

Gut glycoconjugates in *Sparus aurata* L. (Pisces, Teleostei). A comparative histochemical study in larval and adult ages

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Summary. This study examined the gut of the euryaline fish *Sparus aurata*, from the pharynx to the rectum. The specimens were collected from adult animals, both sexes, and several larval and juvenile stages, from 4 to 135 days of age. Histochemical methods to distinguish neutral and acidic glycoconjugates, as well as specific techniques to identify acidic glycoconjugates which contained O-acylated sialic acids were used. The presence and distribution of sugar residues in the oligosaccharide side chain of glycoconjugates were investigated with the use of biotinylated lectins. The pharynx and oesophagus of adult fishes showed the presence of abundant secretory cells which synthesized a large quantity of neutral, as well as sulphated and sialylated glycoconjugates, with different cellular combinations of them in the proximal and distal tract. This may be related to the complex functions carried out by this end of the gut in a marine euryaline fish. Epithelial secretory cells were found in the developing oesophagus during larval life (14 days) earlier than in the stomach and intestine (34 days). The simple columnar epithelium that lined the gastric mucosa of adult fish synthesized a mixture of neutral and acidic glycoconjugates, whereas during larval life it was shown to contain neutral glycoconjugates only. The intestinal goblet cells were shown to secrete both neutral and acidic glycoconjugates, especially sulphated forms. The adherent mucus gel of the gastric and intestinal mucosa contained many sugar residues, as revealed by lectin histochemistry. This work clearly demonstrates that the quality of gut mucosubstances varies in different ages and in regions of the fish alimentary canal. This is possibly caused by changes in environmental conditions and may in turn sustain functional alterations of the digestive apparatus.

Key words: Glycoconjugates histochemistry, Gut, Marine teleosts, Teleost larvae, Lectin histochemistry

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Introduction

The digestive apparatus of fishes shows a marked diversity in its morphology and function. This is related both to taxonomy and to different feeding habits, as well as to the body shape. The gross anatomy and histology of the alimentary tracts of fishes have been well documented for many species (see Kapoor et al., 1975; Smith, 1989, for reviews). These authors also reported the functional correlates of anatomical aspects, when known. Limited information exists about the morphological and functional aspects of development of the digestive apparatus in the larvae of fishes (Blaxter et al., 1983; Connes and Benhalima, 1984; Cousin and Baudin-Laurencin, 1985; Segner et al., 1989; Kjörsvik et al., 1991; Boulhic and Gabaudan, 1992; Bisbal and Bengston, 1995; Kuz'mina, 1996; Sarasquete et al., 1996). It is generally assumed that the alimentary tracts of the larvae are morphologically more simple than in the adults, and that their function is less elaborate (Dabrowski, 1984; Lauff and Hofer, 1984; Govoni et al., 1986; Loewe and Eckmann, 1988; Cousin et al., 1987; Segner et al., 1994).

When reared for intensive aquaculture, some species undergo high larval mortality, whose causes are not yet fully understood. An extremely critical period is evident when the post-hatching larvae start to ingest exogenous food, and also in periods between 20 and 40 days of larval age the percentages of mortality may be severe. Some authors have related this phenomenon to a possible incomplete secretory activity of the gut (Lumare and Villani, 1970; Alessio and Gandolfi, 1975; Barnabé and Billard, 1984).

The glycoconjugates constitute the major component of the gut mucosubstances in vertebrates. The gut glycoconjugates are extensively studied in mammals, where they are known to exert a large variety of functions, from those merely mechanical, through anti-microbial and anti-viral, to "osmotic" ones, in that they may link and transport different ions (Allen, 1981). It has recently been established that in fishes the gut mucosubstances may also exert an osmotic function

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(Smith, 1989). This is especially true for marine fishes. They drink seawater in order to replace the water lost due to the hyperosmotic external environment, and as a result secrete cations, above all excess calcium, in the gut lumen (Smith, 1989; Loretz, 1995). Subsequently, cations are expelled with the feces. Alterations in the osmotic balance, related to vertical migrations of post-hatching sea-bream larvae (Alessio, 1975), are reputed to be the possible cause of early larval mortality in other euryaline species (Kuo et al., 1973; Barnabé, 1976).

The purpose of this study was to describe histochemically the gut glycoconjugates of the sea-bream (*Sparus aurata* L.), a species of high interest in aquaculture, from post-hatching larval to adult ages, in order to verify if the gut mucosubstances are affected by larval development and age. We have also considered that this euryaline fish displays different life styles and feeding habitats at different stages of its life history (Alessio, 1975). Previous studies on the sea-bream have been prevalently histological, and restricted to a limited range of ages (Elbal and Agulleiro, 1986; Cataldi et al., 1987).

This work has been carried out using classical histochemical methods for glycoconjugates, as well as techniques specific for glycoconjugates that contain sialic acids, whose distribution in mammalian alimentary canal varies in species and physiological as well as pathological conditions (Filipe, 1969; Milton et al., 1993). In addition, biotinylated lectins were used to identify specific sugar residues of glycoconjugates (Spicer and Schulte, 1992). We have chosen those lectins which bind to the terminal sugar residues which occur in mammalian gut glycoconjugates (Coggi et al., 1983; Fischer et al., 1984; Suganuma et al., 1984; Kantani-Matsumoto and Kataoka, 1989; Ohara et al., 1993). It is now well known, at least in mammals, that regional variations in lectin binding of intestinal mucosubstances may be related to intestinal diseases (Fischer et al., 1984; Jacobs and Huber, 1985; Carraro et al., 1993).

Materials and methods

Larval, juvenile and adult samples were supplied by the C.I.V.V. (Centro Ittiologico Valli Venete) fish farm in Pellestrina (Venice, Italy).

Larvae and juveniles (ten individuals for each age) were sampled on days: 4, 9, 14, 21, 34, 45, 55, 70, 100 and 135 after hatching. Larvae were fed as follows: from day 4 to 25: protozoa (Rotifera); from day 25 to 45: larvae of *Artemia salina*; from day 45 to 70: larvae of *Artemia salina* plus standard high protein granules; and from day 70: standard high protein granules. Eight male and female adult fishes were used. The males were 20-22 cm long (200-400 g body weight) and the females were 38-42 cm long (1,700-2,400 g body weight). Both larvae and adults were anaesthetized with tricaine methanesulfonate (MS 222, Sandoz, Italy). The adults were killed by decapitation and, after opening the ventral surface, the entire digestive tract was quickly removed. Several small portions of each of the different parts of

the digestive tract (pharynx-oesophagus; cardiac, fundic and pyloric regions of the stomach; pyloric caeca; upper and middle intestine; "ileo-rectal" valve; rectum) were excised. Figure 1 shows the sites of sampling.

The specimens were fixed at 4 °C for 24 h in: a) 10% neutral buffered formalin, b) 10% formol-calcium, c) Bouin's fluid; d) B4G (6% mercuric chloride and 0.1% glutaraldehyde in 1% sodium acetate), according to Kantani-Matsumoto and Kataoka (1989); and e) 2% calcium acetate in 10% formalin. Anaesthetized larvae were decapitated and fixed as above. After fixation, isolated tissues and larvae were dehydrated through a graded series of ethanol and embedded in paraffin wax. Serial sections were cut at 4-6 µm. Larvae were cut in serial sections along longitudinal and transverse planes.

Sections of tissues and larvae fixed in a) and b) were stained with haematoxylin-eosin and Azan trichromic stain for morphology, PAS (periodic acid Schiff's reagent) after Mc Manus (1948) to demonstrate periodate reactive vicinal diols, the AB (alcian blue) pH 2.5/PAS sequence (Mowry, 1963) to distinguish both acidic glycoconjugates and vicinal hydroxyl groups, and their cellular combinations, the PAS/AB pH 2.5 sequence (Yamabayashi, 1987) to reveal some neutral glycoconjugates, and the HID (high iron diamine)/AB pH 2.5 sequence (Spicer, 1965; Reid et al., 1989) to differentiate sulphated (stained brownish-black) from carboxylated, non-sulphated glycoconjugates (stained azure). In order to confirm the presence of sialic acids in the carboxylated, non-sulphated glycoconjugates by the loss or reduction of alcianophilia, the removal of sialic acids was performed by the KOH treatment (saponification) followed by an acid hydrolysis (Culling et al., 1974).

Sections of tissues and larvae fixed in b) were used for the histochemical identification of glycoconjugates which contain O-acylated sialic acids at different carbon positions (Culling et al., 1976; Culling and Reid, 1980). On these sections the following sequences were performed: (i) KOH/PAS: the saponification with 0.5% potassium hydroxide in 70% ethanol for 30 min at room temperature was performed to deacetylate sialic acid

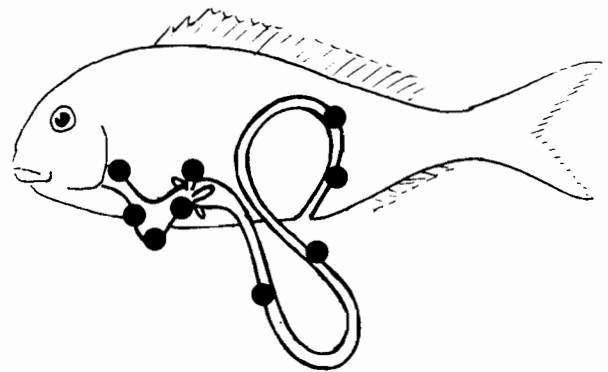


Fig. 1. Sampling sites (black circle) along the digestive tract of *Sparus aurata*, corresponding to list in text.

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residues and was followed by PAS; (ii) PBT (periodate-borohydride-technique)/KOH/PAS: the PAS reactivity of pre-existing tissue vicinal diols was abolished by periodate oxidation followed by sodium borohydride reduction. The KOH/PAS effect was subsequently demonstrated as red staining by saponification followed by the standard PAS technique; (iii) PATS (periodic acid-thionin-Schiff)/KOH/PAS to demonstrate PAS-reactive glycoconjugates in blue, whereas other KOH/PAS-reactive glycoconjugates were shown red, with combinations being purple; and (iv) PBT/PAS to reveal a further type of glycoconjugates in red. These reactions, taken together and with the PAS unreactivity, allowed the identification of one type only of glycoconjugates, those sialylated at carbon in position 8 (C8).

Sections of tissues and larvae fixed in c) were used for morphology and for a control of the presence of neutral glycoconjugates, applying the PAS reaction after malt diastase digestion (Pearse, 1985) for 1 h at 37 °C in a pH 6.0 phosphate buffer. The diastase digestion did not affect the PAS reactivity.

Sections of tissues and larvae fixed in d) and e) were used for lectin histochemistry. Biotinylated lectins were purchased from Vector Laboratories (Labtek, Italy). Table 1 shows the specific names, the common names, the acronyms and the preferred binding specificity of the sugar residues, as well as sugars used in negative controls (blocking sugars).

The sections were incubated in 0.3% H₂O₂ in methanol for 30 min at room temperature to block endogenous peroxidase activity. They were then washed in a pH 7.6 phosphate-buffered saline (PBS) and incubated with biotinylated lectins, 10 µg/ml in HEPES buffer 10 mM, pH 7.5, for 2 h at room temperature. The sections were then washed again in PBS and subsequently treated with an avidin-biotin-peroxidase complex (ABC kit, Vector) for 1 h at room temperature. After a further washing in PBS, the sites of reaction were revealed with diaminobenzidine tetrahydrochloride (DAB), 0.5 mg/ml in Tris buffer 0.1M, pH 7.2, plus 0.02% H₂O₂ for 5-6 min at room temperature.

Controls for lectin binding were made by: (i) omission of the respective lectin; (ii) omission of the ABC treatment; and (iii) incubation with the biotinylated lectins in the presence of respective hapten sugars, 0.2M (see Table 1). No staining was observed.

Considering that WGA is also reputed to bind sialic acid (Danguy et al., 1994), a further control for this lectin was made, performing a prior acid hydrolysis as above mentioned: this did not affect the WGA-labelling.

Results

The epithelium that lined the developing gut showed a weak, diffuse PAS-reactivity in the earliest larval ages examined (4, 9 days). At 14 days of larval age the different organs of the alimentary canal were easily recognisable on morphological and histochemical bases.

Pharynx/oesophagus

Larval and juvenile ages

At 14 days of age a few cells in the epithelium that lined the developing pharynx/oesophagus were shown to be weakly PAS-positive and alcianophilic (Fig. 2a). They were stained brownish-black with the HID/AB pH 2.5 sequence. The later larval ages examined (21, 34 days) did not show any difference in the histochemical pattern of epithelial secretory cells, but they increased in number and size (Fig. 2b). At 45 days of larval age a clear-cut histochemical difference between a proximal, pharynx/oesophageal zone and a distal oesophageal zone was evident (Fig. 2c). The distal oesophageal zone showed the prevalent or unique presence of abundant secretory cells that were intensely red-stained with the AB pH 2.5/PAS sequence (Figs. 2c, 3a). On the contrary, the proximal pharynx/oesophageal zone contained different secretory cell types which were shown at different larval ages. At 45 days of larval age numerous secretory cells were predominantly alcianophilic and weakly PAS-positive (Figs. 2c, 3b), and were stained brownish-black with the HID/AB pH 2.5 sequence (Fig. 3c). Other secretory cells were alcianophilic with both the AB pH 2.5/PAS and HID/AB pH 2.5 sequences and were stained red by the PATS/KOH/PAS (Fig. 3d) and PBT/KOH/PAS sequences, whereas they were PBT/PAS sequence-unreactive. They also showed the KOH/PAS effect. At 55 days of age a new secretory cell type appeared which was intensely red-stained with the AB pH 2.5/PAS sequence and showed a markedly granular cytoplasm. At 70 days of age a further type of secretory cell was recognizable. It was constituted by tall, columnar cells, whose cytoplasm was weakly PAS-reactive and alcianophilic, as well as reactive with the HID/AB pH 2.5 sequence. They were also stained red by

Table 1. Biotinylated lectins employed in this study, their preferred binding specificities and blocking sugars.

LECTIN: <i>Latin name</i> (common name)	ACRONYM	PREFERRED SUGAR RESIDUES BINDING AND BLOCKING SUGARS
<i>Dolichos biflorus</i> (horse gram)	DBA	α-GalNAc
<i>Glycine max</i> (Soybean)	SBA	α,β-GalNAc > Gal
<i>Canavalia ensiformis</i> (Jack bean)	Con-A	α-D-Man
<i>Ricinus communis</i> (caster bean)	RCA-I	β-D-Gal
<i>Ulex europaeus</i> (gorse seed)	UEA-I	L-Fuc
<i>Triticum vulgare</i> (wheat germ)	WGA	GlcNAc

Fuc: fucose; Gal: galactose; GalNAc: N-acetyl-D-galactosamine; GlcNAc: N-acetyl-D-glucosamine; Man: mannose.

the PATS/KOH/PAS and PBT/KOH/PAS sequences, and showed the KOH/PAS effect. The further juvenile ages examined (100, 135 days) did not show any further significant differences in the histochemical pattern of the different secretory cell types, but the general appearance of the epithelium that lined the pharynx-oesophagus became gradually more and more complex (see below, adult ages). With regard to the lectin histochemistry, we found a positivity to DBA and WGA lectins only after 45 days of larval age. The cytoplasmic membranes and the content of many secretory cells appeared stained with DBA (Fig. 2d) and WGA lectins. UEA-I lectin was present in similar sites, but the reactivity was less evident. The pattern of the lectin binding did not change until 100 days of juvenile age, when it became similar to that of the adult (see below).

Adult ages

The epithelium that lined the pharynx and the oesophagus was polymorphic, being simple columnar in some sites and stratified in others (Fig. 2h). It contained a large number of secretory cells which increased in numbers in the distal oesophagus. Three types of epithelial secretory cells were recognizable on the basis of their morphological (Fig. 2h) and histochemical characters. Small secretory cells were present, always superficially located in the epithelium. Their cytoplasm was granular and contained a heavily PAS-reactive material (Fig. 3e-g). Other more numerous secretory cells were large and their cytoplasm was foamy in aspect. They were weakly PAS-positive and alcianophilic (Fig. 3e,f) and stained violet with the PAS/AB pH 2.5 sequence (Fig. 3g). The alcianophilia of the numerous, large, foamy secretory cells was highly variable in intensity in different sites (Fig. 3h), as was their reactivity to the HID/AB pH 2.5 sequence (Fig. 3i). In addition, these secretory cells were stained red by the PATS/KOH/PAS and PBT/KOH/PAS (Fig. 3l) sequences and showed the KOH/PAS effect, whereas they were unstained by the PBT/PAS sequence. A third type of secretory cells was identified, limited to the distal end of the oesophagus. This cell type showed the same histochemical pattern as the numerous, large, foamy cells previously described, but its morphology was different. It was constituted by tall, columnar secretory cells with a homogeneous cytoplasm (Fig. 2h). These

secretory cells had an uneven distribution along the distal oesophageal epithelium and when grouped they formed a simple columnar epithelium. Lectin histochemistry stained only the numerous, large foamy secretory cells. Their content, and sometimes the adherent mucus gel also, was shown to bind to Con-A, RCA-I, UEA-I and WGA (Fig. 2e) lectins. DBA lectin binding was found in the cytoplasmic membranes of these secretory cells (Fig. 2f).

Stomach

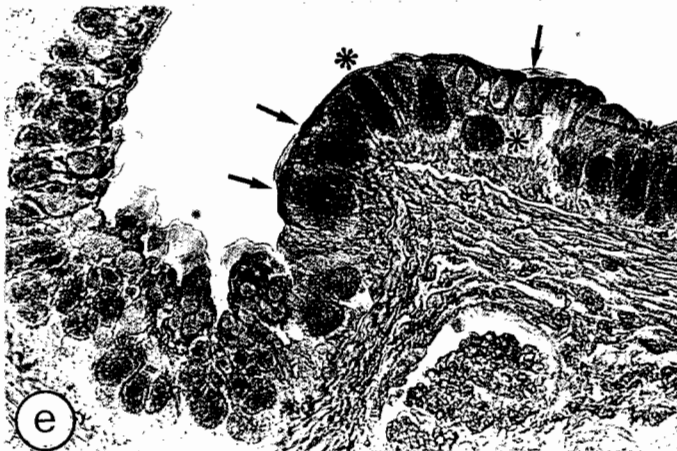
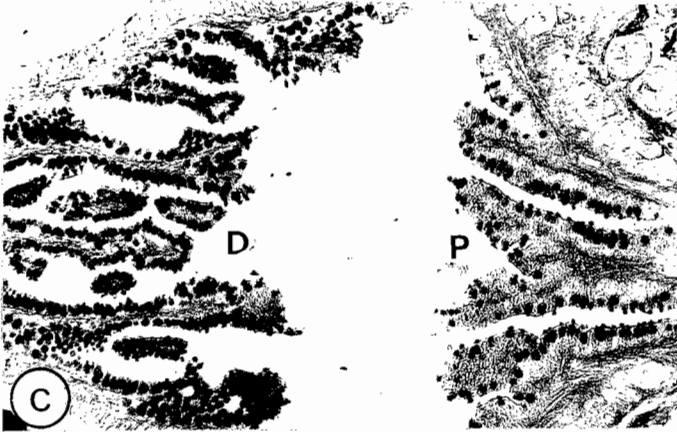
Larval and juvenile ages

The simple columnar epithelium that lined the gastric mucosa was shown to be PAS-reactive from 34 days of larval age. The gastric glands, limited to the actual cardiac and gastric gland regions, were present at 45 days of age, in the form of few simple or branched glandular tubules. The glandular cells were histochemically unreactive, as through the entire larval and juvenile periods (Fig. 3m). At 45 days of larval age the adherent mucus gel was also present and PAS-reactive. At 100 days of age the gastric glands, now as numerous simple or branched tubules, were shown to fill the tunica propria of the mucosa (Fig. 3m). At 135 days of age the epithelium that lined the gastric mucosa and the adherent mucus gel were in some sites stained magenta with the AB pH 2.5/PAS sequence. The purely alcianophilic areas of the secretory epithelium were also stained azure by the HID/AB pH 2.5 sequence. From 45 days of age, binding to DBA and WGA lectins was seen in the glandular cells of the gastric glands and, in some sites, in the adherent mucus gel, too. The binding of these lectins to the glandular cells disappeared at 100 days of age (juvenile). At 135 days the adherent mucus gel was stained by DBA, SBA, Con-A, RCA-I, UEA-I and WGA lectins.

Adult ages

The simple columnar epithelium that lined the gastric mucosa was magenta-stained by the AB pH 2.5/PAS sequence (Fig. 4a). With the HID/AB pH 2.5 sequence it was stained predominantly azure. The epithelial secretory cells also showed a strong KOH/PAS effect (Fig. 4b) and were stained red by the PATS/

Fig. 2. Gut glycoconjugate histochemistry in *S. aurata* (larval and adult ages). **a.** 14 days of larval age, AB pH 2.5/PAS sequence. A few secretory cells which are alcianophilic and PAS-positive are shown in the epithelium of the developing pharynx-oesophagus (arrows). The epithelium that lines the developing intestine (I) does not contain secretory cells. **b.** 34 days of larval age, AB pH 2.5/PAS sequence. Reactive secretory cells of the developing pharynx-oesophagus (OE) are evident (double arrows). In the developing intestine (I) goblet cells (arrows) are now identifiable. **c.** 45 days of larval age, AB pH 2.5/PAS sequence. The epithelial secretory cells of the distal oesophageal zone (D) uniquely produce neutral glycoconjugates, those of the proximal oesophageal zone (P) contain in addition quantities of acidic glycoconjugates. **d.** 45 days of larval age. The cytoplasm of some secretory cells (asterisks) in the epithelium that lines the oesophagus is stained by DBA lectin. **e.** adult fish, oesophagus, WGA lectin. The content of secretory cells is stained (asterisks). In some sites the adherent mucus gel (arrows) is also stained. **f.** adult fish, oesophagus, DBA lectin. The plasmalemmae (arrows) of epithelial secretory cells are stained. **g.** adult fish, pyloric coeca, AB pH 2.5/PAS sequence. Numerous roundish goblet cells are detectable, heavily stained. In some sites (arrowheads), the adherent mucus gel is also reactive. **h.** adult fish, oesophagus, Azan. The epithelium appears polymorphic. On the left side of the picture it is stratified and shows the presence of small granular (asterisks) and large foamy secretory cells (arrowheads). On the right side of the picture it is simple columnar and constituted by tall secretory cells, whose cytoplasm is homogeneous. a-c, x 140; d, x 150; e-h, x 230



KOH/PAS (Fig. 4c) and PBT/KOH/PAS (Figs. 3l, 4d) sequences. They were unreactive with the PBT/PAS sequence. The cardiac gland region usually showed deep and irregular foveolae that were lined by tall secretory cells whose histochemical reactivity was especially intense (Fig. 4a-d). The gastric glands of both the glandular zones were unreactive to the different histochemical methods, as in larval and juvenile ages (Fig. 4a-d). Binding of all tested lectins was identified in the adherent mucus gel.

Intestine

Larval and juvenile ages

The goblet cells were first identified at 34 days of larval age (Fig. 2b). They were localized in the simple columnar epithelium that lined the intestinal mucosa, both in intestinal folds and glands. They were initially few (Fig. 2b), but their number increased with age (Figs. 4e,f). The goblet cells were uniformly magenta-stained with the AB pH 2.5/PAS sequence (Fig. 4e), and resulted in a brownish-black colour with the HID/AB pH 2.5 sequence (Fig. 4f). In addition, they were red-stained by the KOH/PAS, PATS/KOH/PAS and PBT/KOH/PAS sequences. They were unreactive with the PBT/PAS sequence. Among all the lectins tested, only UEA-I binding was detectable in the adherent mucus gel of the intestinal epithelium at 34 days of age. At 70 days of age the adherent mucus gel was also shown to bind DBA, SBA, Con-A, RCA-I and WGA lectins. At the same age, the content of the goblet cells was found to be stained by DBA and WGA lectins.

Adult ages

The goblet cells were abundant along the whole intestinal tract, from pyloric caeca to the rectum (Figs. 2g, 4g,h), with increasing numbers of them towards the rectum. They showed a histochemical pattern similar to that previously shown in the larvae, in that they were magenta-stained with the AB pH 2.5/PAS sequence (Figs. 2g, 4g). In addition, they were red-stained by the

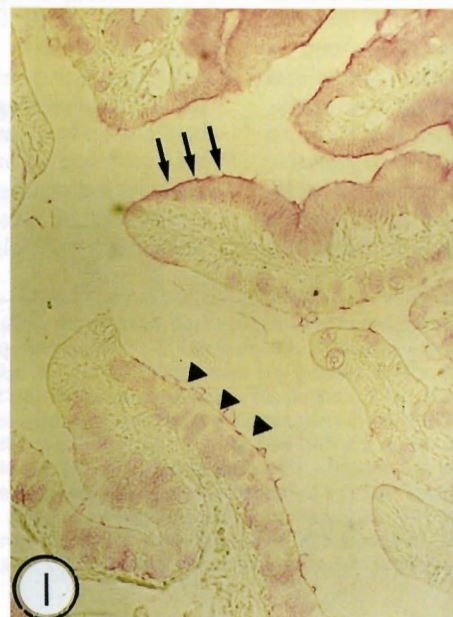
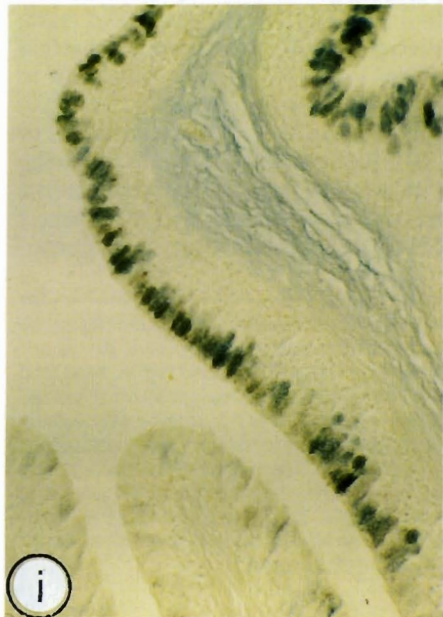
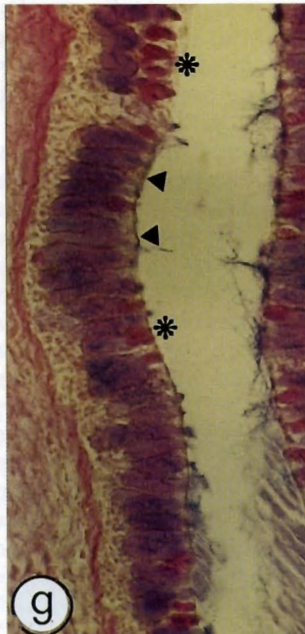
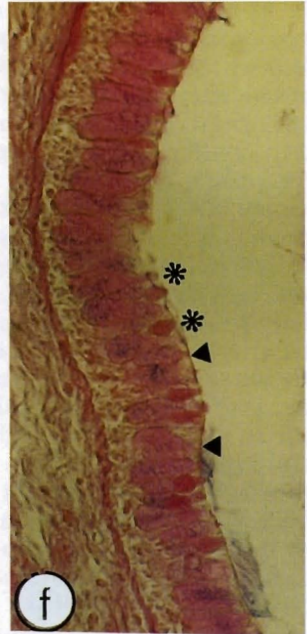
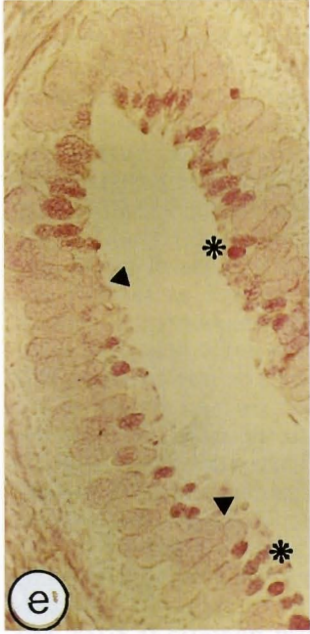
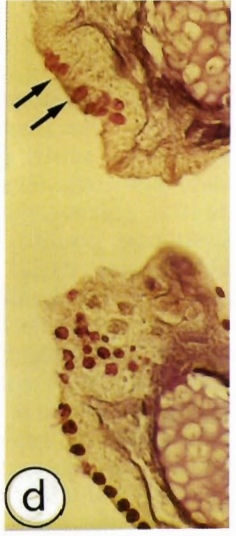
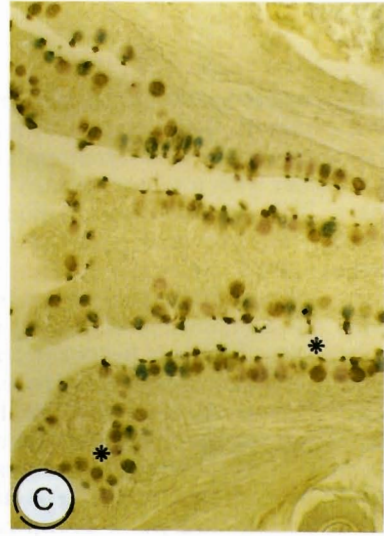
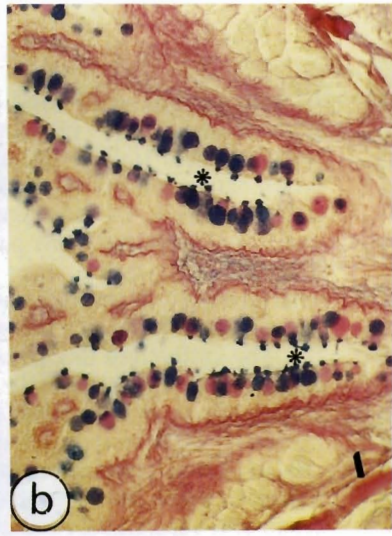
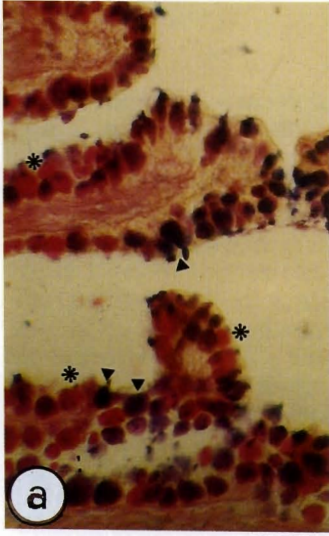
KOH/PAS, PATS/KOH/PAS (Fig. 4h) and PBT/KOH/PAS sequences. They were unreactive with the PBT/PAS sequence. The ileo-rectal valve marked an abrupt increase in the brownish-black reaction colour of the goblet cells of the rectum with the HID/AB pH 2.5 sequence. The adherent mucus gel showed a histochemical reactivity that was similar to that of the goblet cells (Figs. 2g, 4g,h). In addition, it appeared to be stained by all the tested lectins (Fig. 4i,l), including UEA-I. The content of the goblet cells was shown to bind intensely with DBA (Fig. 4i) and weakly with WGA (Fig. 4l) lectins.

Discussion

The secretory activity of the various organs of the alimentary tract in *S. aurata* is identifiable at different times of the larval development, and the pharynx-oesophageal region contains epithelial secretory cells earlier than the stomach and intestine. The pharynx-oesophageal region of adult fish shows a high degree of morphological and histochemical complexity, although it is short. The gut mucosubstances may be differently composed if examined at different ages. The differences in the chemical nature of the gut mucosubstances possibly indicate differences in the luminal chemistry, as well as in the chemical labour of the different organs.

At 4 days of larval age the epithelium that lines the developing gut of *S. aurata* secretes a moderate quantity of neutral glycoconjugates. At this age, even though a small yolk sack is still present, the mouth of the larva is open and its gut shows peristaltic movements (Alessio and Gandolfi, 1975), and the larvae will eat protozoa. The developing gut does not show any morphological differentiation in this and the further larval age examined (9 days), but a secretory function is already present, at least in the form of neutral glycoconjugate secretion. Neutral gut mucosubstances are reputed to cooperate in the enzymatic digestion of food and in transforming it into chyme, as well as in absorptive functions (Clarke and Witcomb, 1980; Anderson, 1986; Grau et al., 1992). At 14 days of age, when the yolk sack is absent, and the developing gut is longer and longer causing a prominent

Fig. 3. Glycoconjugate histochemistry in the pharynx-oesophagus and stomach of *S. aurata* (larval, juveniles and adult ages). **a.** 45 days of larval age, AB pH 2.5/PAS sequence. Abundant secretory cells in the distal oesophageal zone produce predominantly (arrowheads) or uniquely (asterisks) neutral glycoconjugates. **b.** 45 days of larval age, AB pH 2.5/PAS sequence. The secretory cells of the proximal pharynx-oesophageal zone contain predominantly (asterisks) acidic glycoconjugates. **c.** 45 days of larval age, HID/AB pH 2.5 sequence. The sulphated component of the acidic glycoconjugates, stained brownish (asterisks), is substantial. **d.** 45 days of larval age, PATS/KOH/PAS sequence. The secretory cells are stained red (arrows). **e.** adult fish, oesophagus, PAS. There are both small superficial secretory cells, whose cytoplasm is granular and intensely reactive (asterisks), and other more numerous large secretory cells that show a pale, foamy content (arrowheads). **f.** adult fish, oesophagus, AB pH 2.5/PAS sequence. The small secretory cells are uniquely PAS-reactive (asterisks), whereas the large foamy secretory cells are weakly PAS-reactive and alcianophilic (arrowheads). **g.** adult fish, oesophagus, PAS/AB pH 2.5 sequence. The small secretory cells maintain their unique PAS-reactivity (asterisks), whereas the large foamy secretory cells appear predominantly alcianophilic (arrowheads). **h.** adult fish, oesophagus, AB pH 2.5/PAS sequence. The numerous, large foamy secretory cells appear stained predominantly brownish/black. **i.** adult fish, distal oesophagus, PBT/KOH/PAS sequence. The numerous, large, foamy secretory cells appear stained (arrowheads). In the upper part of the picture the epithelium also that lines the gastric mucosa (cardiac gland zone) appears stained (arrows). **m.** 100 days of juvenile age, AB pH 2.5/PAS sequence. The epithelium that lines the gastric mucosa appears PAS-positive (arrows). The foveolae of the cardiac gland zone show sites of weak alcianophilia (asterisks). Histochemically unreactive gastric gland (gl) are present in the lamina propria. a-d, h, i, x 140; e-g, i, x 230; m, x 90

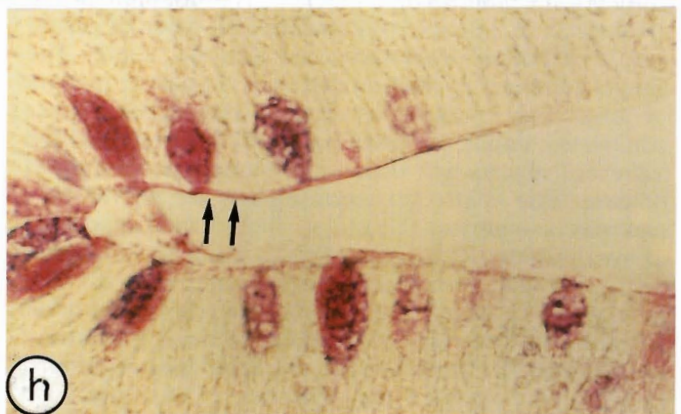
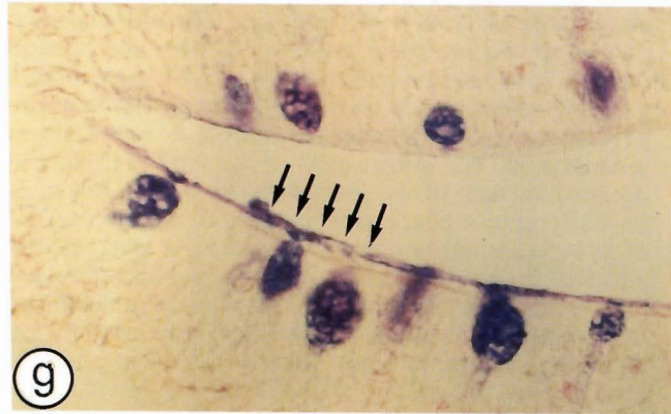
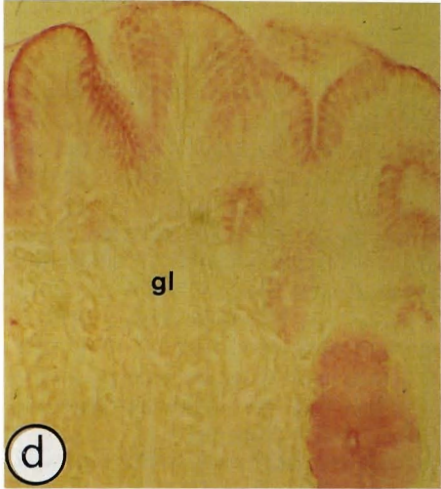
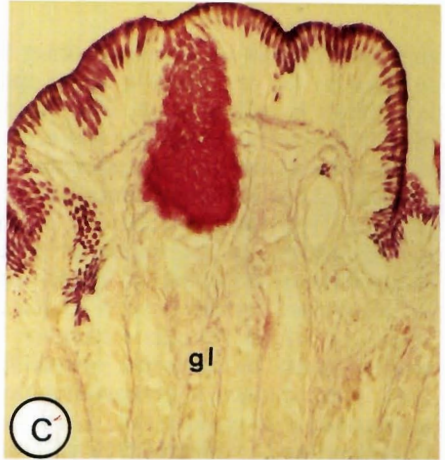
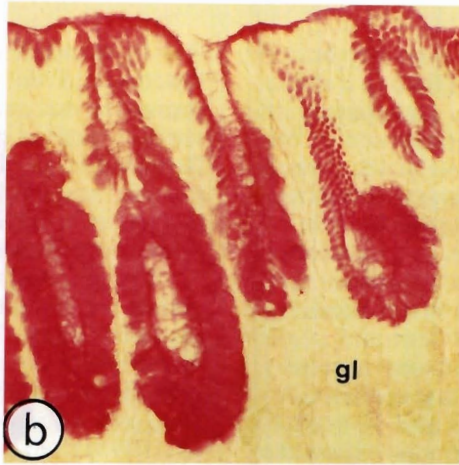
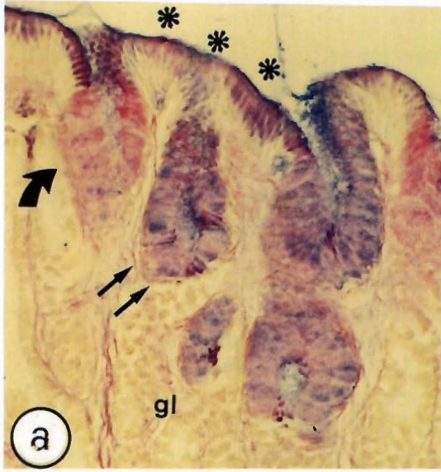


abdomen, secretory cells appear in the epithelium that lines the developing pharynx-oesophagus. They synthesize acidic glycoconjugates with an abundant sulphated component, together with a minor quantity of neutral glycoconjugates. This combination of mucosubstances possibly enables the proximal region of the alimentary canal to respond to changes in the environmental conditions early on. These secretory cells, initially scarce, become more numerous with age, as well as increasing in their size, but their histochemical reactivity does not change when larvae start to eat crustacean larvae at about 25 days. At 45 days of age the metamorphosis begins, in that the swim bladder is now present and the tunica propria of the stomach contains glandular tubules. In this period protein granules are added to the diet. In the developing gut a proximal, pharynx-oesophageal zone shows large secretory cells, whose histochemical patterns are different to those of a distal oesophageal zone. The secretory cells of the distal oesophageal zone prevalently secrete large amounts of neutral glycoconjugates. The secretory cells of the proximal pharynx-oesophageal zone produce, in addition to a minor component of neutral glycoconjugates, a great quantity of acidic sulphated glycoconjugates. A further component of the acidic glycoconjugates contains sialic acids substituted at C8. Using the binding of biotinylated lectins to identify terminal sugar residues in glycoconjugates, we found that the plasmalemmae and, sometimes, the cytoplasm of the secretory cells contained N-acetyl-D-galactosamine and N-acetylglucosamine. L-fucose was also present, but in smaller amounts. The lectin staining, evident from 45 days of larval age, did not change until 100 days, when the staining pattern seen in the adult was reached. As revealed by the binding to UEA-I lectin, L-fucose is believed to occupy its α 1,2 linkage with neighbour sugars (Allen et al., 1977; Spicer and Schulte, 1992). The presence of N-acetyl-galactosamine is revealed by the staining with DBA but not with SBA lectins. This is likely to be related to the position of the N-acetyl-galactosamine in the oligosaccharide branch, as well as to the linkages of this sugar to its neighbours (Spicer and Schulte, 1992). At 55 days of age, when the metamorphosis is finished and fish reach the fry stage, a second secretory cell type appears, showing a granular cytoplasm which contains neutral glycoconjugates only. At 70 days of age, a further secretory cell type is detectable. It is columnar and synthesizes a combination

of both neutral and acidic glycoconjugates. Sulphated glycoconjugates are present with sialoglycoconjugates, which contain sialic acids substituted at C8. At this age, when the juveniles are fed exclusively with standard pelleted food, the pharynx-oesophagus reaches the definitive histochemical pattern of its secretory cells. The later juvenile ages examined showed only morphological modifications in the histological organization of the pharynx-oesophagus.

The epithelium that lines the pharynx-oesophagus of adult fish is almost entirely stratified except for limited areas where it is simple columnar. Three types of secretory cells are recognizable in this epithelium, as one can expect if referring to the larval ages. The granular secretory cells, detectable from 55 days of larval age, are small and superficially located in the epithelium in adult fish, in a generally smaller number than the other secretory cell types. Based on their markedly granular cytoplasm, they might be involved in the synthesis of digestive enzymes. In some fish, digestion is described to start in this proximal part of the gastrointestinal tract and to continue, as usual, in the stomach (Linss and Geyer, 1968; Cataldi et al., 1987). The predominant secretory cell type is constituted by large, roundish cells containing a foamy cytoplasm, which correspond to the type first histochemically seen at 14 days of larval age. These cells synthesize neutral, as well as sulpho- and sialoglycoconjugates. The sialoglycoconjugates contain sialic acids substituted at C8, as in larval ages. The same histochemical pattern is shown by a further secretory cell type, that is distinguishable on the basis of its localization, generally limited to the distal zones of the oesophagus, and morphology. They were observed at 70 days of larval age and in adult fish they are tall columnar secretory cells, which make up a simple epithelium. Columnar cells have also been identified in the oesophagus of *Salmo gairdneri* (Weinreb and Bilstad, 1955), *Esox lucius* (Bucke, 1971), *Anguilla japonica* (Yamamoto, 1978) and *Anguilla anguilla* (Clarke and Witcomb, 1980). Elbal and Agulleiro (1986) and Cataldi et al. (1987) have ultrastructurally described a columnar cell type that constitutes zones of simple epithelium in the distal oesophagus of the *Sparus aurata*. The presence in these cells of numerous short apical microvilli and of a large population of mitochondria, together with the observation of large intercellular spaces, may support the hypothesis of a functional role of them in ion transport across the epithelium. The osmoregulatory function of

Fig. 4. Glycoconjugate histochemistry of the stomach and intestine of *S. aurata* (larval and adult ages). **a.** adult fish, stomach, cardiac gland region, AB pH 2.5/PAS sequence. The simple columnar epithelium appears PAS-reactive and alcianophilic (asterisks). The epithelium that lines the gastric foveolae is either PAS-reactive alone (curved arrow) or PAS-reactive and alcianophilic (arrows). **b.** adult fish stomach, cardiac gland region, KOH/PAS sequence. The columnar cells show the KOH effect. **c.** adult fish stomach, cardiac gland region, PATS/KOH/PAS sequence. The epithelium is stained red. **d.** adult fish stomach, cardiac gland region, PBT/KOH/PAS sequence. The epithelium is stained pale red. In a-d note that the gastric glands (gl) are histochemically unreactive. **e.** 45 days of larval age, AB pH 2.5/PAS sequence. Numerous goblet cells are present in mucosal folds and glands of the developing intestine. Their content is homogeneously stained violet. **f.** 45 days of larval age, HID/AB pH 2.5 sequence. The sulphated component of the acidic glycoconjugates is abundant. **g.** adult fish, rectum, AB pH 2.5/PAS sequence. The goblet cells are stained violet. The adherent mucus gel is also reactive (arrows). **h.** adult fish, rectum, PATS/KOH/PAS. A component of the sialoglycoconjugates is stained red. The adherent mucus gel is also reactive (arrows). **i.** adult fish, upper intestine, DBA. The content of goblet cells and the adherent mucus gel are heavily stained. **l)** adult fish, upper intestine, WGA. The content of goblet cells and the adherent mucus gel are weakly stained. a-d, x 230; e, f, i, l, x 140; g, h, x 760



the oesophagus of the sea-bream is possibly better fulfilled by the organisation of the epithelium in a single layer than if multilayered. The various components of glycoconjugates which we have demonstrated in the epithelial secretory cells (especially their acidic forms) may be related to the desalinization of ingested seawater (Loretz, 1995) and to a general osmoregulatory role of the oesophagus of the sea-bream which is a euryhaline fish with a wide ecological range. This hypothesis may be confirmed by the results of lectin histochemistry, which in adults reveals a number of terminal sugar residues only in the large, roundish, foamy secretory cells. They synthesize glycoconjugates that contain α -D-mannose, β -D-galactose, L-fucose and N-acetylglucosamine. In addition, their cell membranes show the presence of N-acetyl-galactosamine, and it is now well known in mammals that the binding of lectins to plasmalemmal glycoconjugates is indicative of a role of these molecules in the regulation of movements of ions and fluids (Spicer and Schulte, 1992).

If pharynx-oesophageal tract contains epithelial secretory cells early on, the epithelium that lines the gastric mucosa was shown to be secretory at 34 days of age, and gastric glands appear again later, when the larvae are close to metamorphosis. The secretory cells that constitute the simple columnar epithelium synthesize neutral glycoconjugates until juvenile age, when they show the additional presence of acidic glycoconjugates, limited to some sites. As is commonly found in most teleosts (Smith, 1989), the gastric glands are present in cardiac and fundic regions, and the glandular cells are histochemically unreactive. On the contrary, in mammalian species glycoconjugates are extensively identifiable in glandular cell types of gastric mucosa (Kantani-Matsumoto and Kataoka, 1989). The simple columnar epithelium that lines the gastric mucosa of adult fish synthesizes neutral and acidic glycoconjugates, unlike the situation found in larvae, and the adherent mucus gel shows the same histochemical pattern. The acidic glycoconjugates are prevalently sialoglycoconjugates (C8). It is likely that components of gastric glycoconjugates perform transport processes associated with gastric acid secretion and with dilution of ingested seawater. The results here reported in adult fish are in some respects different to those previously obtained in the same species by Elbal and Agulleiro (1986), who showed the presence of sulphoglycoconjugates in the columnar secreting cells of the epithelium lining the gastric mucosa. This may be related to the different histochemical methods employed in this study, which allowed a more specific differentiation of the two components of the acidic glycoconjugates. Comparison of the glycoconjugate composition with that in other species of teleosts reveals a remarkable variety between species (Reifel and Travill, 1978; Sis et al., 1979; Stroband and Kroon, 1981; Ostos Garrido et al., 1993a). To our knowledge, this variety can be attributed not only to different feeding habits, but possibly to different taxonomic positions. We are aware

that in mammals the protective and autoprotective functions of the gastric mucosa are reflected in the secretion not only of glycoconjugates but also of surface-active phospholipids (Hills et al., 1983; Bernhard et al., 1994). In fish too the composition of gastric mucosal barrier possibly includes other molecules in addition to glycoconjugates. This will be examined in future works.

The glandular cells which compose the gastric glands are histochemically unreactive, as in larval and juvenile ages. They are named by most authors "oxyntopeptic cells" as they are presumed to secrete both enzymes and hydrochloric acid (Barrington, 1942; Ferri and Hernández-Blazquez, 1984; Ostos-Garrido et al., 1993a). As is believed usual for piscine species (Suganuma et al., 1981; Oinuma et al., 1991), we did not find mucous neck cells in the gastric glands of the adult sea-bream, either with the histochemical methods, or with the lectin histochemistry. On the contrary, N-acetyl-galactosamine and N-acetyl-glucosamine could be identified in the gastric glands for a limited larval period, and were not seen at 100 days of age, probably because they were then in a sub-terminal position in the oligosaccharide branch. Only when the juvenile stage was reached, the adherent mucus gel appears composed by glycoconjugates that contain N-acetyl-galactosamine, α -D-mannose, β -D-galactose, L-fucose and N-acetylglucosamine, in a pattern similar to that of adult fish. These sugars probably occupy a terminal position in the glycoconjugates only when they are secreted to form the adherent mucus gel of the gastric mucosa.

As for the glycoconjugate secretion in the stomach, the secretion in the goblet cells of intestinal mucosa is identifiable at 34 days of larval age. The histochemical pattern of the intestinal goblet cells does not change through larval and juvenile periods to adult ages. Goblet cells synthesize neutral glycoconjugates as well as acidic glycoconjugates whose sulphated component is predominant. The goblet cells of the intestinal mucosa do not bind any of the lectins tested, until 70 days of juvenile age, when N-acetyl-galactosamine and N-acetyl-glucosamine can be detected. At this age the adherent mucus gel reaches its definitive histochemical pattern, showing the binding of all the lectins employed in this study. The adherent mucus gel contains L-fucose at 34 days of age, in coincidence with the beginning of secretion in the goblet cells. These results of lectin binding are to some extent different to those obtained by Pajak and Danguy (1993) in the rainbow trout. This is possibly related to different feeding habits, which influence the intestinal enzyme activities. The lectin binding results only partially similar obtained by Madrid et al. (1989) upon the intestinal goblet cells of adult *S. aurata* are possibly to be referred to different husbandry conditions of fish, or to different fixation procedures of fish specimens.

The intestinal mucosa of the adult sea-bream shows a large number of goblet cells, especially in the rectum, as in other piscine species (Grau et al., 1992; Murray et

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al., 1996). The increasing numbers of goblet cells in the rectum may respond to an increased need of lubrication related to the the expulsion of feces. The goblet cells of the sea-bream synthesize neutral and acidic glycoconjugates. The acidic glycoconjugates are made by both sialo- and sulphoglycoconjugates. The sialoglycoconjugates contain sialic acids substituted at C8. It is thus evident that the unique type of sialylated glycoconjugates present in the alimentary canal of the sea-bream is that substituted at C8, without differences within ages. On the contrary, this type of glycoconjugate shows a peculiar limited localization to colonic mucosubstances in the majority of mammals, where it is reputed that it may be easily cleaved by bacterial products (Culling and Reid, 1980). The sulphoglycoconjugates constitute the most abundant component, above all in the goblet cells of the rectum. In *Scophthalmus maximus* (Segner et al., 1994) and in *Pleuronectes americanus* (Murray et al., 1996) have also shown that sulphated glycoconjugates are abundant in the goblet cells of the rectum.

The intestine in some teleosts may exert complex functions, some of which are linked to the osmoregulation, above all in the marine fishes which drink sea water for osmotic purposes. In this context, it is significant that fecal fluids are isosmotic with sea water (Smith, 1989). This is obtained by active epithelial transport of NaCl and subsequent water uptake from the lumen (Loretz, 1995). Numerous marine teleosts show the presence of gut regions involved in osmoregulatory functions (Hirano and Mayer-Gostan, 1976; Cataldi et al., 1988; Ciccotti et al., 1993; Abaurrea-Equisoain and Ostos-Garrido, 1996). In addition, differently from other vertebrates, in the fish the enterocytes of the rectum or distal intestine may be responsible for pinocytotic uptake of dietary as well as exogenous proteins, which are subsequently digested intracellularly (Noillac-Depeyre and Gas, 1979; Stroband and Van der Veen, 1981; Albertini-Berhaut, 1987; Sire and Vernier, 1992; Abaurrea et al., 1993; Segner et al., 1994). On the other hand, in most fishes the proximal intestinal regions absorb lipids or aminoacids by diffusion (Ezeasor and Stokoe, 1981; Sire et al., 1981; Sheridan, 1988; Ostos-Garrido et al., 1993b). These functionally different segments show ultrastructurally different absorptive cells (Vernier, 1990; Murray et al., 1996), and experimental treatments or diets differently affect the middle or distal intestine (Nonnotte et al., 1995; Baevefjord and Krogdahl, 1996).

The ability to ingest and digest proteins via a pinocytotic pathway is commonly believed to be a primitive mechanism of ingesting nutrients. This process is well known in its significance and duration for newborn mammals, in which it is important for developing immunity. In fishes, it may be regarded as an adaptation of the alimentary canal for the optimal use of dietary proteins, both in agastric and in gastric fishes (Sire and Vernier, 1992). This is of a special significance in these vertebrates in which the continuous growth, for example

in the skeletal muscle (Veggetti et al., 1990; Rowleron et al., 1995), needs a quantity of dietary aminoacids for protein synthesis as well as for major energy sources (Guillaume, 1987; Sire and Vernier, 1992). Finally, the ingestion of protein as macromolecules may be linked to local "intestinal" immune responses to exogenous proteins (or fragments of them), via the "gut-associated lymphoid tissue" (GALT) (Davina et al., 1982; Temkin and MacMillan, 1986; Rombout and van der Berg, 1989; Dorin et al., 1993).

Just recently, Ribelles et al. (1995) have shown that the presence of the detergent sodium dodecyl sulphate deeply affects the histochemical reactivity of intestinal goblet cells in *S. aurata*, especially in their sulphoglycoconjugate content. This is a demonstration that the quality of gut mucosubstances is directly related to the environmental conditions, which in turn may indirectly affect the functions of the alimentary tract. Our hypothesis is that the various components of intestinal mucosubstances, specially those sulphated of the rectum, possibly regulate the transfer of proteins or fragments of them, as well as of ions and fluids. This is supported by our observation that the adherent mucus gel (which in the intestinal mucosa includes the brush border) is shown to contain N-acetyl-galactosamine, α -D-mannose, β -D-galactose, L-fucose and N-acetyl-glucosamine. In fishes as in mammals the adherent mucus gel constitutes a highly protective mucosal barrier. The cytoplasm of goblet cells, on the other hand, is shown to contain N-acetyl-galactosamine and, in a minor quantity, N-acetyl-glucosamine. As we previously hypothesized for the gastric mucosa, in the intestinal mucosa the sugars possibly change their position in the oligosaccharide branch during the process of secretion, and the possibility of revealing them also varies.

The present study shows that in *S. aurata* the proximal and distal tracts of the gut show a noticeable complexity in the composition of the glycoconjugates, which is gradually acquired during larval ages. This is likely to be related to influences exerted by these tracts on epithelial movements of fluids and ions, especially in this euryaline species. The possible osmoregulatory significance of this histochemical pattern is further supported by the observation that secretory cells occur in the epithelium that lines the developing pharynx-oesophagus at an earlier larval age than in the other tracts of the gut. It is possible to hypothesize that the pharynx-oesophageal zone may be affected in glycoconjugate composition and secretion by different environmental conditions, both in larval and adult ages. This in turn may sustain alimentary and metabolic disorders which are considered possible causes of the high larval mortality of sea-bream in aquaculture conditions. Among the acidic glycoconjugates, those of the sialylated type substituted at C8 are present along the entire gut, whereas the sulphated type is detectable in secretory cells of the pharynx-oesophagus and in the intestinal goblet cells, especially those of the rectum. This is to be regarded as a high protective mechanism of

the mucosa towards exogenous conditions, even if the constancy of the presence of one type only of sialoglycoconjugates does not approach these vertebrates to the high complexity of the gut of mammals.

The present histochemical study reveals additional information about the morphological and functional organization of the alimentary tract of the sea-bream in relation to the age and environmental characteristics, and may provide the basis for future nutritional studies.

Acknowledgements. This work was supported by grants from the Italian Ministero Università Ricerca Scientifica Tecnologica (MURST- 60%).

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Accepted September 11, 1997