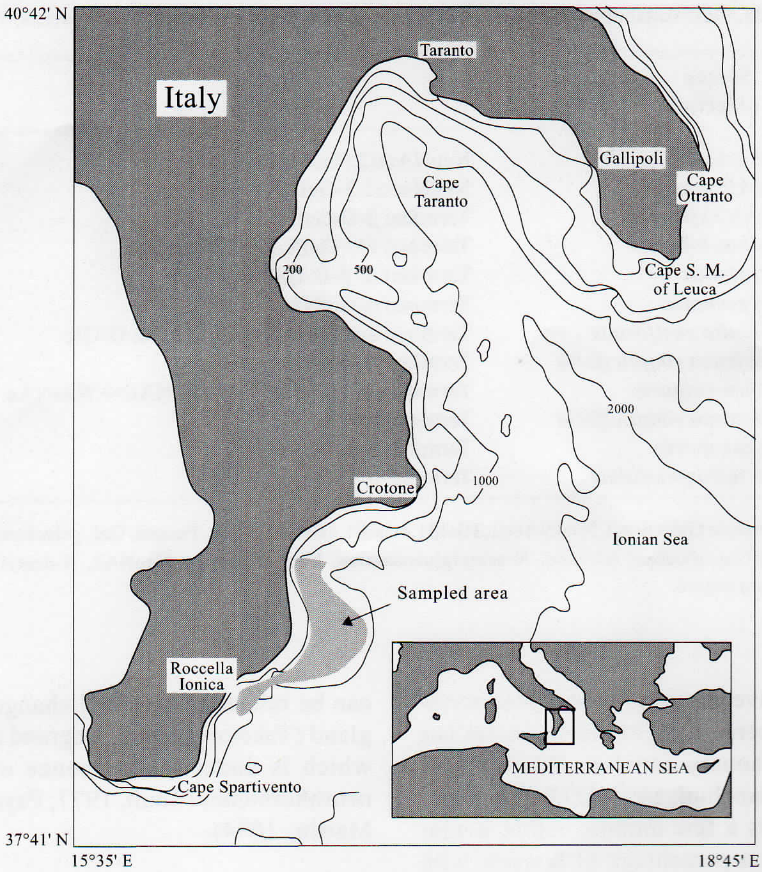

Fig.1. Map of Ionian Sea (Mediterranean Sea) with indication of the sampled area.

distinguish clearly the cell surface from the cytoplasm, therefore the material stained outside the nuclear region we sometimes indicate as cytoplasm.

The lectin binding pattern of testicular (T) and hemispermatophore (H) spermatozoa are summarized in Table 2. SNA weakly bound to the T spermatozoa surface in spring, the whole spermic cell in March and a few in July (Fig. 3a); this lectin moderately marked the surface and the cytoplasm of H spermatozoa (Fig. 3b). MAA moderately marked the surface of T spermatozoa (Fig. 3c) and also the cytoplasm of H spermatazoa (Fig. 3d). PNA did not find binding sites on T spermatozoa but weakly reacted with the cytoplasm of H spermatozoa. KOH-sialidase (si)-treatments revealed cryptic binding sites in the nucleus of H spermatozoa. DBA did not show binding sites on T spermatozoa, whereas the lectin gave a faintly visible reaction in the nuclear region of rare spermatozoa in the spring H, and a weak staining in the cytoplasm of spermatozoa contained in summer H. DBA affinity was increased by KOH-si-treatments only in cytoplasm of H sperm mass. HPA did not mark T spermatozoa, whereas it showed weak reactions in the cytoplasm of H spermatozoa. Con A showed staining on the surface and in the cytoplasm of T and H spermatozoa (Fig. 3e); the H spermatozoa displayed binding sites also in the nucleus region (Fig. 3f). The KOH-si-WGA procedure intensely marked the surface of T spermatozoa (Fig. 3g), and gave a faintly visible reaction in the nucleus and the cytoplasm of H spermatozoa (Fig. 3h). GSA II did not react with T spermatozoa, whereas in H showed binding sites both for the cytoplasm and the nucleus in spring spermatozoa, and it stained only the cytoplasm in summer ones. SBA, GSA I-B_4, UEA I, and LTA did not show binding patterns.

Discussion

The present study was performed on testes and hemispermatophores from mature males of *A. foliacea* collected in the Mediterranean Sea during the major

TABLE 1. Lectin used, their sugar specificities and inhibitory sugar used in control experiments.

Lectin abbreviation	Source of lectin	Sugar specificity	Inhibitory sugar (0.2 M)*
SNA	*Sambucus nigra*	NeuNAcα2,6Gal/GalNAc	NeuNAc
MAA	*Maackia amurensis*	NeuNacα2,3-Gal/ β1,4-GlcNac	NeuNAc
PNA	*Arachis hypogea*	Terminal β-D-Gal(1-3)-GalNAc	Galactose
DBA	*Dolichos biflorus*	Terminal FP>GalNAcα1,3GalNAc	GalNAc
SBA	*Glycine max*	Terminal α/ β-D-GalNAc	GalNAc
HPA	*Helix pomatia*	Terminal α-GalNAc	GalNAc
Con A	*Canavalia ensiformis*	Terminal and internal α-D-Man>α-D-Glc	Mannose
GSA I-B4	*Bandeiraea simplicifolia*	Terminal α-D-Gal	Galactose
WGA	*Triticum vulgaris*	Terminal and internal β-D-GlcNAc>> NeuNAc	GlcNAc
GSA II	*Bandeiraea simplicifolia*	Terminal D-GlcNAc	GlcNAc
UEA I	*Ulex europaeus*	Terminal α-L-Fuc	Fucose
LTA	*Lotus tetragonolobus*	Terminal α-L-Fuc	Fucose

FP, Forssman pentasaccharide GalNAcα1,3GalNAcα1,3Galβ1,4Galβ1,4GlcNAc; Fuc, Fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuNAc, N-acetyl neuraminic acid; *, concentration of inhibiting sugars.

mating and reproductive periods. In the Mediterranean Sea, the highest percentage of mature males has been found between January and July (Mura *et al.*, 1992; Ragonese and Bianchini, 1995; D'Onghia *et al.*, 1998). Coupling occurs a few months before ovulation because the highest percentage of females with spermatophores in the thelycum has been observed during March and May (D'Onghia *et al.*, 1998), whereas females with mature ovaries (Levi and Vacchi, 1988; Desantis *et al.*, 2001) occur from May to September with the highest percentage during August.

As in other Decapoda (Kaestner, 1970), the testes of *A. foliacea* are a pair of tubes in which the male reproductive cells are produced. In this shrimp the testicular activity appeared to be discontinuous. In late winter, the testes are inactive, since they do not show meiotic activity and the seminiferous epithelium consists of interkinetic spermatogonia and spermatozoa. These spermatozoa could represent the remains of a previous spermiogenesis period. The highest spermiogenetic activity occurs in spring when the seminiferous epithelium consisted mainly of primary spermatocytes and spermatozoa. In summer the testicular activity appears to have stopped again: testes containing both spermatocytes and spermatozoa are lacking. Seasonal changes of testicular activity have been reported in other crustaceans (King, 1948; Meusy, 1963; Sreekumar and Adiyodi, 1983; Du *et al.*, 1988; Pochon-Masson, 1994) as well as in the companion species *Aristeus antennatus* collected in the same basin (Desantis *et al.*, 1999). These changes can be related to seasonal changes in the androgenic gland (Taketomi, 1986; Legrand and Juchault, 1994), which is under the influence of the protocerebral neurohormones (Touir, 1977; Payen and Amato, 1978; Martin, 1994).

The glycoproteins fall into two main categories according to the attachment of the oligosaccharide to the peptide (Kornfeld and Kornfeld, 1985). The two types include those in which a reducing terminal GalNAc is linked O-glycosidically to the hydroxyl of serine or threonine and those in which a reducing terminal of GlcNAc is bound N-glycosidically to the epsilon amine of asparagine. Among the lectins used in this study PNA, DBA, or HPA specifically identify the many O-linked oligosaccharides (Spicer and Schulte, 1992), whereas Con A visualize specifically the glycoproteins containing N-linked oligosaccharides (Bernhard and Avrameas, 1971). The surface of intratesticular spermatozoa reacted with SNA, MAA, Con A and KOH-si-WGA showing the presence of N-linked oligosaccharides terminating or not with sialic acid, besides in March and July intratesticular spermatozoa showed nuclear and cytoplasmic binding sites for SNA and Con A, respectively. This similar lectin reactivity strengthens the assumption that late winter and summer testicular spermatozoa could represent the remains of a previous period of spermiogenesis, whereas spring spermatozoa are a recent spermiogenic product. In hemispermatophore the spermatozoa displayed a more complex glycoprotein pattern than in testis because they also

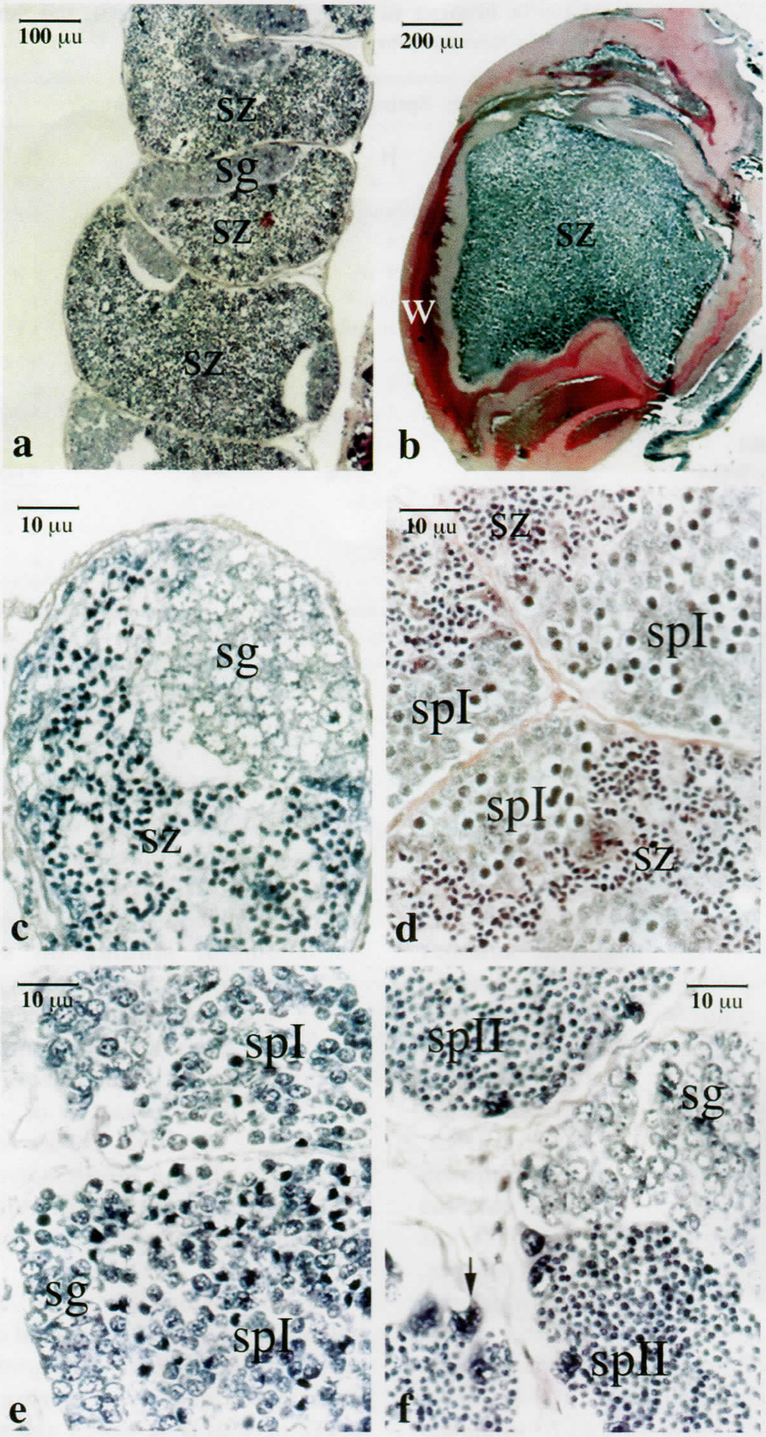

Fig. 2. Hematoxylin-eosin staining. Seminiferous tubules of *Aristaeomorpha foliacea* collected in March (**c**), April (**d**), July (**a**, **e**, **f**); (**b**), cross section of hemispermatophore. sg, spermatogonia; spI, primary spermatocytes; spII, secondary spermatocytes; sz, spermatozoa; w, wall of hemispermatophore; ⟶, somatic cell.

TABLE 2. Summary of lectin binding to the sperm mass in testis and hemispermatophore of *Aristaeomorpha fliacea.*

	Late Winter–Spring		Summer	
Lectin	T	H	T	H
SNA	+s/+wMr	++	+s/++w*	++
MAA	++s	++	++s	++
PNA	–	+	–	+
KOH-si-PNA	–	+ w	–	+w
DBA	–	-/±n*	–	+
KOH-si-DBA	–	±/+n*	–	++
SBA	–	–	–	–
HPA	–	+	–	+
Con A	++s/+Mr	++/+n	+++s/+	+/+n
GSA I-B4	–	–	–	–
KOH-si-WGA	++s	++s/±w	++s	++s/±w
GSA II	–	++w	–	++
UEA I	–	–	–	–
LTA	–	–	–	–

H, hemispermatophore; Mr, March; n, nucleus; s, surface; si, sialidase (neuraminidase); T, testis; w, whole cell; *, rare positive reaction; –, negative reaction; ±, faintle visible reaction; +,++,+++, weak, moderate, intense positive reactions. Where not specified, the reactions concern both the surface and the cytoplasm.

reacted with PNA, DBA, HPA (specific for O-linked oligosaccharides), and GSA II. In addition, the staining with DBA, KOH-si-DBA, and GSA II showed differences between the hemispermatophore late winter-spring spermatozoa and the summer hemispermatophore. During the late winter-spring the spermatozoa showed a nuclear affinity for DBA, increased after KOH-sialidase procedure, and Con A. They were wholly stained by GSA II. In summer the hemispermatophore spermatozoa displayed surface and/or cytoplasm affinity for DBA, KOH-si-DBA, GSA II. These findings suggest that the glycoprotein sperm pattern is seasonally different and that the spermatozoa mature as they transit from the vas deferens to the terminal ampulla. In other species, such as *Inachus falangium* (Diesel, 1989), it was found that mature spermatozoa are formed in the medial portion of the vas deferens. The function of the terminal ampulla is connected to the maturation of spermatozoa in *Penaeus setiferus* and *P. vannamei* (Chow *et al*., 1991). The referred cytoplasmic lectin binding pattern can hide surface glycoproteins or oligosaccharides contained in vesicles. The former could be involved in the interaction with the oocyte and the latter may represent an acrosome-like structure since no acrosomal structure is recognizable in *A. foliacea* (Medina, 1995). To our knoweledge lectin histochemical studies on spermatozoa from crustaceans are lacking, but in vertebrates it is well known that spermatozoa undergo changes in their surface and acrosome glycoconiugates as they transit through extratesticular ductus (Arya and Vanha-Perttula, 1984; Burkett *et al*,, 1987; Eddy, 1988; Vreeburg *et al.*, 1992; Labate *et al.*, 1997; Ueda *et al.*, 1997). These modifications are of critical importance since the glycoconjugates are involved in inter- and intracellular processes of fertilization.

In conclusion, this study shows that in mature male of *A. foliacea* 1) seasonal changes of testicular activity occur, 2) sperm glycoprotein pattern of active spermiogenesis period (spring) is different from locked periods (late winter and summer), 3) spermatozoa undergo maturative changes in glycoconjugates during their transit from testis to hemispermatophore.

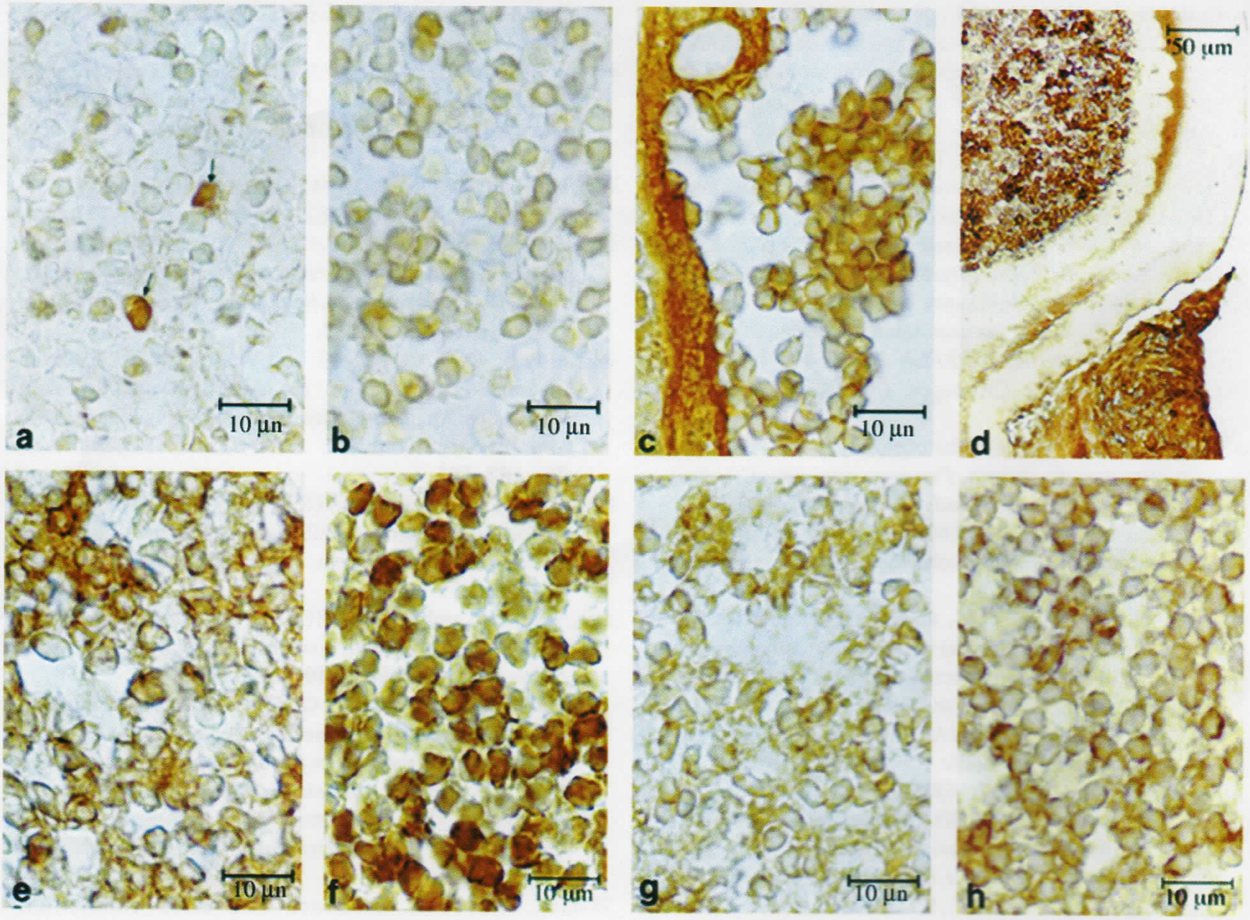

Fig. 3. Lectin histochemistry. **(a)** summer testis and **(b)** hemispermatophore stained with SNA; **(c)** spring testis and **(d)** summer hemispermatophore incubated with MAA; **(e)** late winter testis and **(f)** spring hemispermatophore stained with Con A; **(g)** spring testis and **(h)** hemispermatophore incubated with KOH-si-WGA procedure. ⟶, wholly stained spermatozoa.

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