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A morphological, glycohistochemical and ultrastructural study on the stomach of adult Rainbow trout *Oncorhynchus mykiss*

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Abstract

In this study the morphology, the conventional glycohistochemistry and the ultrastructure of adult rainbow trout (*Oncorhynchus mykiss*) stomach is reported. For this purpose, stomach belonging to 10 adult female rainbow trout, weighting 500 ± 20 gr and 32 ± 2.50 cm long, were collected and processed. *Oncorhynchus mykiss* has a siphonal stomach, with a J letter shape, showing a descending portion (cardiac region), an ascendant one (pyloric region), connected through the most represented middle portion (fundic region). Morphologically gastric wall shows an overlay of four typical vertebrate tunicae: mucosa, sub-mucosa, muscularis and serosa. Tunica mucosa, lacking of *muscularis mucosae*, is raised into longitudinal folds. The tunica propria submucosa is of considerable thickness and receives the bodies of many gastric glands whose lumen is lined with oxyntopeptic cells. Nervous cells, organized in ganglion, that can be compared to Mammals plexus submucosus, are noted. Glycohistochemistry was performed by staining with Periodic acid–Schiff (PAS), Alcian blue (AB) pH 2.5, AB pH 1 and AB-PAS. Histochemical analysis revealed a consistent presence of glycoconjugates produced by epithelial lining and gastric pit cells. Ultrastructural studies, performed at scanning electron microscopy, showed a mucosa that rises in folds with a geometrically well-defined shape and an abundance of gastric pits in the fundic region. Taken together our results integrate the anatomical description of rainbow trout stomach and will be helpful for future studies related to digestive physiology, development of appropriate feeding strategies and gastric disease control of this teleostean species.

Keywords: *Oncorhynchus mykiss*, digestive tract, conventional glycohistochemistry, SEM

Introduction

The digestive apparatus of fishes displays a remarkable diversity in its morphology and function among species. This is correlated to taxonomy, feeding habits, body size, shape and even sex (Kapoor et al. 1975; Smith 1989). Thereby different studies have been conducted to define the structure of the digestive apparatus in various fish species (Carrassón et al. 2006; Chatchavalvanich et al. 2006; Cao & Wang 2009; Faccioli et al. 2014; Kasozi et al. 2017; Moawad et al. 2017; de Oliveira et al. 2019). Although significant differences can be observed macroscopically, the basic histological structure of the digestive tract is similar among species (Wilson &

Castro 2010). Several authors have described the presence of mucosubstances in digestive system of many Teleosts (Scocco et al. 1996, 1997, 1998; Pedini et al. 2004; Domeneghini et al. 1998; Domeneghini et al. 2005; Diaz et al. 2008; Dos Santos et al. 2015; Ghosh & Chakrabarti 2015). Glycoconjugates are correlated with different functions, such as lubrication, protection of the epithelium against proteolytic degradation, inhibition of microorganisms, buffering of intestinal fluid, link and transport of ions (Reifel & Travill 1979; Allen 1981; Reid et al. 1988).

The stomach is the organ of the digestive apparatus were the differences among fish species are

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clearly marked. In fact, the stomach is absent in some Teleosts or modified as intestinal bulb. When it is present as true organ, it generally consists of three regions (or portions), named cardiac, fundic and pyloric regions (Wilson & Castro 2010). The stomach is characterized by the presence of gastric glands, showing a single cell type secreting both hydrochloric acid and pepsinogen (Diaz et al. 2003; Gallagher et al. 2005). The efficiency of the gastric digestion depends, mainly, on the pH and the mucous secretions that provide a suitable environment to the activity of gastric enzyme (Garrido et al. 1998; Saadatfar et al. 2010; Namulawa et al. 2014). The function of mucous substances in the stomach is generally thought to be linked to processes that increase and improve the digestive efficiency, to lubricate the cavity of the organ and defend it from pathogens (Reifel & Travill 1977; Murray et al. 1994; Pedini et al. 2005).

The structure and the function of fish gut represent a complex system. That is why, in-depth characterization of fish's alimentary canal tract is fundamental to better understand digestive physiology and delineate nutritional researches.

Rainbow trout (*Oncorhynchus mykiss*) is a carnivorous fish belonging to Salmonidae family. It represents the most-widely cultivated cold freshwater fish in the world and an important model species for many research areas, including basic biological disciplines like comparative physiology and toxicology (Mennigen & Zhang 2016). Although its intestinal morphology has been already investigated (Ezeasor & Stokoe 1981; Bielek 2002; Banan Khojasteh et al. 2009; Heidarieh et al. 2012; Ramos et al. 2015; Shabanzadeh et al. 2015; Berillis & Mente 2017; Nofouzi et al. 2019; Verdile et al. 2020), few study are dedicated to the histology of trout stomach (Weinreb & Bilstad 1955; Ezeasor 1981; Ostos Garrido et al. 1993; Marchetti et al. 2006; Mireşan et al. 2012). However, most recent publications analyze the morpho-histology of stomach in young trout reared in indoor standardized condition (Ostos Garrido et al. 1993); other authors analyze sexually immature subjects by conventional and lectin histochemistry (Marchetti et al. 2006) or adult subjects by means of morphological staining (Mireşan et al. 2012), but among them there isn't a glycohistochemical study of stomach on adult trout reared in natural-like condition and none of them focused on scanning electron microscopy (SEM). SEM uses a focused beam of electrons to scan a surface of a sample to create a high-resolution image, that can show information on sample's surface composition and topography (Choudhary & Choudhary 2017).

The purpose of present study is to integrate the description of the morphology of adult rainbow trout stomach and the characterization and the distribution of complex carbohydrates in this organ. These data could contribute to establish a complete morpho-histochemical picture to take into account as comparative base point for future studies aimed to understand the nutritional physiology of this species.

Materials and methods

Animals and sampling

A total number of ten adult female rainbow trout (*Oncorhynchus mykiss*), weighting $\sim 500 \pm 50$ gr and 32 ± 2.5 cm long, were used for this study. The animals, intended for human consumption, were obtained by a local agro-fish company (Val Sele, Eboli, Sa). Fishes were bred in natural-like conditions, feed twice day with a commercial diet, according the instruction of manufacturer (Oregon OP-2). Samples were collected fresh at the morning after 24 hours from the last meal. The animal care procedures were in accordance with Legislative Decree No. 146, implementing Directive 98/58/EC of 20 July 1998 concerning the protection of animals kept for farming purposes. Fish were manipulated and sacrificed using the MS-222 (Coyle et al. 2004).

An incision was made at the ventral aspect of the body from the anal opening to the interbranchial membrane to take the stomach. Small pieces (0.5 cm \times 0.5 cm) from descending, bottom and ascending parts of stomach (cardiac, fundic and pyloric regions; see Figure 1 for details) were obtained and immediately fixed by immersion in:

- a. Bouin's fluid fixative for 24 hours (h) to evaluate the morphology;
- b. Carnoy's fluid for 24 h followed by post-fixation in 2% calcium acetate–4% paraformaldehyde solution (1:1) for 3 h at room temperature for conventional histochemistry;
- c. 2.5% glutaraldehyde solution in phosphate buffer (PBS) pH 7.4 for 24 h at 4°C for scanning electron microscopy (SEM).

Morphological and conventional glycohistochemical treatments

Specimens for optical analysis were dehydrated through a series of graded ethanol, cleared in xylene,

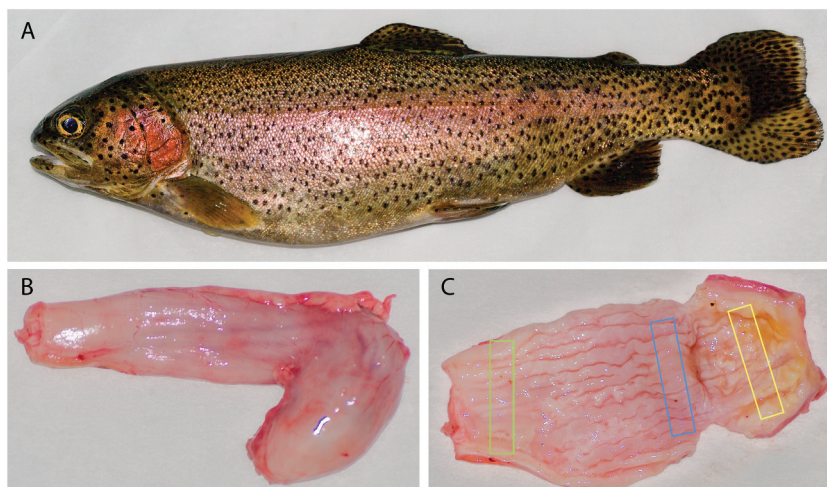


Figure 1. Rainbow trout. Adult female (a). Outer surface of stomach (b), note the J shape with the short descending segment and the longest ascending one. Inner surface of stomach (c), note the wall longitudinal folds. Rectangles (c) indicate the sampling sites: green rectangle for cardiac region, blue one for fundic region and yellow one for pyloric region.

embedded in paraffin wax and cut into 5- μ m-thick serial sections at microtome Reichert-Jung 2050.

For morphological study at optic level, sections were stained with Harris's hematoxylin-eosin (HE) and Toluidine Blue (TB) (Melis et al. 1992). Complex carbohydrate characterization was performed using the established screening staining procedures, carrying out the following histochemical reactions: Periodic acid-Schiff (PAS), Alcian blue (AB) pH 2.5, AB pH 1, AB/PAS (Pearse 1985; Bancroft & Gamble 2008; Mercati et al. 2020). PAS reaction was followed by counterstaining with hematoxylin. Staining solutions were purchased by Bio-Optica Milano SPA: AB pH 2.5 code 05-M26003; AB pH 1 code 05-M26005; PAS code 04-130802A; AB/PAS code 04-163802. All reactions were carried out according to the manufacturer's instructions.

Sugar moieties visualized by different histochemical treatments are shown in Table I.

All stained sections were observed and photographed under Leica DM 1000 light microscope. The digital raw images were optimized for image resolution, contrast, evenness of illumination and background by using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA). For each animal, three independent observers, unaware of the treatments carried out, evaluated five microscopic fields of each intestinal tract and the intensity of the staining was graded in arbitrary units as follows: negative (-), weak (\pm), moderate (+), strong (++) and very strong (+++).

Table I. Sugar moieties visualized by the histochemical treatments carried out.

HISTOCHEMICAL TREATMENTS	SUGAR MOIETIES VISUALISED
AB pH 2.5	Acidic groups: Sialylated glycoproteins (SGPs), carboxylated (Hyaluronic acid, Chondroitin) and sulphated (Chondroitin-sulphates A/B/C, Heparin, Heparansulphate) Glycosaminoglycan (GAG)-like material
AB pH 1	Sulphated GAG-like material (Chondroitin-sulphates A/B/C, Heparin, Heparansulphate)
PAS	Vicinal hydroxyls (neutral and sialylated glycoproteins, GAG-like material)
AB/PAS	Acidic groups and vicinal hydroxyls

AB: Alcian Blue; PAS: Periodic Acid Schiff; GAG: Glycosaminoglycan.

Scanning electron microscopy (SEM)

Specimens of 5 \times 5 mm were postfixed with 1% osmium tetroxide in PBS for 6 h at 4°C in the dark. After three washes in PBS, the specimens were dehydrated first through graded series of ethanol and subsequently in CO₂ atmosphere using a critical point-dried EMITECH K850 (Emitech, Asford, UK). They were attached to aluminum stubs facing upwards, covered with carbon tabs, and then the samples were sputtered with gold-palladium (AGARIB 7340, Agar Scientific Ltd, Stansed, UK). The sections were examined with a scanning electron microscopy ZEISS

EVO40 (20.000KU EHT, 11.5 mm < ω D < 16.5 and 90x < magnification < 8k).

Statistical analyses

Arbitrary units of glycohistochemical reactivity sorted from 0 (negative) to 4 (very strong), were converted into ranks to run the statistical analysis (Scocco et al. 2017; Dall'Aglio et al. 2020a). To test the null hypothesis that the location shift between histochemical treatments was equal to zero, we performed paired t-test if the variables satisfied conditions for parametric tests or Wilcoxon signed rank test when they did not. Normality was tested using the Shapiro-Wilk test; homogeneity of variance using the Levene test. In both the analyses, we applied the Holm's correction for multiple comparisons to avoid type I error.

Statistical elaborations were performed using the R version 3.5.3 (R Core Team 2019), the stats R-package, version 3.5.3 (shapiro.test, t.test, wilcox.test functions), and the car R-package, version 3.0–2 (leveneTest function).

Results

Oncorhynchus mykiss has a siphonal stomach, with a J letter shape, showing a descending portion whose early tract represents the cardiac region and an ascendant one whose last tract represents the pyloric region, connected through the main fundic portion. The inner wall is raised in several longitudinal folds (Figure 1) (Mireşan et al. 2012).

The wall of the organ shows the vertebrate typical overlapping of four tunicae, but the *muscularis mucosae* is lacking. The epithelial lining is represented by a simple columnar epithelium that deepens to form mucous gastric pits on whose bottom the tubular gastric glands open. The epithelium lies on a thick tunica propria-submucosae of loose connective richly occupied by the gastric glands (Figure 2). The epithelium, supported by the conspicuous underlying loose connective hosting the tubular gastric glands, everts out to form the above-described folds. Tubular gastric glands showed an epithelial wall constituted of a single cell type, as previously described in the literature (Ezeasor 1981). The tunica muscularis consists of smooth muscle tissue organized in two layers, with an inner consistent circular and an outer slender longitudinal fiber's pattern. Between these two muscle layers, there is a consistent layer of connective tissue, in which blood vessels, nerve fibers and ganglia are observed

(Figure 2). The tunica serosa of peritoneal derivation is represented by a thin mesothelium supported by a thinner connective layer.

Stomach wall organization does not show any difference among the three analyzed segments; also as far as conventional histochemistry is concerned, no differences were observed among the three analyzed segments of trout stomach (see Figure 1 in Supplementary material).

PAS showed very strong positivity in the supranuclear region and cell coat of the epithelial lining cells and in the gastric pits (Figure 3(a)). AB pH 2.5 showed a similar binding pattern distribution at epithelial lining level, while showed a positivity ranging from moderate to strong in gastric pits. AB reactive sites decreased with pH 1 treatment, above all in gastric pits (Figure 3(b)). AB/PAS were positive in epithelium and gastric pits, all reactive structures showed a prevalently PAS affinity particularly evident in gastric pits (Figure 3(c)). In gastric glands no binding patterns were present for any histochemical treatment.

The above results for trout stomach structures to conventional histochemistry are summarized in Table II.

Comparison between AB pH 2.5 and AB pH 1 showed statistically significant differences in the response of the reactive structures, except for gastric glands, to the two sequential treatment (Table III). The most significant differences were identified in the supranuclear region and gastric pits (Table III).

To better understand the results, it should be considered that the specific complex carbohydrate kinds are mainly identified taking into account the reactivity difference by comparing sequential treatments (Dall'Aglio et al. 2020b). So, the difference between the AB pH 2.5 and pH 1 reactivity is ascribable to carboxylated complex carbohydrates, while the AB pH 1 residual reactivity is due to sulphated complex carbohydrates with the exception of highly sulphated-like material since the lack of metachromasia to toluidine blue staining. In addition, the prevalently PAS positive response to AB/PAS treatment of some structures indicates the presence of neutral glycoproteins.

On the basis of the above considerations, glycohistochemical evidences allow to hypothesize that in rainbow trout the stomach structures of produce the complex carbohydrate types listed in Table IV, in descending semi-quantitative order.

Scanning electron microscope studies showed that the mucosa lifts up to form longitudinal folds supported by a connective axis. The luminal surface is

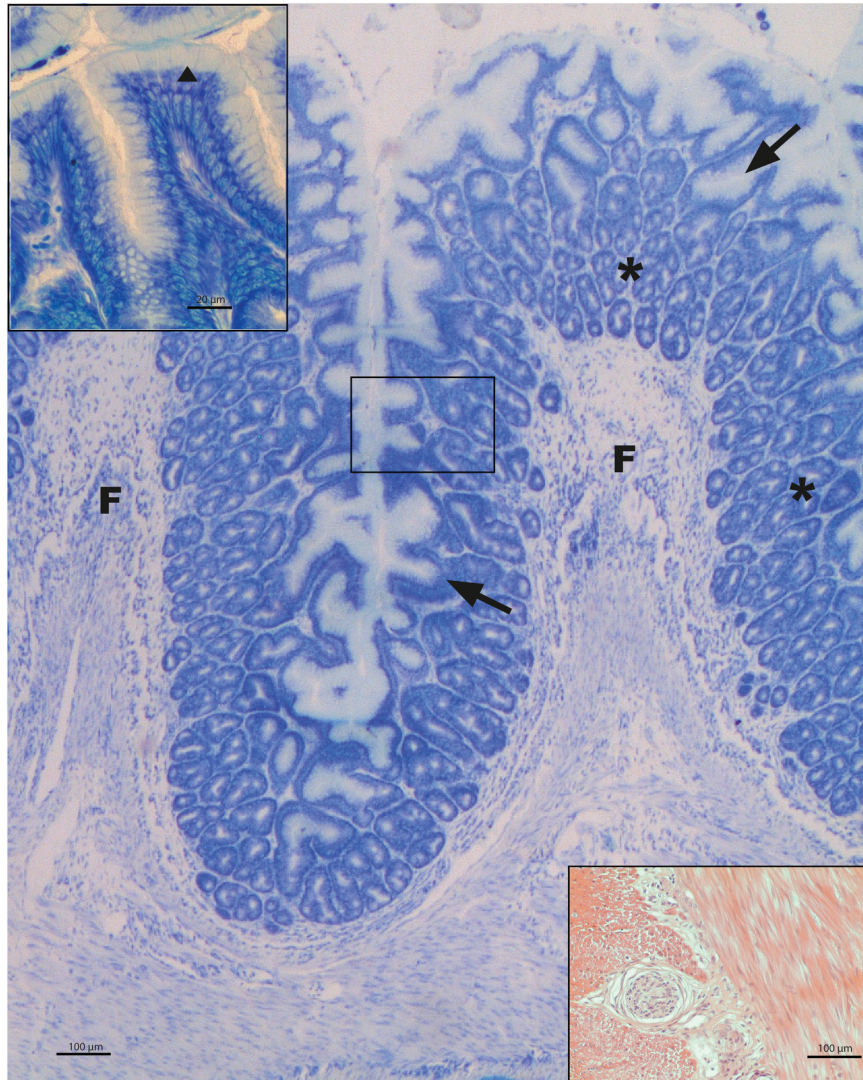


Figure 2. Morphology of rainbow trout fundic portion of stomach at optical level. Toluidine blue staining shows the simple columnar epithelium that deepens to form the gastric pits (↑); on the floor of these, tubular gastric glands open (*). Nor lining epithelium (▲) neither gastric pits show metachromatic behavior (see enlarged upper left corner). Between the two muscular tunica layers a nervous net with ganglia is present (down right corner; hematoxylin-eosin stained). F = folds.

characterized by a large number of opening related to numerous gastric pits (Figure 4(a,b)). The lining epithelium of the mucosa is of a simple prismatic type, with high cells that at the apical membrane level show a thin cell coat (Figure 4(c)) and which rest on a connective layer thickly occupied by tubular gastric glands (Figure 4(c)).

Discussion

This study provided more in-depth information on the morphological and glycohistochemical aspects of rainbow trout stomach in adult subjects reared in

natural-like conditions, completing the anatomical description by SEM technique.

Rainbow trout stomach showed a J-shaped, that is the configuration most commonly found in fish species (Wilson & Castro 2010). Presumably, this shape may help in extending the duration that food stays in the stomach, thus promoting better enzymatic digestion. Rainbow trout, as other salmonid species, is a carnivore fish: its diet is composed by insects, crustaceans and small fishes, and the presence of wall inner folds allows trout's stomach to be easily extendable enabling the fish to swallow large prey whole and increases the surface area allowing efficient mixing of food with gastric juice.

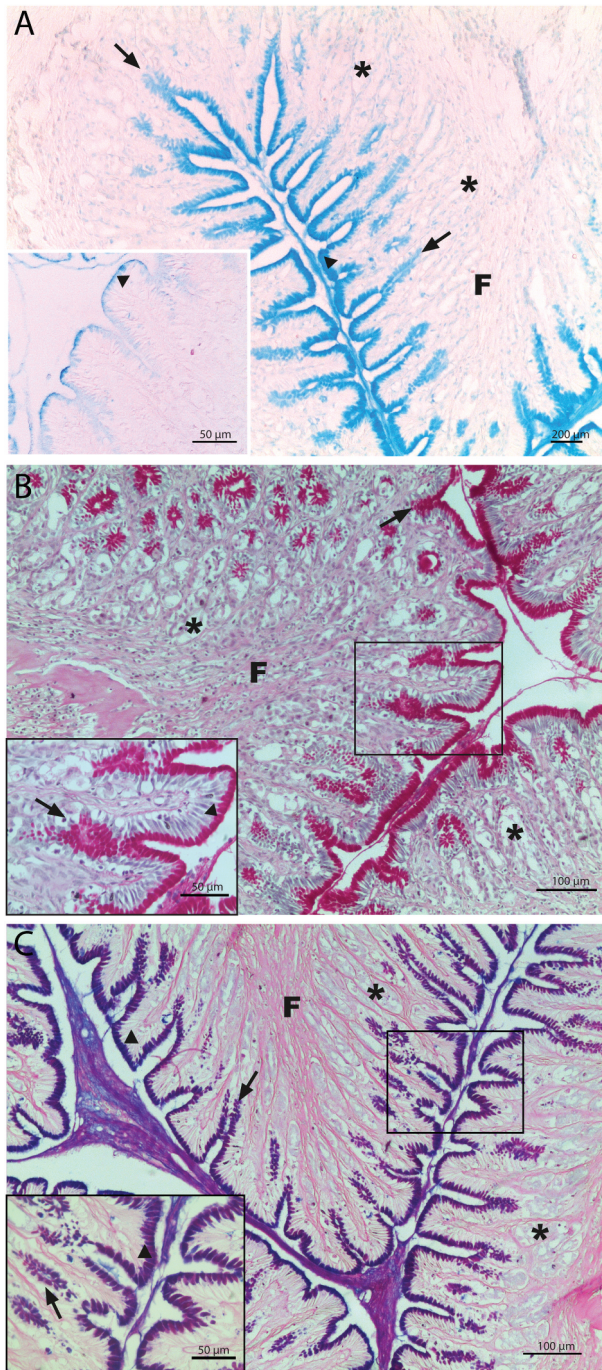


Figure 3. Rainbow trout fundic portion of stomach. AB staining pH 2.5 (a) show reactive binding sites at epithelial lining, cell coat and gastric pit level. The same treatment at pH 1 strongly decrease the reaction response, above all as epithelial supranuclear region and gastric pits regards (down left corner). PAS staining (b) evidence very strong response by epithelium and gastric pits; down left corner is the enlargement of the box in the photo. AB/PAS staining (c) show that epithelial cells are prevalently AB positive at both cell coat and cytoplasm level, while gastric pits show a prevalently PAS positivity to the histochemical treatment; down left corner is the enlargement of the box in the photo. Epithelial lining (▲), gastric pit (†), gastric glands (*), F = folds.

Table II. Histochemical reactivity of stomach structures.

Histochemical treatments	Epithelial lining		Gastric pits	Gastric glands
	Supranuclear region	Cell coat		
PAS	+++	+++	+++	-
AB pH 2.5	+++	+++	+ / +++	-
AB pH 1	+	++	±	-
AB/PAS	B++/R+++	B++/R+++	B+/R++	-

AB: Alcian Blue; PAS: Periodic Acid Schiff; B = blue; R = red.

Table III. Statistical significance of differences (*p* values) between histochemical treatments AB pH 2.5 and AB pH 1, as performed by Wilcoxon signed rank test and paired t-test. *p* values were adjusted for multiple testing using the Holm correction.

	Test	<i>p</i>
Supranuclear region	Paired t-test	8.574×10^{-11}
Cell coat	Wilcoxon signed rank test	5.761×10^{-3}
Gastric pits	Paired t-test	2.200×10^{-8}
Gastric glands	-	-

AB: Alcian Blue.

Although the structure of the wall has the same stratification noted in the majority of other Teleosts, there are some variations that are mostly related to the species' feeding habits. We observed four layers: mucosa in which the *muscularis mucosae* is not present, in contrast to what described by Weinreb and Bilstad (1955), propria-submucosa, muscular and serosa. Like in other Teleosts, the lining epithelium of gastric mucosa is represented by a simple layer of prismatic cells showing the same morphology along the entire wall of the organ. The supranuclear portion of these epithelial cells evidenced the production of a variety of complex carbohydrates. The mucosal epithelium invaginates to form gastric pits at bottom of which gastric glands open. These are common characteristics of carnivorous fish species (Vieira-Lopes et al. 2013). The single cell type presents in the gastric glands is described as oxyntopeptic cell, because it is generally assumed that they synthesize both hydrochloridric acid and pepsinogen, that in Mammals are produced by the oxyntic and chief cells respectively (Diaz et al. 2003). The presence of a consistent submucosal connective layer might have a mechanical function mostly limiting the damage to tissues in any moments of extreme fullness organ. In the stomach wall localizes a nervous control system formed by two different patterns that intertwine giving rise to two plexuses and, therefore, the development of an intramural network with cells that are organized to form small ganglia. They can be compared to a real small autonomous system with

Table IV. Complex carbohydrate types produced by stomach structures.

Epithelial lining	Supranuclear region	Carboxylated mucins (Hyaluronic acid/Chondroitin-like GAGs) Neutral glycoproteins
	Cell coat	Sulphated mucins (Chondroitinsulphate A/B/C-like GAGs) Acid glycoproteins with sulphated glucidic residues Neutral glycoproteins
Gastric pits		Carboxylated mucins (Hyaluronic acid/Chondroitin-like GAGs) Neutral glycoproteins Sulphated mucins (Chondroitinsulphate A/B/C-like GAGs)

GAGs: Glycosaminoglycans.

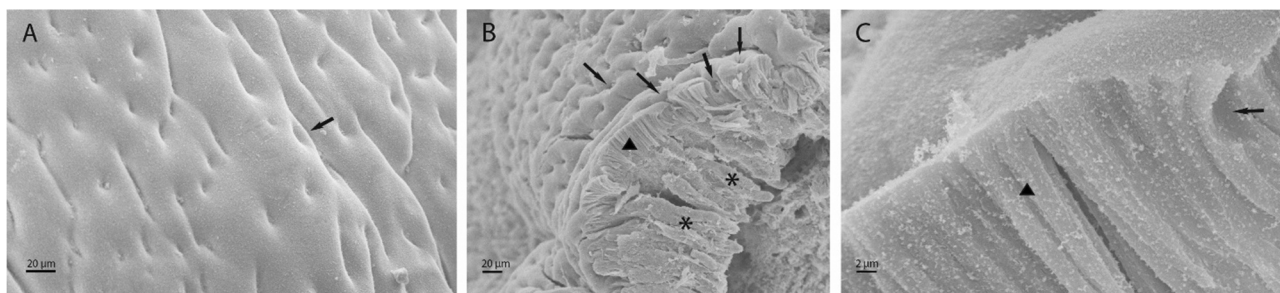


Figure 4. SEM images of fundic portion of stomach inner surface (a) in which the outlets of the gastric pits are well visible (↑). SEM cross section of stomach mucosae (b) showing the epithelial columnar lining (▲), the gastric pits (↑) and the tubular gastric glands (*). In C it is showed an enlargement of epithelium (▲) with a gastric pit (↑).

action on the smooth muscle similar to mammalian myenteric plexus of Auerbach.

Based on conventional glycohistochemical treatments response from tunica mucosae pertaining structures, we provided also the histochemical profile of rainbow trout stomach. Findings allow to argue that epithelial lining cells produce both surface and secretion glycoconjugates represented by glycoproteins and glycosaminoglycan-like carbohydrates, while gastric pits produce secretion glycosaminoglycan-like material. In particular, at epithelial cell coat level, a consistent amount of surface glycoproteins was evidenced. The strong positivity to AB pH 1 and the prevailing PAS positivity to AB/PAS treatments allows us to hypothesize that in the cell coat, the glycoproteins are mainly acid, showing the oligosaccharidic chain in which many glucosidic residues linked sulphated groups, and in lesser quantity neutral glycoproteins. Indeed, previous research (Marchetti et al. 2006) evidenced on the epithelial cell coat of trout stomach, the presence of both acid sialylated and neutral fucosylated glycoproteins in addition to oligosaccharidic chain with terminal residues represented by β -galactose and α -N-acetylgalactosamine, two sugar residues proved to be linked to sulphated groups (Parillo & Verini-Supplizi 2001).

The acid glycoconjugates could likely confer a permanent negative charge to the cell plasma

membranes, so protecting the mucosae epithelium from the highly acid gastric juice action (Scocco et al. 1996; Marchetti et al. 2006). In this last function, surface glycoconjugates are largely supported by the similar action carried out by secretion glycoconjugates produced by both stomach epithelial lining, which also acts like a secreting lamina, and gastric pits. These structures prevalently secrete carboxylated glycosaminoglycans like to hyaluronic acid and/or chondroitin, in addition to lesser amount of both chondroitinsulphate A/B/C-like glycosaminoglycans and neutral glycoproteins. Heparin and/or heparansulphated-like glycosaminoglycan production can be excluded by the lack of metachromatic response of toluidin blue treated samples. The great amount of glycosaminoglycans draws large quantities of water to form a viscoelastic barrier able to guarantee a good mucosae hydration degree, in addition to protect the epithelial apical membrane from both HCl and peptidase action (Mercati et al. 2020; Dall'Aglio et al. 2020a). Finally, an important involvement of produced complex carbohydrates in the defense of rainbow trout stomach mucosae from pathogen agents have to be considered; in fact, the several kinds of glycoconjugates could hamper the attach of viruses and/or bacteria to the stomach mucosae, acting as hapten-like sites and enveloping

the pathogen agents as hypothesized in fish and in different animal species (Scocco & Pedini 2006; Scocco et al. 2017; Dall'Aglio et al. 2020b), in addition to prevent some pathogen enzymatic strategies (Ohya & Kaneko 1970; Yamada & Hirano 1973; Hanaoka et al. 1989; Zimmer et al. 1992; Nieuw-Amerongen et al. 1995; Scocco et al. 1997, 1998).

In conclusion, our work integrates the morphology description of adult rainbow trout's stomach and defines its glycohistochemical profile. Our findings offer the baseline to further researches aimed to monitor modifications linked to environmental changes and to assess the best feed strategies in aquaculture. However, more studies should be carried out for a deeper understanding of the digestion process and nutrient absorption of these fish.

Disclosure statement

No potential conflict of interest was reported by the authors.

Author contributions

Conceptualization, A.C. and P.S.; methodology and validation, E.D.F. and A.C.; formal analysis, F.M.T.; investigation, A.C. and C.M.A.B.; data curation, A.P., D.G. and C.M.A.B.; writing—original draft preparation, E.D.F. and P.S.; writing—review and editing, E.D.F.; supervision, A.C. and P.S. All authors have read and agreed to the published version of the manuscript.

Supplementary material

Supplemental data for this article can be accessed [here](#).

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