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Histochemical study of glycoconjugates in the toadfish *Halobatrachus didactylus* oesophagus epithelium

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Summary. The carbohydrate expression in the epithelium lining the oesophagus of the toadfish *Halobatrachus didactylus* was studied by means of conventional and lectin histochemistry. The stratified epithelium was constituted by basal cells, polymorphous cells in the intermediate layer, pyramidal and flattened cells in the outer layer and contained two types of large secretory cells: goblet cells and sacciform cells. PAS, Alcian blue pH 2.5 and pH 1.0 stained very strongly the goblet cells, weakly the surface of the other epithelial cells but did not stain the sacciform cells. The goblet cells cytoplasm contained oligosaccharides with terminal Galß1,3GalNAc, α/ßGalNAc, Galß1,4GlcNAc, αL-Fuc and internal ßGlcNAc residues (PNA, SBA, RCA_{120}, UEA I, LTA and KOH-sialidase-WGA affinity). Galß1,4GlcNAc, αL-Fuc and internal ßGlcNAc were also found in the glycocalyx. The sacciform cells expressed sialyloligosaccharides terminating with Neu5Acα2,3Galß1,4GlcNac, Neu5Acß2,6Gal/GalNAc, Neu5AcForssman pentasaccharide (MAL II, SNA, KOH-sialidase-DBA staining) as well as asialo-glycoconjugates with terminal/internal αMan (Con A affinity) and with terminal Galß1,3GalNAc, Forssman pentasaccharide, Galß1,4GlcNAc, GalNAc (HPA and SBA reactivity), αGal (GSA I-B_4 reactivity), D-GlcNAc (GSA II labelling), αL-Fuc. The basal cells cytoplasm exhibited terminal/internal αMan and terminal Neu5Acα2,6Gal/GalNAc, Galß1,4GlcNAc, α/ßGalNAc, αGal, GlcNAc, αL-Fuc. Intermediate cells showed oligosaccharides with terminal/internal αMan and/or terminating with Neu5Acα2,6Gal/GalNAc, Galß1,4GlcNAc in the cytoplasm and with Neu5Acα2,3Galß1,4GlcNac, α/ßGalNAc, αGal, GlcNAc, αL-Fuc in the glycocalyx. The pyramidal cells expressed terminal/internal αMan and terminal Neu5Acα2,6Gal/GalNAc, α/ßGalß1,4NAc, αGal, αL-Fuc in the entire cytoplasm, terminal Neu5Acα2,3 Galß1,4GlcNac and Forssman pentasaccharide in the apical extension, internal ßGlcNAc and/or terminal αL-Fuc in the luminal surface, Neu5acα2,3Galß1,4GlcNac, Neu5Acα2,6Gal/GalNAc, Galß1,4GlcNAc, αGal in the basolateral surface. The flattened cells displayed glycans with terminal/internal αMan and terminal Neu5Acα2,6Gal/GalNAc, α/ßGalNAc, αGal, D-GlcNAc in the entire cytoplasm, glycans terminating with Galß1,3GalNAc and/or internal ßGlcNAc in the sub-nuclear cytoplasm.

Key words: Glycoconjugates, Lectin histochemistry, Oesophagus, Toadfish

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Introduction

The morphology of the oesophagus in teleosts has been documented for many species and shows a marked diversity in relation to the taxonomy, the physical characteristics of food and the feeding habits (Kapoor et al., 1975; Reifel and Travill, 1977; Hirji, 1983; Martin and Blaber, 1984; Elbal and Agulleiro, 1986; Grau et al., 1992; Gargiulo et al., 1996).

In fishes, the oesophagous is almost entirely lined by a stratified epithelium with the exception of limited areas where it is simply columnar in some species (Weinreb and Bilstad, 1955; Bucke, 1971; Reifel and Travil, 1977; Yamamoto and Hirano, 1978; Clarke and Witcomb, 1980; Elbal and Agulleiro, 1986; Cataldi et al., 1987; Grau et al., 1992; Domeneghini et al., 1998).

In addition, fish oesophageal epithelium presents secretory cells which release large amounts of neutral and acidic glycoconjugates (Kapoor et al., 1975; Reifel and Travill, 1977; Domeneghini et al., 1998, 2005; Scocco et al., 1998; Gisbert et al., 1999; Sarasquete et al., 2001) which could have a role in the lubrication of the epithelium (Domeneghini et al., 1999), in the interaction between mucosa and viruses and/or bacteria (Hanaoka et al., 1989; Zimmer et al., 1992), in the execution of the protective functions of mammalian

saliva (Scocco et al., 1998), as well as in carbohydrate absorption (Sarasquete, 2001). Finally, in some fish, digestion has been observed to start in the oesophagus and to go on in the stomach (Linss and Geyer, 1968; Kapoor et al., 1975; Cataldi et al., 1987).

Oligasaccharides establish the ligand-receptor part of information transfer of the sugar code (Reuter and Gabius, 1999; Gabius, 2000; Solis et al., 2001) and are involved in many biological activities including cell proliferation and differentiation, cell to cell interaction, and regulation of secreted macromolecules (Spicer and Schulte, 1992; Varki, 1999; Fukuda, 2000). In addition, in mammals the different mucoadhesive properties of materials at level of oesophagus has been correlated with the differential charbohydrate exposition in the mucosa (Accili et al., 2004).

Lectins have a specific binding affinity for the sugar residues of glycoconjugates.Therefore, they are a useful tool to investigate glycans distribution as well as cell differentiation and maturation (Spicer and Schulte, 1992). Lectins have been successfully used to evaluate the composition of the oligosaccharides in the oesophageal epithelium of some fishes such as *Acipenser baeri* (Sarasquete et al., 2001), *Anguilla anguilla* (Domeneghini et al., 2005), *Solea senegalensis* (Sarasquete et al., 2001), *Sparus aurata* (Domeneghini et al., 1998, 2005; Sarasquete et al., 2001), *Tilapia spp*. (Scocco et al., 1998), *Umbrina cirrosa* (Parillo et al., 2002; Pedini et al., 2004), as well as of other vertebrate classes such as amphibians (Ferri et al., 2001; Liquori et al., 2002), reptiles (Ferri and Liquori, 1992) and mammals (Poorkhalkali et al., 1999) including human in normal (Yamaguchi et al., 1985) and pathological (Akamatsu et al., 1986; Kannan et al., 2003) conditions.

The aim of this study was to investigate the structural features as well as the glycoconjugate composition by means of conventional and lectin histochemistry of the toadfish *Halobatrachus didactylus* oesophagus epithelium. *Halobatrachus didactylus* is the only species of Batrachoididae found in the Iberian Peninsula. It is distributed from the Bay of Biscay to Ghana and the West Mediterranean (Roux, 1986) and it represents an important component for fishing communities in the Bay of Cádiz (Arias, 1976). This species has received special attention in recent years because of its use as a laboratory animal in toxicological and cardiological experiments (see Palazón et al., 2001, for references).

Materials and methods

Tissue preparation

Adult toadfish *Halobatrachus didactylus* were acquired from artisanal fishermen's catches in Cádiz Bay (South-Western Spain) and maintained in running seawater at the Instituto de Ciencias Marinas de Andalucia. Five fishes were sacrificed with an overdose (0.3% V/V) of fenoxy-ethanol and their oesophagus was quikly removed and fixed in Bouin's fluid for 24 h at room temperature (RT), dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin wax. Serial sections (4-μm thick) were cut and, after de-waxing with xylene and hydration in an ethanol series of descending concentrations, were stained with Haematoxylin-Eosin (HE) and Haematoxylin-VOF type III G.S. (Sarasquete and Gutiérrez, 2005) for morphology, and by means of conventional histochemical procedures or the lectin histochemistry according to Desantis et al. (2003).

Conventional histochemistry

Sections were treated with: 1) periodic acid-Schiff (PAS) reaction for neutral glycoconjugates (GCs) (Mc Manus, 1948); 2) Alcian blue 8GX at pH 2.5 (AB 2.5) for testing simultaneously sulphate esters and carboxyl groups in GCs; 3) Alcian blue 8GX at pH 1.0 (AB 1.0) for the characterization of sulphated GCs (Spicer et al., 1967; Pearse, 1968). In order to reveal cellular combinations of both acidic and neutral glycoconjugates, the AB 2.5/PAS and AB 1.0/PAS staining sequences were performed.

Lectin histochemistry

The lectins used are listed in Table 1. The PNA, DBA, RCA_{120}, SBA, HPA, Con A, WGA, and UEA-I lectins were HRP-conjugated. They were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). MAL II, SNA, GSA-II, and GSA I-B_4 were biotinylated lectins and were purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

De-waxed and re-hydrated 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 (Table 1) for 1 h at room temperature (RT). After 3 rinsings in TBS, the 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 (MAL II, SNA, GSA I-B_4, and GSA II) were rinsed 3 times with 0.05 M phosphate-buffered saline (PBS) and were incubated in streptavidin/peroxidase complex (Vector Lab. Inc., USA) for 30 min at RT. After washing in PBS, peroxidase was developed in a DAB-H_2O_2 solution as above.

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 (0.2-0.5 M in Tris buffer).

Enzymatic and chemical treatments

Before staining with MAL II, SNA, PNA, DBA, and WGA some sections were incubated at 37°C for 16 h in 0.86 U/mg protein of sialidase (Type V, from

Clostridium perfringens) (Sigma Chemicals Co.) 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 sialidase digestion procedure, sections were incubated in the enzyme-free buffer solution under the same experimental conditions.

Results

The tunica mucosa of the oesophagus was lined by a stratified epithelium in which two types of large secreting cells (goblet cells and sacciform cells) were abundant (Fig. 1). Occasional taste buds were also found within the epithelial layer. The goblet cells exhibited a foamy cytoplasm and were stained in Blue-Purple (Methyl Blue affinity) when stained with Haematoxylin-VOF Type III.G. The sacciform cells were full of an acidophilic material showing Eosin or Light Green affinities when stained with HE and H-VOF type III G.S, respectively. Both these cells contained a flattened and peripheral nucleus. The secretory cells of the lining epithelium were intermingled with basal cells, intermediate cells, pyramidal cells, and flattened cells. Basal cells were small cubic cells with a high nucleus/cytoplasm ratio. Intermediate cells were polyhedral-shaped (polymorphous) cells and were placed between the basal and surface zones of epithelium. The latter was consisted of flattened cells and pyramidal cells. Pyramidal cells showed a wide apical extension zone overlooking the lumen characterized by irregular luminal surface protrusions (Fig. 1) .

Conventional histochemistry

Combination of AB/PAS 2.5 and AB/PAS 1.0 methods, which resulted in purple-violet staining, marked strongly the goblet cells, whereas stained discontinuously and very weakly the luminal surface of the lining epithelium and the small perinuclear granules of flattened cells. The sacciform cells were not stained (Fig. 2). AB 2.5 and AB 1.0 procedures gave a similar staining intensity of goblet cells.

Lectin histochemistry

The results of lectin histochemistry are summarized in Table 2.

MAL II gave a faintly visible staining with the glycocalyx of the basal cells and intermediate cells, a moderate affinity of the apical zone in the pyramidal cells (Fig. 3). The lectin showed an intense and moderate reactivity for the peripheral cytoplasm and the content of sacciform cells, respectively. Saponification, followed by neuraminic acid cleavage (KOH-sialidase), abolished staining.

SNA showed moderate staining of the basal cells, weak reaction with the cytoplasm of intermediate cells, flattened cells and pyramidal cells (Fig. 4). The latter showed an intense reactivity with the luminal surface epithelium. This lectin stained strongly the sacciform cells (Fig. 4). After KOH-sialidase treatment no positive reaction was observed.

PNA displayed a weak reaction with the goblet cells, a moderate reaction with the sub-nuclear zone of the flattened cells and with the sacciform cells content. This latter showed a more intense staining in the peripheral cytoplasm band (Fig. 5). KOH-sialidase pre-treatment did not affect the PNA positivity.

DBA gave a faintly visible reaction for the apical zone of pyramidal cells and the cytoplasm of rare flattened cells, and stained moderately sacciform cells (Fig. 6a). After KOH-sialidase treatment the lectin DBA revealed cryptic binding sites in the flattened cells and in the sacciform cells (Fig. 6b).

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

Lectin abbreviation	Source of lectin	Concentration (μg/ml)	Sugar specificity	Inhibitory sugar
MAL	*Maackia amurensis*	15	Neu5acα2,3Gaß,1,4GlcNac	NeuNAc
SNA	*Sambucus nigra*	10	Neu5Acα2,6Gal/GalNAc	NeuNAc
PNA	*Arachis hypogea*	20	Terminal Galß1,3GalNAc	Galactose
DBA	*Dolichos biflorus*	20	Terminal FP>GalNAcα1,3GalNAc	GalNAc
RCA120	*Ricinus communis*	15	Terminal Galß1,4GlcNAc	Galactose
SBA	*Glycine max*	10	Terminal α/ßGalNAc	GalNAc
HPA	*Helix pomatia*	15	Terminal αGalNAc	GalNAc
Con A	*Canavalia ensiformis*	15	Terminal and internal αMan>αGlc	Mannose
WGA	*Triticum vulgaris*	15	Terminal and internal ßGlcNAc>>NeuNAc	GlcNAc
GSA I-B_4	*Bandeiraea simplicifolia*	15	Terminal αGal	Galactose
GSA II	*Bandeiraea simplicifolia*	15	Terminal D-GlcNAc	GlcNAc
UEA I	*Ulex europaeus*	20	Terminal αL-Fuc	Fucose
LTA	*Lotus tetragonolobus*	25	Terminal αL-Fuc	Fucose

Fuc, Fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; FP, Forssman pentasaccharide: GalNAcα1,3GalNAcα1,3Galß1,4Galß1,4GlcNAc; Man, mannose; NeuNAc, N-acetyl neuraminic (sialic) acid.

Table 2. Lectin staining pattern of the epithelium lining the oesophagus mucosa of the toadfish *Halobatrachus didactylus* .

Lectin	Basal cells	Intermediate cells	Pyramidal cells	Flattened cells	Goblet cells	Sacciform cells
MAL II	±s	±s	+a/++s	-	-	++/+++p
KOH-si-MAL II	-	-	-	-	-	-
SNA	++	+	+/+++s	+	-	++++
KOH-si-SNA	-	-	-	-	-	-
PNA	-	-	-	++sn	+	++/+++p
KOH-si-PNA	-	-	-	++sn	+	++/+++p
DBA	-	-	±a	±*	-	++
KOH-si-DBA	-	-	±a	+++	-	+++
RCA120	±/+++*	±	++s	-	+/+s	+/+++p
SBA	±	±s	++++	+++	+	+/+++p
HPA	+	+s	++	++	-	+++/++++p
Con A	+/++s	+/++s	++	++	-	++/+++p
KOH-si-WGA	-/++	-	+ls	++sn	+++/+s	++/+++p
GSA I-B_4	±	+s	+/+++s	++	-	++/+++p
GSA II	+	+s	+s	+	-	+++/++++p
UEA I	±/+s	+s	±/+++ls	-	±/±s	±/+++p
LTA	±	±s	++ls	-	±/±s	±/+++p

a, apical zone; ls, luminal cell surface; p, peripheral cytoplasm; s, entire cell surface; si, sialidase; sn, sub-nuclear zone; *, rare; -, negative reaction; ±, faintly visible reaction; +, ++, +++, ++++, weak, moderate, intense, strong positive reactions. Where non specified, the reactions concern the cytoplasm.

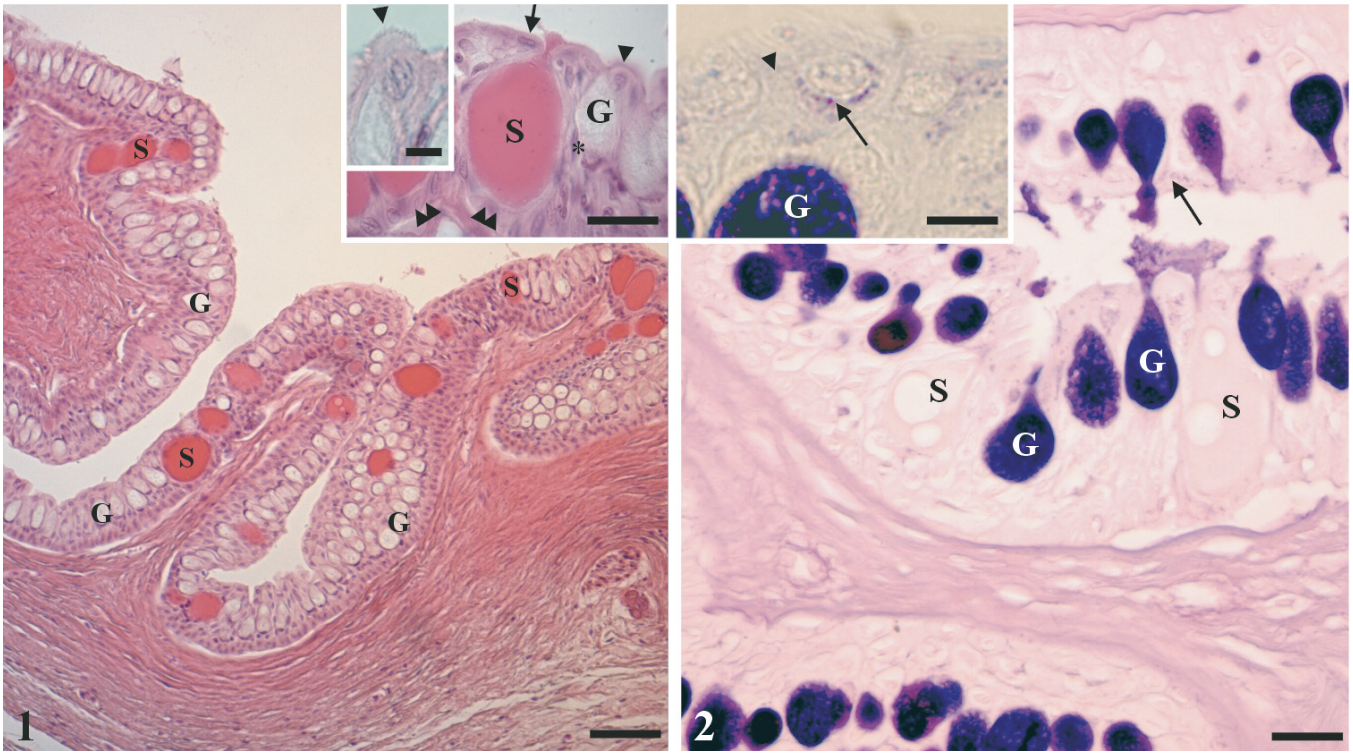


Fig. 1. Epithelial layer of the oesophagus of *Halobatrachus didactylus*. The oesophagus was lined by a stratified epithelium containing numerous goblet cells and sacciform cells. Mayer's hematoxylin-eosin staining. G, goblet cells; S, sacciform cells; arrow, flattened cells; arrowhead, pyramidal cells; double arrowheads, basal cells; asterisk, intermediate cells. Scale bar: 350 μm. Scale bar in insets: 130 μm.

Fig. 2. Epithelial layer of the oesophagus of *Halobatrachus didactylus*. Alcian blue 2.5/PAS reaction. The goblet cells and small granules in flattened cells were purple-violet stained. G, goblet cells; S, sacciform cells; arrow, flattened cells. Scale bar: 175 μm. Scale bar in inset: 70 μm.

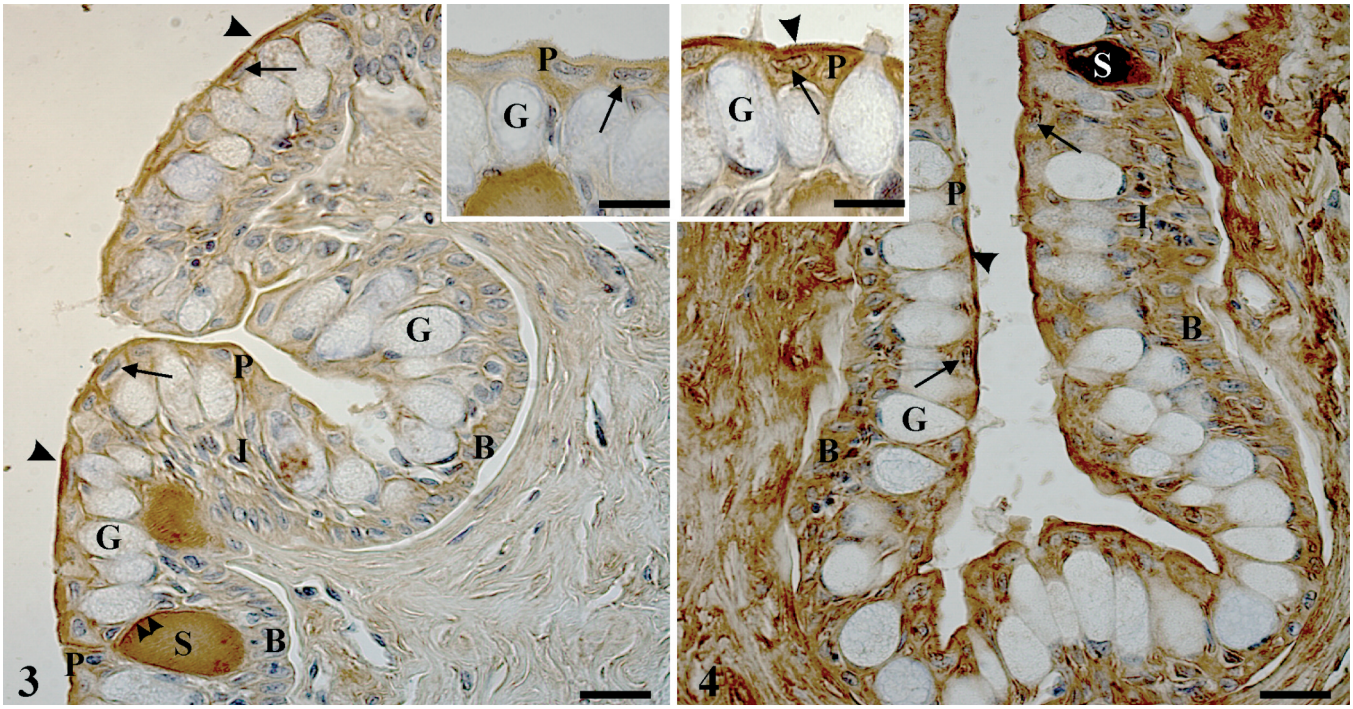


Fig. 3. MAL II reactivity in the oesophagus of *Halobatrachus didactylus*. MAL II stained weakly the surface of basal cells and intermediate cells, moderately the apical zone and surface of pyramidal cells, intensely and moderately the peripheral zone and the content of sacciform cells. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; arrowhead, luminal surface of pyramidal cells; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm. Scale bar in inset: 70 μm.

Fig. 4. SNA binding pattern in the oesophagus of *Halobatrachus didactylus*. SNA labelled the basal cells, intermediate cells, flattened cells, pyramidal cells (which showed intense staining in the luminal surface), and sacciform cells. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; arrowhead, luminal surface of pyramidal cells. Scale bar: 175 μm. Scale bar in inset: 70 μm.

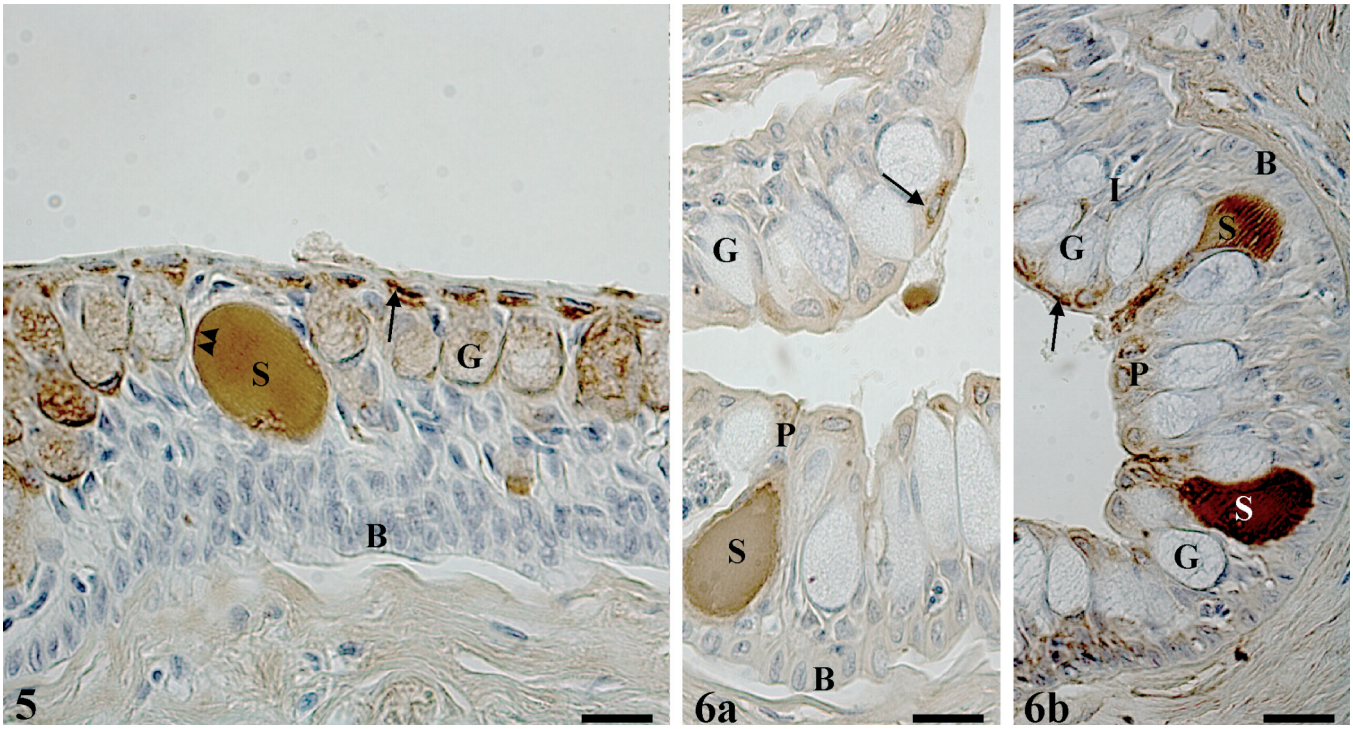
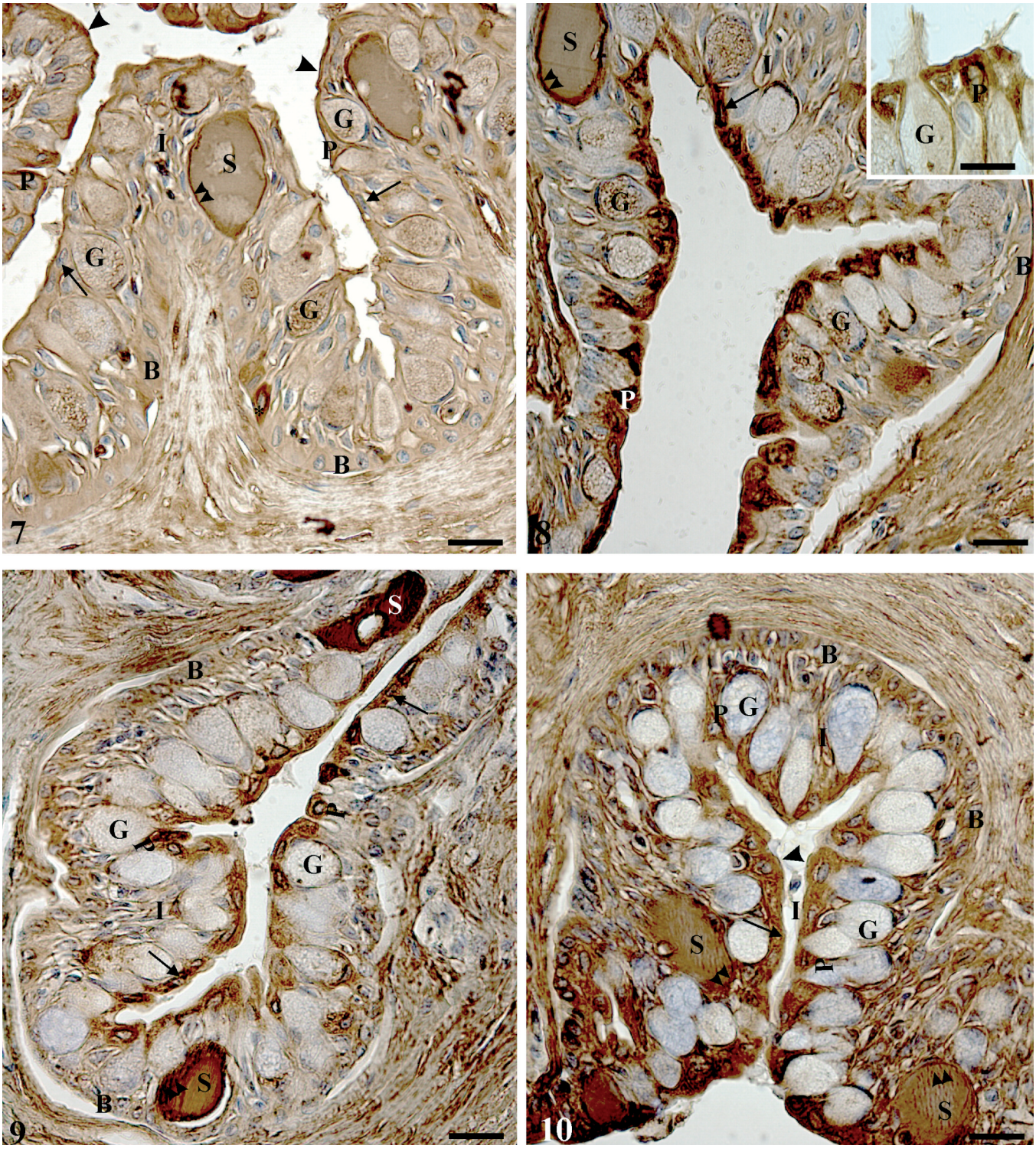


Fig. 5. PNA staining in the oesophagus of *Halobatrachus didactylus*. PNA stained weakly the goblet cells, moderately the sub-nuclear zone of flattened cells, the content of sacciform cells, and more intensely the peripheral zone of sacciform cells. B, basal cells; G, goblet cells; S, sacciform cells; arrow, flattened cells; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm.

Fig. 6. DBA **(a)** and KOH-sialidase-DBA **(b)** staining in the oesophagus of *Halobatrachus didactylus*. **a.** DBA stained very weakly the apical zone of pyramidal cells and the cytoplasm of some flattened cells, moderately the sacciform cells. **b.** KOH-sialidase/DBA procedure increased staining of the flattened cells and the sacciform cells. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells. Scale bar: 175 μm.

RCA_{120} displayed a faintly visible reaction with the basal cells, few of them were intensely stained, and with intermediate cells. In addition, this lectin gave a moderate reaction with the luminal surface of pyramidal cells, a weak staining of goblet cells and content of sacciform cells (Fig. 7). The latter showed intense



reactivity in the peripheral cytoplasm.

SBA showed a faintly visible reaction of the basal cells cytoplasm and the intermediate cells glycocalyx, a strong and intense affinity with the pyramidal cells and the flattened cells, respectively (Fig. 8). The lectin stained weakly the goblet cells and the content of

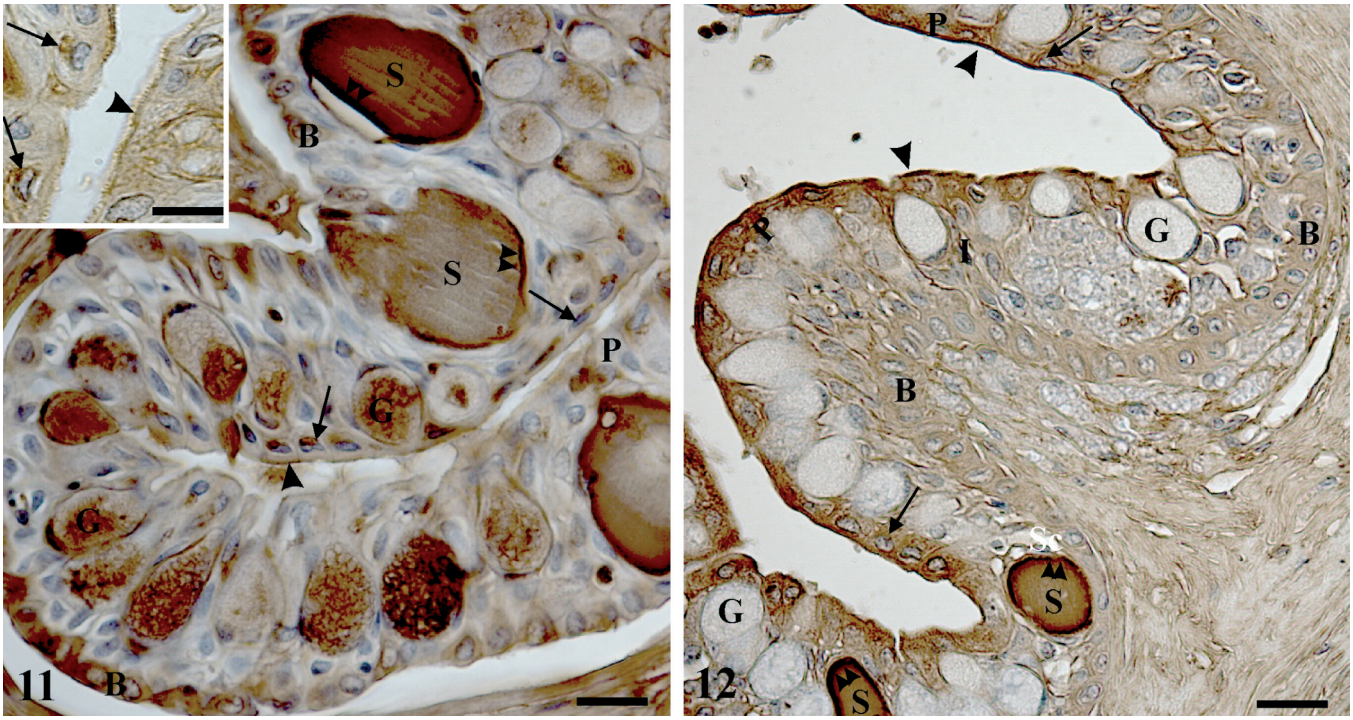


Fig. 11. KOH-sialidase/WGA staining of the oesophagus of *Halobatrachus didactylus*. WGA binding sites were present in the some basal cells, luminal surface of pyramidal cells, sub-nuclear zone of flattened cells, goblet cells and sacciform cells which reacted more strongly in the peripheral zone. B, basal cells; G, goblet cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; arrowhead, luminal surface of pyramidal cells; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm. Scale bar in inset: 70 μm.

Fig. 12. GSA I-B_4 labelling of the oesophagus of *Halobatrachus didactylus*. Staining was present in the surface of intermediate cells and pyramidal cells, in the cytoplasm of basal cells, pyramidal cells, flattened cells and sacciform cells which label more strongly in the peripheral zone. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; arrowhead, luminal surface of pyramidal cells; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm.

Fig. 7. RCA_{120} binding pattern in the oesophagus of *Halobatrachus didactylus*. RCA_{120} stained intensely some basal cells, weakly intermediate cells, moderately the surface of pyramidal cells, weakly the goblet cells and the content of sacciform cells which displayed a more intense reaction in the peripheral zone. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; arrowhead, luminal surface of pyramidal cells; asterisk, intensely stained basal cell; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm.

Fig. 8. SBA staining of the oesophagus of *Halobatrachus didactylus*. SBA labelled the basal cells, intermediate cells surface, pyramidal cells, flattened cells, goblet cells and sacciform cells which react more intensely in the peripheral zone. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm. Scale bar in inset: 70 μm.

Fig. 9. HPA staining of the oesophagus of *Halobatrachus didactylus*. HPA binding sites were located at the level of cytoplasm of basal cells, glycocalyx of intermediate cells, cytoplasm of pyramidal cells, peripheral cytoplasm of sacciform cells which reacted more intensely than the cell content. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm.

Fig. 10. Con A binding pattern in the oesophagus of *Halobatrachus didactylus*. Con A stained the glycocalyx and cytoplasm of basal cells and intermediate cells, the cytoplasm of pyramidal cells, flattened cells and, more strongly, the peripheral zone of saccifom cells. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; arrowhead, luminal surface of pyramidal cells; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm.

sacciform cells, which displayed an intense staining in the peripheral cytoplasm.

HPA gave a weak staining of the basal cell cytoplasm and the intermediate cell glycocalyx, a moderate staining of the pyramidal cells, a strong and intense affinity with the content and periphery of sacciform cell cytoplasm, respectively (Fig. 9).

Con A showed a moderate staining of the glycocalyx of basal cells and intermediate cells. Both these cell types displayed a weak reactive cytoplasm (Fig. 10). Con A stained moderately the pyramidal cells, flattened cells and the content of sacciform cells. This latter cell type showed intense positive reaction in the peripheral cytoplasm (Fig. 10).

KOH-sialidase-WGA treatment (performed to highlight ßGlcNAc, but not sialic acid) showed negative or moderately reactive basal cells, weak affinity with the luminal surface of pyramidal cells, moderate reactivity with the sub-nuclear zone of flattened cells, intense and weak staining of the content and glycocalyx of goblet cells, as well as moderate and intense staining of content and the peripheral cytoplasm of the sacciform cells (Fig. 11).

GSA I-B_4 showed a faintly visible reaction with the basal cell cytoplasm, a weak staining of the intermediate cell glycocalyx and pyramidal cells cytoplasm (Fig. 12). This latter displayed an intense staining of the luminal surface. GSA I-B_4 reacted moderately with the cytoplasm of the flattened cells and with the content of the sacciform cells which also showed a strong affinity in the peripheral zone (Fig. 12).

GSA II displayed a weak staining of the basal cells, of the glycocalyx of intermediate cells, of the luminal surface of pyramidal cells and of the flattened cell

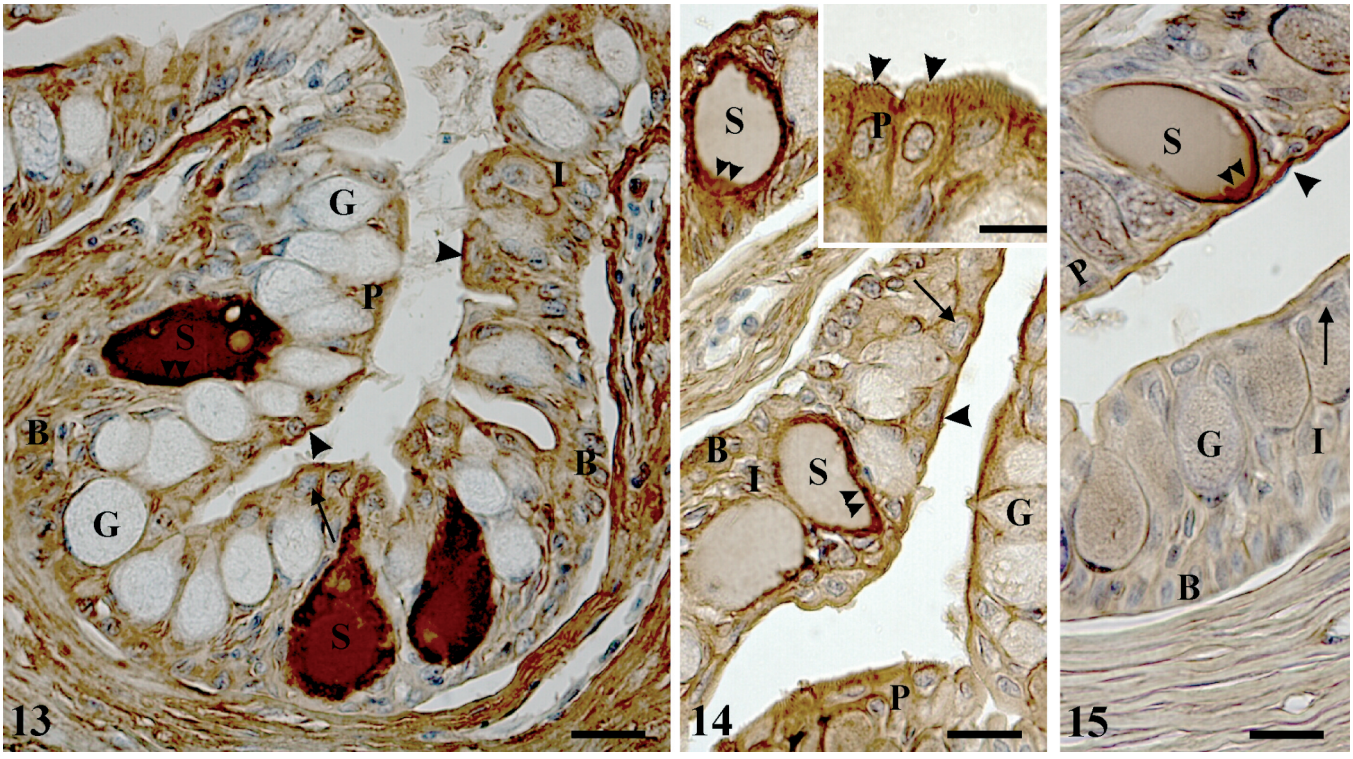


Fig. 13. GSA II binding pattern of the oesophagus of *Halobatrachus didactylus*. GSA II stained the cytoplasm of basal cells, flattened cells, sacciform cells (which reacted more strongly in the peripheral zone) as well as the surface of intermediate and pyramidal cells. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; arrowhead, luminal surface of pyramidal cells. Scale bar: 175 μm.

Fig. 14. UEA I binding pattern in the oesophagus of *Halobatrachus didactylus*. UEA labelled the basal cells, the goblet cells and sacciform cells and the pyramidal cells which reacted strongly in their luminal surface. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; arrowhead, luminal surface of pyramidal cells; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm. Scale bar in inset: 70 μm.

Fig. 15. LTA binding pattern in the oesophagus of *Halobatrachus didactylus*. LTA stained very weakly the basal cells, intermediate cells, goblet cells, the content of sacciform cells and more strongly the luminal surface of pyramidal cells and the peripheral cytoplasm of sacciform cells. B, basal cells; G, goblet cells; I, intermediate cells; P, pyramidal cells; S, sacciform cells; arrow, flattened cells; arrowhead, luminal surface of pyramidal cells; double arrowheads, peripheral cytoplasm. Scale bar: 175 μm.

cytoplasm (Fig. 13). This lectin reacted intensely and strongly with the content and the peripheral cytoplasm of the sacciform cells, respectively (Fig. 13).

UEA I showed a faintly visible reaction with the basal cells, pyramidal cells, the goblet cells and the sacciform cell cytoplasm (Fig. 14). In addition, this lectin stained weakly the glycocalyx of basal cells, intermediate cells, and goblet cells and it marked intensely the luminal surface of pyramidal cells as well as the peripheral cytoplasm of the sacciform cells (Fig. 14).

LTA stained very weakly the basal cells, the goblet cells and the sacciform cell cytoplasm, as well as the glycocalyx of the intermediate cells and goblet cells (Fig. 15). In addition, LTA reacted moderately and intensely with the luminal surface of the pyramidal cells and the peripheral cytoplasm of the sacciform cells, respectively (Fig. 15).

Discussion

This study describes structural aspects of the mucosal epithelium lining the oesophagus of the toadfish *Halobatrachus didactylus* and investigates the glycoconjugate pattern of the cell types that constitute the epithelium.

The two secretory unicellular glands (goblet cells and sacciform cells) showed a different tinctorial affinity when stained with Haemoxylin-Eosin (eosinophilia for sacciform and unstained for goblet cells) and Haematoxylin VOF Type IIIG.S, (Light green/sacciform and Methyl Blue goblet cells affinities) thus suggesting differences in acidic groups of the glycoconjugate contents, as already observed in different fish species (Sarasquete and Gutiérrez, 2005). These authors indicated that the polychromatic/metachromatic property of goblet cells (blue-purple colour/Methyl Blue affinity) found in the digestive tract (oesophagus and intestine) of different fish species could be attributed to glycoconjugates containing strongly ionised sulphated groups, which have negative charges but are also extremely hydrophilic, attracting large volumes of water and cations.

The mucosal epithelium is a stratified epithelium showing cuboidal cells in the basal layer, polymorphous cells in the intermediate layer, pyramidal and flattened cells in the outer layer. As already observed in tilapiine fish (Gargiulo et al., 1996), there are no cells extending from the basal lamina to lumen.

Conventional histochemistry showed that the goblet cells contained both neutral and acidic glycoconjugates, as revealed by PAS, AB 2.5 and AB 1.0 stainings. Since AB 2.5 and AB 1.0 did not show an appreciable difference in the staining intensity of goblet cells, it is possible to infer that these cells produce both sialylated and sulphated glycoconjugates in equal amounts. Mucous cells containing a mixture of neutral and acidic glycoconjugates have been seen in the oesophagus epithelium of several teleostean species (Kapoor et al., 1975; Reifel and Trevil, 1977; Sarasquete et al., 2001; Pedini et al., 2002; Domeneghini et al., 2005). An abundance of glycoconjugate secretion in fish oesophagus is probably linked to lack of salivary glands (Scocco et al., 1998). The neutral GCs and acidic glycans secreted by oesophagus goblet cells could play several roles such as lubrication (Reifle and Travil, 1977) and protection of the mucosa from damage caused by ingested materials (Ezeasor and Stokoe, 1980; Humbert et al., 1984; Uehara and Miyoshi, 1988), giving high viscosity to the mucous to trap the small particles and their aggregation into boluses (Tibbets, 1997). The goblet cells expressed reactivity with PNA and SBA in the cytoplasm and with RCA_{120}, UEA I, LTA and KOH-sialidase-WGA in both cytoplasm and cell surface. This finding indicates the production of O-linked oligosaccharides terminating with Galß1,3GalNAc and α/ßGalNAc only in the cytoplasm and with Galß1,4GlcNAc, αL-Fuc and/or with terminal or internal ßGlcNAc in both the cytoplasmic and glycocalyx. Absence of Con A reactivity was found also in the mucous cells of *Tilapia sp*. (Scocco et al., 1998), *Sparus aurata* and *Solea senegalensis* (Sarasquete et al., 2001) and *Umbrina cirrosa* (Pedini et al., 2004). It is well known that Con A binds to a range of N-linked glycans from high-Man, through intermediate/hybrid, to small bi-antennary complex type, irrespective of bisection (Goldstein and Hayes, 1978; Debray et al., 1981). Therefore, the absence of Con A binding sites indicates that the foamy cells carbohydrate residues belong to O-linked oligosaccharides. O-linked oligosaccharides (mucyn-type glycans) are typical secretory moieties (Spicer and Schulte, 1992). Mucyn-type glycoproteins contain more complex and heterogeneous charbohydrates than N-linked types (Fukuda, 2000). Thus, O-linked glycans could play a greater role than N-linked glycans in the functions of the oesophagus epithelium. The cell surface glycoconjugates terminating with Galß1,4GlcNAc and αL-Fuc and/or containing terminal/internal ßGlcNAc might be involved in the transport of ions (Spicer and Schulte, 1992; Domeneghini et al., 1998) and/or the interaction with other cells constituting the mucosal epithelium of the oesophagus. The oligosaccharide sequences of mucous foamy cells of toadfish *Halobatrachus didactylus* oesophagus reveal that they secrete specific glycoconjugates when compared with the oesophageal mucous cells from other teleosteans in which lectin histochemistry was applied (Domeneghini et al., 1998, 2005; Scocco et al., 1998; Sarasquete et al., 2001; Pedini et al., 2004).

The sacciform cells were not stained with PAS, AB 2.5 or AB 1.0 whereas they were stained with all the used lectins. Lectin binding was stronger in the peripheral cytoplasm probably because of the presence of organules involved in the synthesis of glycoconjugates such as rough endoplasmic reticulum and Golgi apparatus. Lectin reactions indicate that the peripheral cytoplasm as well as the homogeneous

content of sacciform cells are made of sialo- and asialoglycoconjugates belonging both to O- and N-linked glycans as revealed by PNA, DBA and HPA (lectins identifying the many O-linked oligosaccharides containing GalNAc) and by Con A which bind the many N-linked oligosaccharides (Spicer and Schulte, 1992). The sialyloligosaccharides terminate with Neu5acα2,3Galß1,4GlcNac, Neu5Acα2,6Gal/GalNAc and sialic acid linked to Forssman pentasaccharide (this was highlighted by the increase in DBA staining after KOH-sialidase treatment which revealed cryptic binding sites penultimate to sialic acid). The asialoglyco conjugates end with Galß1,3GalNAc, 1,3Galß1, 4Galß1,4GlcNAc (Forssman pentasaccharide), Galß1,4GlcNAc, α/ßGalNAc, αGal, D-GlcNAc, αL-Fuc glycoconjugates. Lectin binding of sacciform cells is not in contrast to PAS procedure failure, because terminal disaccharide Galß1,3GalNAc, and terminal αßGalNAc, αGal, D-GlcNAc, αL-Fuc have been found in many PAS-negative cells. Cells consistently PAS negative are reactive with PNA (Schulte and Spicer, 1983; Spicer et al., 1987), DBA (Spicer et al., 1987), GSA I-B_4 (Schulte and Spicer, 1983; Flint et al., 1986; Spicer et al., 1987), GSA II (Hennigar et al., 1986), UEA I and LTA (Spicer and Schulte, 1992). Staining of acidic groups with basic dyes can be obstructed by associated proteins in cells with serous secretion (Spicer and Schulte, 1992). Thus, the affinity with MAL II, SNA, KOH-sialidase-DBA in unreactive Alcian blue sacciform cells could be related to the presence of acidic glycoconjugates such as sialyloligosaccharides highlighted by the three above-mentioned lectins. The role of these sacciform cells is obscure. However, the presence of sialoglycoconjugates provides a negative charge to secretions. Sialo glycoconjugates in oesophageal secretory cells have been observed in the mucous cells of *Tilapia sp*. (Scocco et al., 1998) and *Umbrina cirrosa* (Pedini et al., 2004). The sialyloligosaccharides in these two species are different when compared to those observed in *Halobatrachus didactylus* because those species show glycans terminating with sialic acid linked to Galß1,3GalNAc (revealed by an increase in the PNA reactivity after sialidase treatment). KOH-sialidase treatment did not reveal any cryptic PNA binding sites in *Halobatrachus didactylus* and sialoglycoconjugates were evidenced with MAL II and SNA, specific for Neu5acα2,3Galß1,4GlcNac and Neu5Acα2, 6Gal/GalNAc, respectively. The presence of sialyloligosaccharides prevents viruses from recognizing their receptor determinants and also protects the mucosae from attack by sialidase produced bacteria (Hanaoka et al., 1989; Zimmer et al., 1992). Sacciform cells are considered to be involved in the secretion of bioactive proteins. Moreover, they are also supposed to secrete mucus lectins which exhibit hemagglutinic activity. These bioactive substances are assumed to be involved in defense mechanisms (Roberts, 1981).

The cells constituting the mucosal epithelium which lines the oesophagus of toadfish showed a very weak staining with the conventional histochemical methods. The combination of PAS/AB methods stained the baso-lateral cell surfaces very weakly thus indicating the presence of neutral and acidic glycoconjugates. Lectin histochemistry highlighted some changes in the oligosaccharide pattern during cell development from the basal cells.

The basal cells exhibited cytoplasmic binding to SNA, RCA_{120}, SBA, HPA, Con A, GSA I-B4, GSA II, UEA I, and LTA. This suggests the presence of O- and N-linked oligosaccharides (the latter with high-Man content revealed with Con A) terminating with Neu5Acα2,6Gal/GalNAc, Galß1,4GlcNAc, α/ßGalNAc, αGal, GlcNAc, and αL-Fuc. The cytoplasmic staining could indicate both synthesis and processing of glycoproteins in the endoplasmic reticulum. Numerous rough endoplasmic reticulum (RER) cisternae have been observed in the basal cells of oesophagus epithelium of tilapiine fish by transmission electron microscopy (Gargiulo et al., 1996). The observed surface labelling with MAL II, Con A, and UEA I demonstrates the presence of sialylated N-linked oligosaccharides terminating with Neu5acα2,3Galß1,4GlcNac and/or αL-Fuc in the glycocalyx. These glycoconjugates might be involved in the interaction basal cells-the basal lamina and/or with the adhesion to intermediate cells, as well as in the transport of ions. In mammals, the plasmalemma glycoconjugates could be involved in the movements of ions and fluids (Spicer and Schulte, 1992). Since KOH-sialidase-WGA stained the cytoplasm of some basal cells, it is possible to infer the existence of two subpopulations of basal cells, some of them containing oligosaccharides with internal GlcNAc.

The intermediate cells expressed reactivity for SNA, RCA_{120} and Con A in the cytoplasm and for MAL II, SBA, HPA, Con A, GSA I-B_4, GSA II, UEA I, and LTA in the glycocalyx. The lectin-binding pattern demonstrated the absence of O-linked glycans and the presence of N-linked oligosaccharides containing terminal/internal Man terminating with Neu5Acα2, 6Gal/GalNAc, Galß1,4GlcNAc in the cytoplasm and the presence of O- and N-linked glycans terminating with Neu5acα2,3Galß1,4GlcNac, α/ßGalNAc, αGal, GlcNAc, and αL-Fuc in the glycocalyx. O-linked oligosaccharides were highlighted by the presence of terminal αGalNAc residues (Spicer and Schulte, 1992). Since oligosaccharides terminating with α/ßGalNAc, αGal, and GlcNAc are also present in the cytoplasm of basal cells, these glycans may be supposed to be synthesized in the basal cells and move onto the surface during transition to intermediate cells. Cell surface glycoproteins could regulate cell-to-cell adhesion as well as allow for the survival of the cells through differentiation (Akama et al., 2002).

The cytoplasm of pyramidal cells from the base to the apical region expressed binding sites to SNA, SBA, HPA, Con A, GSA I-B_4, and UEA I thus suggesting the presence of N-linked glycans containing terminal/internal Man (Con A reactivity), as well as O-

linked glycans terminating with GalNAc (SBA and HPA affinity). Both O- and N-linked oligosaccharides could terminate with Neu5Acα2,6Gal/GalNAc, αGal, and αL-Fuc (SNA, GSA I-B_4, and UEA I staining). This cytoplasmic lectin labelling could be related to structural glycoproteins. The apical cytoplasm extension of pyramidal cells showed the presence of glycans terminating with Neu5acα2,3Galß1,4GlcNac and Forssman pentasaccharide as evidenced by MAL II and DBA, respectively. The irregular luminal surface, probably made of short microvilli, reacted with KOH-sialidase-WGA, UEA I, and LTA thus indicating the presence of oligosaccharides with terminal/internal ßGlcNAc as well as terminating with αL-Fuc. The glycans highlighted in both luminal surface and apical extension of the pyramidal cells could be associated with the presence of absorptive and digestive apparatuses. Ultrastructural investigations in the oesophagus of other teleosts showed that the columnar cells contain numerous apical short microvilli and the cytoplasm contains RER cisternae, a distinct Golgi complex, vesicles and/or granules surrounded by a membrane (Elbal and Agulleiro, 1986; Gargiulo et al., 1996). The baso-lateral surface of pyramidal cells exhibited binding sites to MAL II, SNA, RCA_{120}, GSA I-B_4, and GSA II, thus suggesting the existence of oligosaccharides terminating with Neu5acα2,3Galß1,4GlcNac, Neu5Acα2,6Gal/GalNAc, Galß1,4GlcNAc, αGal, and D-GlcNAc. In mammals, the glycoproteins expressed at epithelium glycocalyx are generally believed to be involved in cell adhesion, lubrication of epithelial surfaces, transport of metabolites and ions across the plasmalemma (Jeanloz and Codington, 1976; Jentoff, 1990; Devine and McKenzie, 1992; Spicer and Schulte, 1992). Therefore, these cells could play a role in the desalination of ingested seawater and, accordingly, confer an osmoregulatory role to the oesophagus, as is thought for the columnar cells of other teleosts oesophagus (Elbal and Agulleiro, 1986; Loretz, 1995; Domeneghini et al., 1998; Radaelli et al., 2000).

The flattened cells showed binding for SNA, DBA (occasionally), SBA, HPA, Con A, GSA I-B_4, GSA II in the entire cytoplasm. KOH-sialidase procedure revealed cryptic DBA binding sites in the cytoplasm of many flattened cells. A granular reactivity with PNA and KOH-sialidase-WGA occurred in the sub-nuclear zone. These findings suggest the presence of: a) both O- and N-linked glycans terminating with Neu5Acα2,6Gal/GalNAc, Forssman pentasaccharide, NeuAc-Forssman pentasaccharide, GalNAc, αGal, and D-GlcNAc in the entire cytoplasm, b) O-linked glycans terminating with Galß1,3GalNAc and containing internal ßGlcNAc in the sub-nuclear cytoplasm. These results are consistent with conventional histochemistry which revealed neutral and acidic (sulphated) glycans in small granules of flattened cells. The presence of a granular binding pattern in the sub-nuclear zone could be related to the presence of the apparatus involved in the synthesis of glycoprotein destined to secretion. If this is the case, the findings of this study indicate that the flattened cells concur, together with goblet cells and sacciform cells, in the molecular composition of *Halobatrachus didactylus* oesophageal epithelium secretions and, consequently, play a role in the mucoadhesive characteristic of the oesophagus. Finally, large intra-specific differences in glycoconjugate composition of oesophageal mucous cells across fish (*Solea senegalensis*, *Sparus aurata*, *Hipoglossus hypoglossus*, *Acipenser baeri, Acipenser transmontanu, Anguilla anguilla*) including *Halobatrachus didactylus*, are known to exist (Arellano and Sarasquete, 2005; Ottesen and Olafsen, 2000; Arellano et al., 1999; Domeneghini et al., 1998; 1999, 2005, Sarasquete et al., 2001) thus suggesting possible differences in their physiological functions and/or in their maturation stages (neutral, carboxylated, sulphated).

In conclusion, the present histochemical study provides an insight into the oligosaccharide sequences of the secretory and non-secretory cells that line the mucosal epithelium of toadfish *Halobatrachus didactylus*. In addition, the results provide information about the changes of glycoconjugate pattern taking place in the epithelial cells during their different maturation stages.

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