Lectin-binding pattern of Senegalese sole *Solea senegalensis* (Kaup) testis

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Key words: testis, spermatogenesis, glycoconjugates, lectins, teleost, Pleuronectiformes

Running title: glycoconjugates in the Senegalese sole testis

Summary

The localization and characterization of oligosaccharide sequences in the testis of Senegalese sole *Solea senegalensis* was investigated using 12 lectins in combination with KOH saponification and sialidase digestion (K-s). The interstitial compartment contained all the sugar residues investigated, those bearing oligosaccharides terminating with sialic acid (Neu5ac) α2,3Galβ1,4GlcNAc, Neu5acGalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1 and GalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1 being more abundant in the medullar region than in the cortex. The melano-macrophage centres found in the interstitial compartment displayed glycans terminating with Galβ1,3GalNAc. The basal lamina separating the germinal and interstitial compartments exhibited glycans with terminal/internal mannose, internal βGlcNAc, and terminal Neu5Acα2,6Gal/GalNAc, and Neu5acGalβ1,3GalNAc, Galβ1,3GalNAc (PNA), Galβ1,4GlcNAc, GalNAc, αGal, and αL-Fuc. In the germinal compartment, the Sertoli cells expressed only glycans terminating with Neu5acα2,3Galβ1,4GlcNAc in the apical and supra-nuclear lateral surface of the spermatonial cysts located in the distal part of the seminiferous lobules. Primary spermatocytes exhibited oligosaccharides terminating with Galβ1,3GalNAc and αGalNAc in the cytoplasm and nucleus, respectively. The spermatids contained highly mannosylated glycans terminating with GalNac, αGal, and αL-Fuc. The head of spermatozoa expressed a more complex glycosylation pattern characterized by the additional presence of oligosaccharides terminating with Neu5acα2,3Galβ1,4GlcNAc, Neu5acGalβ1,3GalNAc, Neu5acGalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1, GalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1. The comparison with previous lectin histochemical studies carried out in other fish species reveals a specific glycosylation pattern of Senegalese sole testicular structures and spermatozoa head.

Introduction

Senegalese sole *Solea senegalensis* is a target species for the aquaculture industry in Southern European countries (Spain and Portugal, mainly). The complete development of its intensive culture, however, is hampered by among other factors, reproductive problems in captive broodstocks, which often fail to spawn or produce small quantity of eggs (García-López et al., 2005). Recently, the finding that (i) several hormonal treatments, which were effective in the induction of ovulation, were basically ineffective stimulating sperm production and spermiation (Agulleiro et al., 2006), and (ii) spontaneous spawning had normally low to null fertilization rates (Anguis and Cañavate, 2005; Guzmán et al., 2008), has pointed the male contribution as one of the main factors contributing to the reproductive dysfunctions reported (Cabrita et al., 2006). Despite this, available information to date on Senegalese sole testicular development and spermatogenesis is still scarce. Recently, the spermatogenetic process has been reported in Senegalese sole, showing evidence of a semi-cystic mode of spermatogenesis: spermatids are released into the lobule lumen where they differentiate in successive batches into mature spermatozoa (García-López et al., 2005, 2006). Within the testis, two differentiated regions are observed: the cortex containing the seminiferous lobules where germ cells proliferate in spermatocyts, and the medulla containing spermatic ducts that collect and store the spermatozoa (García-López et al., 2005). Although spermatogonia cysts occur along the entire length of testicular lobules, indicating an unrestricted spermatogonial growth type (Grier et al., 1980; Selman and Wallace, 1986), most type A spermatogonia are located at the distal part of the lobules (García-López et al., 2005).

During spermatogenesis, germ cells undergo profound morphological, molecular and biochemical changes to give rise to the highly specialized mature spermatozoon. Among other molecules, the contents and profiles of different glycoproteins significantly change during spermatogenesis, suggesting the important role of such components in the process (Jones et al., 1988; Anakwe and Gerton, 1990; Martínez-Menárguez et al., 1992). Since lectins have specific binding affinities for the sugar residues of glycoconjugates, they constitute useful tools to investigate glycoconjugate distribution during germ cell differentiation and sperm maturation (Spicer and Schulte, 1992; Sharon and Lis, 2003). Lectin histochemistry has been successfully used to investigate changes in carbohydrate moieties during male gamete differentiation and in the testicular interstitium of several mammalian species (Arya and Vanha-Perttula 1984; Calvo et al. 2000; Pastor et al., 2003; Desantis et al., 2006), including humans (Arenas et al., 1998). Lectin-binding pattern has also been reported in the testis of non-mammalian species (Ballesta et al., 1991; Labate and Desantis, 1995; Sáez et al., 2000, 2004), as well as in the spotted ray *Torpedo*

marmorata (Liguoro et al., 2004) and in the teleost fishes Nile tilapia *Oreochromis niloticus niloticus* and medaka *Oryzias latipes* (Tokalov and Gutzeit, 2007).

Conventional protein and carboxylated mucosubstance/glycoprotein histochemistry, as well as 3β-hydroxysteroid dehydrogenase enzymatic activity, have recently been investigated in the testis of Senegalese sole (García-López et al., 2005), but information on the glycoconjugate pattern changes occurring in germ and somatic cells during spermatogenesis is lacking. Therefore, in order to provide additional data on male reproductive biology of this species, which could potentially help to solve the aforementioned reproductive problems in captivity, the present study reports the identification and cellular localization of glycoprotein oligosaccharide sequences in sexually mature Senegalese sole testis by means of 12 lectins in combination with enzymatic and chemical treatments.

Materials and methods

Two adult Senegalese sole males at mid-late spermatogenesis stage (García-López et al., 2006) were euthanized in January by overexposure to 600 ppm 2-phenoxiethanol (total body weight: 0.67-0.87 kg; gonadosomatic index: 0.07-0.09 %). Testes were quickly dissected, fixed in Bouin's fluid for 24 h and embedded in paraffin wax according to conventional protocols. Transversal sections of 4-μm thickness were obtained for morphologic inspection after haematoxylin and eosin (H-E) staining and for lectin histochemistry (Table 1) following previously reported protocols (Desantis et al., 2006).

Briefly, tissue sections were immersed in 3% v/v H_2O_2 in methanol for 10 min, rinsed in 0.05 M Tris-HCl buffered saline (TBS) pH 7.4, and incubated with each lectin (see Table 1 for dilutions) for 1 h. Then, sections were either incubated with streptavidin/peroxidase complex (Vector Lab. Inc.; Burlingame, CA, USA) for 30 min and subsequently with 0.05% 3,3'-diaminobenzidine (DAB) plus 0.003% H_2O_2 in 0.05 M TBS (pH 7.6) for 10 min (for biotinylated lectins; Table 1) or directly developed in DAB-H_2O_2 solution (for horseradish peroxidase-conjugated lectins; Table 1). Controls for lectin staining included (i) incubation with lectin-free substrate medium, and (ii) incubation with each lectin in the presence of its hapten inhibitory sugar (0.2-0.5 M in Tris buffer; see Table 1).

In order to identify the carbohydrate residues linked to terminal sialic acids, some sections were digested at 37 °C for 16 h with 0.86 U sialidase (Type V, from *Clostridium perfringens*; Sigma Chemicals Co., St. Louis, MO, USA) in 0.1 M sodium acetate buffer pH 5.5 containing 10 mM

$CaCl_2$ and thereafter subjected to the lectin staining procedures described above. Prior to sialidase digestion the samples were saponified with 0.5% w/v KOH in 70% ethanol for 15 min (Reid et al., 1978) to detach acetyl substituents contained in the acetylated groups on C_4 of the pyranose ring of sialic acid residues, thus rendering the residues susceptible to sialidase digestion (Moschera et al., 1975). As controls of the enzymatic digestion, sections were incubated in enzyme-free buffer.

Results

The Senegalese sole testis presents two main regions, a cortical one with seminiferous lobules where germ cells proliferate in spermatocysts (germ/Sertoli cells units), and a medullar one with spermatic ducts that collect and store the spermatozoa (Figs. 1-5) (for major morphological details on Senegalese sole testicular structure and spermatogenesis see García-López et al., 2005). The results of lectin staining in Senegalese sole testis sections are summarized in Table 2.

In the interstitial compartment, reactivity to MAA II was mainly observed in the stroma located in the medullar region of the testis, whereas the apical and lateral extensions of the cytoplasm of Sertoli cells located in the cortical region and the head of spermatozoa occupying the efferent ducts were the only MAA II positive elements in the germinal compartment (Fig. 6). Saponification, followed by neuraminic acid cleavage (hereinafter indicated as K-s treatment), abolished MAA II reactivity.

SNA I stained moderately the interstitial stroma and weakly the basal lamina separating the interstitial and germinal compartments. The SNA I reactivity disappeared after K-s treatment.

Reactivity to PNA was found in the interstitial stroma, basal lamina, cytoplasm of meiotic cells, and in the melano-macrophages centres (MMC's), which are nodular structures mainly located within the medullar stroma (Fig. 7). In addition, a strong reaction was observed in the cytoplasm of the cells covering the wall of the efferent ducts (Fig. 8). Such a staining pattern was conserved after K-s treatment, except that PNA cryptic binding appeared in the head of spermatozoa located in the efferent ducts (inset of Fig. 8).

Weak DBA staining was observed in the medullar interstitial tissue and in the head of spermatozoa stored in the efferent ducts, while a moderate reactivity was found in the cytoplasm of cells covering the wall of the efferent ducts. K-s treatment revealed cryptic binding sites in the interstitial tissue of the medullar region, head of spermatozoa, and cytoplasm of the cells covering the wall of the efferent ducts, whereas Leydig cells were still negative (Fig. 9).

RCA_{120} displayed weak reactivity in the interstitial stroma and in the basal lamina of seminiferous lobules. K-s treatment did not modify the RCA_{120} staining pattern.

SBA reactivity was detected in the interstitial stroma, basal lamina, and in the cytoplasm of spermatids, which in addition presented granular areas with moderate staining (Fig 10). Spermatozoa showed moderate reactivity to SBA as well. K-s treatment did not reveal cryptic binding sites.

Staining for HPA revealed weak reactivities in both the interstitial tissue and the basal lamina of seminiferous epithelium (Fig. 11). In the germinal compartment, HPA binding sites were found in the nucleus of primary spermatocytes (inset a in Fig. 11) and in the cytoplasm of spermatids, which also displayed strongly stained granular regions (inset b in Fig. 11). HPA reactivity was also found in the cytoplasm of cells covering the wall of the efferent ducts and in the spermatozoa head. K-s treatment increased the staining intensity in the head of spermatozoa.

Similar staining patterns were obtained for GSA I-B_4 and Con A binding sites. The interstitial stroma, basal lamina, and the cytoplasm of spermatids displayed weak reactivities, while granular areas within the spermatid cytoplasm (Fig. 12) and the head of spermatozoa located in the efferent ducts showed moderate to strong staining (Fig. 13).

K-s treatment in combination with WGA (performed to highlight βGlcNAc, but not sialic acid) showed binding sites in the interstitial stroma and in the basal lamina (Fig. 14).

Binding sites for GSA II were hardly present in Senegalese sole testis tissue; only weak staining was observed in the interstitial tissue.

Similar binding patterns were obtained for UEA-I and LTA. Binding sites for both lectins were found in the interstitial tissue, basal lamina, cytoplasm of spermatids where moderately stained granules were observed (Fig. 15), cytoplasm of the epithelial cells lining the efferent ducts, and in the head of spermatozoa (Fig. 16).

Leydig cells, identified as round cells organized in small groups within the interstitial tissue, were not stained with any of the lectins tested (*e.g.* Fig. 9).

Discussion

In order to provide additional data on Senegalese sole testicular development and spermatogenesis, which could eventually help to solve the reproductive problems in captivity found in this species (García-López et al., 2005; Anguis and Cañavate, 2005; Agulleiro et al., 2006; Guzmán et al., 2008), the present study reported the identification and cellular localization of glycoprotein oligosaccharide sequences reacting with several specific lectins in testicular tissue obtained from sexually mature Senegalese sole specimens. Lectin-binding sites were observed in both interstitial and germinal compartments, as well in the basal lamina separating both compartments. In the interstitial stroma, a different glycosylation pattern was detected between

cortical and medullar regions of the testis, possibly reflecting the different developmental processes taking place in each testicular area (García-López et al., 2005). In addition, the lectin-binding pattern of germ line changed as spermatogenesis progressed, in accordance with the profound morphological, molecular and biochemical changes that germ cells undergo to give rise to mature spermatozoa.

The connective tissue constituting the interstitial stroma presented reactivity to all twelve specific lectins used in the current study, although different reaction intensities were observed for each one. These results indicate that the interstitial stroma has a very complex glycoprotein composition, including asialo- as well as sialoglycans in N- and O-linked oligosaccharides, as indicated by the MAA II, PNA, HPA, DBA, and K-s-DBA reactions (Spicer and Schulte, 1992) or N-linked glycans, ranging from high-Man to small bi-antennary complex type, which bind Con A irrespective of bisection (Goldstein and Hayes, 1978; Debray et al., 1981). Such a complex glycosylation pattern may be related to the intense glycoprotein compound trafficking occurring from the blood, which irrigates the testicular tissue through an intricate vascular system located in the interstitial compartment to both the cellular components of the interstitial tissue and the germinal compartment, thus ensuring the continuous supply of substances required for germ cell development. Interestingly, disparate glycosylation patterns were registered in the interstitial stroma depending on the testicular region analysed. Specifically, MAA II, DBA and K-s-DBA reactions demonstrated higher abundance of Neu5acα2,3Galβ1,4GlcNac, and GalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1 penultimate or not to sialic acid in the medullar region of the testis in comparison to the cortical area. While germinal activity, i.e. germ cell proliferation and differentiation within spermatogenetic cysts, is restricted to the cortical region of the testis, the final sperm maturation seems to take place in the efferent ducts of the medullar region, which serves as spermatozoa storage prior to spawning (García-López et al., 2005). Therefore, since such developmental processes may have distinct glycoprotein requirements, we hypothesise that differential trafficking patterns from the vascular system to each testicular region would be the origin of the disparate stromal glycosylation patterns observed between the cortical and the medullar regions of Senegalese sole testis.

The presence of MMC's has been reported in the testis of several teleosts (Grier and Taylor, 1998; Cinquetti and Dramis, 2003) including Senegalese sole (García-López et al., 2005). MMC's are composed of different types of cells, mainly macrophages (Agius and Roberts, 2003; Lo Nostro et al., 2004) and granulocytes (Grier and Taylor, 1998), which generally accumulate pigments like hemosiderin, lipofuscin, ceroids and melanin (Sarasquete and Gutiérrez, 1984; Wolke, 1992). In this study MMC's contained binding sites to PNA, indicating the presence of oligosaccharides

terminating with Galβ1,3GalNAc. The role of these glycans in Senegalese sole testis tissue is not known since this is the first study showing the presence of lectin-binding sites in MMC's of any vertebrate species.

To our surprise, Senegalese sole Leydig cells, the androgen-producing cells within sole testis (García-López et al., 2005), were unreactive to all the lectins tested in the current study. Nevertheless, Leydig cell lectin binding pattern has been reported to be highly variable among different species, both mammalian and non mammalian. Among mammalian Leydig cells disparate lectin binding descriptions have been reported; while boar and domestic cat Leydig cells present simple glycosylation patterns (Calvo et al., 2000; Desantis et al., 2006), those observed in human, mouse or hamster Leydig cells are much more complex (Arenas et al., 1998; Zhou et al., 1992; Pastor et al., 2003). On the other hand, Leydig cells did not bind any of the five lectins (SBA, PNA, RCA-I, WGA, GSA-II) assayed in adult horse testicular sections (Verini-Supplizi et al., 2000). As regards the three fish species studied prior to this report, several differences in the Leydig cell lectin binding pattern were also observed; while Leydig cells of spotted ray have binding sites for Con A (Liguoro et al., 2004), Nile tilapia and medaka Leydig cells bind WGA in addition to Con A (Tokalov and Gutzeit, 2007). Therefore, it can be concluded that the glycosylation pattern of Leydig cells presents a highly species-specific behaviour and, thus, the absence of lectin binding sites described in the present report for Senegalese sole Leydig cells may be characteristic for this species. Unfortunately, similar studies in closely-related species, like other sole or flatfish species, are not available to compare our results. However, we do not rule out that further studies including some other lectins among the wide range available from lectin histochemistry could reveal some glycosylation pattern in the Senegalese sole Leydig cells.

The basal lamina separating the germinal and the interstitial compartments of Senegalese sole testis showed reactivity with a wide range of lectins tested in this study, suggesting the presence of a complex glycosylation pattern constituted mainly by mannosylated glycans with internal GlcNAc (as indicated by the positive reaction to K-s-WGA), oligosaccharides terminating with Galβ1,3GalNAc, Galβ1,4GlcNAc, βGalNAc, αGal, αL-Fuc, Neu5Acα2,6Gal/GalNAc, and sialic acid linked to Galβ1,3GalNAc. Such a complex lectin-binding pattern could be related to the presence of a meshwork composed of several large glycoconjugate components, such as laminins, collagen IV, perlecan, and nidogen, which are responsible for many of the biological functions attributed to basal laminas (Timpl, 1993; Malinda and Kleinman, 1996; Timpl and Brown, 1996).

Senegalese sole Sertoli cells expressed only binding sites to MAA II. The finding that such reactivity was located in the apical extensions and supra-nuclear lateral surface of the spermatocysts located in the distal part of the seminiferous lobules suggests that Sertoli cells sialoglycans

terminating with Neu5acα2,3Galβ1,4GlcNac could have a role in the organization of spermatogenetic cysts, including the establishment of interactions between adjacent spermatocysts. Different from mammals, fish spermatogenesis takes place within spermatocysts, which are functional units composed of a germ cell clone (derived from an unique early spermatogonia and all in the same stage of development) surrounded by the cytoplasmic extensions of Sertoli cells. At the beginning of the annual testicular cycle the establishment of successive spermatocysts along the seminiferous lobules originates a continuous germinal epithelium (Brown-Peterson et al., 2002; García-López et al., 2005; 2006), which becomes discontinuous as germ cells are released to the lumen to complete their differentiation prior to spawning. Different from Senegalese sole, the Sertoli cells of other fish species, such as spotted ray, Nile tilapia and medaka, display a more complex glycosylation pattern, but sialoglycans seem to be lacking in those species (Liguoro et al., 2004 Tokalov and Gutzeit, 2007). Sialoglycans have been found in the Sertoli cells of reptiles (Labate and Desantis, 1995), amphibians (Sáez et al., 2004) and several mammals (Jones et al., 1992b, 1993; Arenas et al., 1998; Calvo et al., 2000; Pastor et al., 2003; Desantis et al., 2006). In addition to structural elements, it has been suggested in the mammalian testis that lectin-labelled glycoproteins found in Sertoli cells may be secreted, acting as paracrine factors in the modulation of germ cell development (see below) and peritubular and Leydig cells functioning (Skinner, 1993; Sáez, 1994; Petersen and Söder, 2006). Although the roles of sialoglycoproteins detected in Sertoli cells are not clearly elucidated, in rat Sertoli cells it has been reported that they form part of the secretory sulfated glycoprotein-2 (clusterin) (Sensibar et al., 1993).

The current study has shown that the glycosylation pattern of Senegalese sole germ cells changes as spermatogenesis progresses, reaching in mature spermatozoa a highly complex composition, as previously reported in other vertebrate species (Calvo et al., 2000; Pastor et al. 2003; Desantis et al., 2006; Labate and Desantis, 1995; Sáez et al., 2004), including fish (Liguoro et al., 2004; Tokalov and Gutzeit, 2007).

While spermatogonia were unreactive to all the lectins tested in Senegalese sole testicular sections, primary spermatocytes exhibited cytoplasmic PNA reactivity and nuclear HPA binding sites, suggesting the presence of oligosaccharides terminating with Galβ1,3GalNAc and αGalNAc, respectively. The appearance of lectin-binding sites in germ cell nuclei at the time of meiosis seems to be a common feature in vertebrate spermatogenesis, and thus it has been suggested as a marker for early meiotic processes in rat (Tanii and Yoshinaga, 2002). Germ cell nuclear glycoproteins have been associated with the chromatins playing a role in the regulation of transcription and in the control of cell cycle (Varki et al., 1999; Lowe and Marth, 2003).

The cytoplasm of spermatids reacted with Con A, PNA, SBA, HPA, GSA I-B_4, UEA I and LTA. This suggests the presence of N-linked glycans containing high-Man residues, as well as oligosaccharides terminating with α/βGalNAc, αGal and αL-Fuc. These glycoproteins, except for PNA binding ones, were also found in fine granular structures found in the cytoplasm of spermatids. It is important to mention here that Senegalese sole spermatozoa, like those of the majority of teleost fish species, lack an acrosome (Medina et al., 2000), and thus such structures are not attributable to the initial development of such an organelle. Rather, we suggest that such granular structures observed in the current light microscopy study could originate from aggregation of multiple vesicles, as observed in several ultrastructural investigations during teleost fish spermiogenesis, or form part of the well developed endoplasmic reticulum and Golgi apparatus reported in fish spermatids (Billard, 1983; Quagio-Grassiotto and Carvalho, 2000; Quagio-Grassiotto et al., 2003; Wu et al., 2008). The presence of abundant lectin-binding sites indicates that these cytoplasmic masses have the machinery needed for glycoprotein synthesis and/or processing.

The head of Senegalese sole spermatozoa presented a highly complex glycosylation profile, which includes oligosaccharides terminating with αGalNA and with sialic acid linked to Galβ1,4GlcNAc Galβ1,3GalNAc, and αGalNAc. Such pattern differs from that observed in spotted ray (Liguoro et al., 2004), Nile tilapia and medaka (Tokalov and Gutzeit, 2007), especially in the presence of sialoglycoproteins in sole spermatozoa, which have not been reported previously in any of the three fish species mentioned. Nevertheless, the presence of sialoglycoproteins has been demonstrated in spermatozoa of several mammals (Burkett et al., 1987; Jones et al., 1992; Arenas et al., 1998; Desantis et al., 2006) and reptiles (Labate and Desantis, 1995). Due to their negative charges sialic acids are involved in the inhibition of intermolecular and intercellular interactions (Varki, 1997), and in addition they can increase solubility, as well as affect the conformation of glycoproteins and cell adhesive properties (Hilkens et al., 1992; Arenas et al., 1998). In Nile tilapia a LCA- and Con A-positive glycoprotein localized on the surface of spermatozoa acts as a sperm immobilizing factor (Mochida et al., 1999). In salmonid fish, the testis spermatozoa are immotile, acquiring the motility during their passage through the sperm duct (Miura et al., 1992). Accordingly, it is suggested that the high-mannose glycoproteins (revealed with Con A) in Senegalese sole spermatozoa could keep the spermatozoa immotile while still in the testis.

The inner surface of Senegalese sole efferent ducts is covered by epithelial cells showing oligosaccharides terminating with Neu5acGalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1, Galβ1,3GalNAc, αGalNAc, and αLFuc. Although the nature and functions of such cells in Senegalese sole testicular development are unknown at present, we speculate that these cells are Sertoli cell-derived, as suggested in other fish species belonging to the Order Atheriniformes and to

the family Blenniidae (Lahnsteiner et al., 1990; Grier, 1993; Manni and Rasotto, 1997) and in the amphibian urodele *Pleurodeles waltl* (Sáez et al., 2004). This suggestion is supported by the presence of a common basal lamina and the expression of the WT1 gene (Del Rio-Tsonis et al., 1996). The different glycosylation pattern observed in Sertoli cells and duct cells could be related to their different functions. In particular, Neu5acGalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1, Galβ1,3GalNAc, αGalNAc are revealed by PNA, DBA and HPA, which identify the many O-linked oligosaccharides (mucyn-type glycans) containing GalNAc, which are typical secretory moieties (Spicer and Schulte, 1992). Electron microscopy and histochemical studies in these species, some of which present semi-cystic spermatogenesis like Senegalese sole, have suggested that such cells play two main functions, synthesis and secretion of glycoproteins and phagocytosis of degenerating germ cells. The complex glycosylation pattern of efferent duct cells reported in the current study may support the secretory function of such cells in Senegalese sole as well. Accordingly, efferent duct cells may synthesize and secrete into the lumen substances required for final germ cell maturation. This assumption would indicate that the efferent duct system found in the testis of the aforementioned fish, including Senegalese sole, plays similar functions to those reported in mammals for extra-testicular ducts, such as efferent ductuli and ductus epididymis (Labate et al., 1997; Tulsiani et al., 1998).

In conclusion, the *in situ* characterization of the glycoconjugates present in Senegalese sole testis by means of lectin histochemistry demonstrated a species-specific glycosylation pattern of both somatic and germ cells. In addition, differential lectin-binding profiles were observed in the interstitial stroma according to the testicular region analysed, which is likely related to the spatial segregation of developmental processes observed within Senegalese sole testis. Further studies have to analyse if the germ cell glycosylation pattern differs between specimens spawning successfully and those with reproductive dysfunctions, in order to evaluate the usefulness of lectin histochemistry as a tool for the development of effective therapies for controlled spawning from captive Senegalese sole broodstocks.

Acknowledgments. The authors wish to thank Mrs. Annunziata Marinelli and Mr. Vincenzo Pesola, and Mrs Isabel Viaña for their technical assistance. This research was funded by the Spanish Ministry of Education and Science-MEC- (Project AGL2006-13777)

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Table 1. Lectins used, their sugar specificities and inhibitory sugars used in control experiments

Acronym	Source of lectin	Concentration (μg/ml)	Sugar specificity	Inhibitory sugar
MAA II*	*Maackia amurensis*	15	Neu5acα2,3Galβ1,4GlcNAc	NeuNAc
SNA I*	*Sambucus nigra*	15	Neu5Acα2,6Gal/GalNAc	NeuNAc
PNA	*Arachis hypogaea*	20	Terminal Galβ1,3GalNAc	Gal
DBA	*Dolichos biflorus*	20	Terminal GalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1	GalNAc
RCA_{120}	*Ricinus communis*	25	Terminal Galβ1,4GlcNAc	Gal
SBA	*Glycine max*	15	Terminal α/βGalNAc	GalNAc
HPA	*Helix pomatia*	20	Terminal αGalNAc	GalNAc
GSA I-B_4*	*Griffonia simplicifolia*	25	Terminal αGal	Gal
Con A	*Canavalia ensiformis*	20	Terminal/internal αMan>αGlc	Man
WGA	*Triticum vulgaris*	20	Terminal/internal βGlcNAc>>NeuNAc	GlcNAc
GSA II	*Bandeiraea simplicifolia*	20	Terminal D-GlcNAc	GlcNAc
UEAI	*Ulex europaeus*	25	Terminal L-Fucα1,2Galβ1,4GlcNAcβ	Fuc
LTA	*Lotus tetragonolobus*	25	Terminal αL-Fuc	Fuc

Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuNAc, N-acetyl neuraminic (sialic) acid. Biotinylated lectins (Vector Laboratories Inc.) are labelled with an asterisk; horseradish peroxidase-conjugated lectins (Sigma Chemicals Co) are not labelled.

Table 2. Lectin staining pattern in Senegalese sole *Solea senegalensis* testis sections

Lectins	Interstitial compartment	Basal lamina	Seminiferous lobules			Efferent ducts
			Sertoli Cells	Primary spermatocytes	Spermatids	
MAA II	±cs/++ms	-	++	-	-	+++sz
K-s-MAA II	-	-	-	-	-	-
SNA I	++s	+	-	-	-	-
K-s-SNA I	-	-	-	-	-	-
PNA	+s/++MCCs	±	-	±	±	+++ec
K-s-PNA	+s/++MCCs	+	-	±	±	+++ec/+++sz
DBA	+ms	-	-	-	-	++ec/+sz
K-s-DBA	++ms	-	-	-	-	+++ec/++sz
RCA_{120}	+s	+	-	-	-	-
K-s-RCA_{120}	+s	+	-	-	-	-
SBA	+s	+	-	-	+/++g	++sz
K-s-SBA	+s	+	-	-	+/++g	++sz
HPA	+s	-	-	++	+/+++g	+++ec/+sz
K-s-HPA	+s	-	-	++	+/+++g	+++ec/++sz
GSA I-B_4	+s	+	-	-	+/+++g	+++sz
Con A	+s	+	-	-	+/++g	+++sz
K-s-WGA	+s	++	-	-	-	-
GSA II	+s	-	-	-	-	-
UEA I	++s	±	-	-	+/++g	+ec/++sz
LTA	++s	±	-	-	+/++g	+ec/++sz

K-s indicates that sections were saponified and treated with sialidase prior to specific lectin reactions. Reaction intensity: -, negative; ±, very weak; +, weak; ++, moderate; +++, strong. cs, stroma of the cortical region; ec, efferent duct wall cells; MCCs, Melano-macrophages centres; ms, stroma of the medullar region; s, whole stroma; g, granular region; sz, spermatozoa.

Captions

Figs. 1-5. Photomicrographs showing the morphology of Senegalese sole testis at different magnifications. **Fig. 1.** Low magnification view depicting the two testicular regions, the cortex, at the periphery of the organ, and the medulla, in the inner part of the testis. **Fig. 2.** Medium magnification view of the cortical area showing germ cells at different developmental stages. **Fig. 3.** Medium magnification view of the medullar area showing numerous efferent ducts filled with spermatozoa and a group of Leydig cells in the interstitial compartment. **Fig. 4.** High magnification view of a seminiferous lobule in the cortical area of the testis. **Fig. 5.** High magnification view of an efferent duct in the medullar area of the testis. Note the presence of several epithelial cells covering the wall of the duct and a Leydig cell in the interstitial compartment. ec, efferent ducts wall cells; L, Leydig cells; S, Sertoli cells; sd, spermatids; sgA, type A spermatogonia; sgB, type B spermatognia; spI, primary spermatoytes; sz, spermatozoa; double arrow, tunica albuginea. Haematoxylin-eosin staining. Scale bars: Fig. 1 = 100 μm; Figs 2, 3 = 40 μm; Figs. 4,5 = 10 μm.

Fig. 6. MAA II reactivity in the Senegalese sole testis. Note the positive reactions in the interstitial stroma, in the Sertoli cell cytoplasm extensions in the cortical region, and in the head of spermatozoa occupying the efferent ducts (inset). s, interstitial stroma; sz, spermatozoa; arrowhead, Sertoli cell extensions. Scale bars: 6 μm.

Figs. 7,8. PNA reactivity in Senegalese sole testis. Fig. 7 shows a general view of the tissue section. Observe the positive staining in the interstitial stroma of both cortical and medullar regions (insets a,b), basal lamina (inset **a**) and cytoplasm of primary spermatocytes (inset a), melano-macrophages centres (inset b), and in the cytoplasm of the cells covering the wall of the efferent ducts (Fig. 8). Note the cryptic PNA binding appearing in the head of spermatozoa (sz) after KOH-sialidase treatment (inset in Fig. 8). M, melano-macrophages centre; s, interstitial stroma; sg, spermatogonia; spI, primary spermatocytes; sz, spermatozoa; asterisk, efferent duct wall cells; double arrow, basal lamina. Scale bars: Fig. 7 = 180 μm; inset a = 13 μm; inset b= 28 μm; Fig. 8 = 8 μm; inset in Fig. 8 = 60 μm.

Fig. 9. KOH-sialidase-DBA staining of Senegalese sole testis. This high magnification picture shows positive reactions to DBA of the interstitial stroma, whereas Leydig cells were unreactive. L, Leydig cells; s, interstitial stroma. Scale bar = 8 μm.

Fig. 10. SBA reactivity in Senegalese sole testis. This high magnification picture shows the weak reactions in the interstitial stroma, basal lamina, and in the cytoplasm of spermatids which contain a granular positive region. s, interstitial stroma; sd, spermatids; spI, primary spermatocytes; arrow, granule in spermatid; double arrow, basal lamina. Scale bar = 10 μm .

Fig. 11. HPA staining in Senegalese sole testis. Note the positive reactions in the interstitial stroma,, primary spermatocytes (inset **a**), cytoplasm of spermatids and its granular regions (inset **b**). s, interstitial stroma; sd, spermatids; short arrow, granule in spermatid; large arrow, primary spermatocytes. Scale bars: = 25 μm; inset a = 9 μm; inset b = 4 μm .

Figs. 12,13. GSA I-B_4 labelling of Senegalese sole testis. Reactivity was observed in the interstitial stroma, basal lamina, cytoplasm of spermatids and its granular regions , and in the head of the efferent duct spermatozoa. s, interstitial stroma; sd, spermatids; sz, spermatozoa; arrow, granule in spermatid;. Scale bars = 4 μm.

Fig. 14. K-s-WGA staining in Senegalese sole testis. Reactivity was detected in the interstitial stroma and in the basal lamina. s, interstitial stroma; double arrow, basal lamina. Scale bar = 21 μm.

Figs. 15,16. LTA binding pattern in Senegalese sole testis. Fig. 15 shows reactivity in the interstitial stroma, basal lamina, cytoplasm of spermatids and its granular regions (inset). Fig. 16 demonstrates binding sites in the epithelial cells lining the efferent ducts and in the head of spermatozoa. s, interstitial stroma; sd, spermatids; sz, spermatozoa; arrow, granule in spermatid; double arrow, basal lamina; asterisk, efferent duct wall cells. Scale bars: Fig. 15 = 18 μm; inset in Fig. 15 = 4 μm; Fig. 16 = 6 μm; inset in Fig. 16 =10 μm.