Research Article

Histochemical and Immunohistochemical Analysis of the Stomach of *Rhinella icterica* (Anura, Bufonidae)

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The stomach of *Rhinella icterica* was analyzed at light microscopy, employing histochemical techniques, lectin histochemistry, and immunohistochemistry for identifying enteroendocrine cells (EC). Although the stomach was composed of fundic and pyloric regions, its wall is formed by mucosa, submucosa, muscularis, and serosa. The mucosa was lined by a simple columnar mucous epithelium, supported by loose connective tissue. Several tubular, simple glands were composed of mucous neck cells, containing oxynticopeptic cells and EC cells. The mucous neck cells were rich in neutral glycoconjugates. The oxynticopeptic cells were predominant in fundic glands, exhibiting weaker alcianophilic reaction at their apical cytoplasm. Serotonin (5-HT) immunoreactive (IR) cells occurred throughout the entire stomach, preferentially located among mucous cells at upper part of the fundic glands. The muscularis mucosae, formed of smooth muscle, separated the mucosal layer from the submucosa, both of which were constituted by loose connective tissue, but without glands. Lymphoid modules occurred in the mucosa at the boundary at the stomach and the gut. In addition, the muscularis was constituted by two sublayers, the circular internal and the longitudinal external, being recovered by the connective tissue of the serosa.

1. Introduction

The structural organization of a tissue and/or an organ represents a close association between life habits and physiology of the animal; thus, the tissue structure can reflect their lifestyle and provide information for understanding the population declination and fluctuations [1, 2].

Morphological studies on the anuran gastrointestinal tube have been performed by histochemical [3–5], ultrastructural [6], and immunohistochemical techniques [7, 8], showing histological peculiarities to the stomach. These differences are more evident considering mainly the exocrine gastric glands, whose morphology varies according to the gastric region.

In mammals, the stomach is made up of mucosa, submucosa, muscularis, and adventitia or serosa. The gastric surface is recovered by a thin biofilm of mucous that is secreted by superficial mucous and mucous neck cells [9]. This mucous layer forms a highly viscous barrier that protects the stomach surface, being formed by acid and neutral glycoconjugates. Histochemical studies indicate differences regarding the qualitative expression of neutral, sulfated, and carboxylated mucosubstances [10, 11]. The mucosubstances, which phylogenetically first appeared in mucous neck cells of the gastric glands of anurans [12], have already been reported for *Rana aurora aurora* [13], *Bufo viridis* [14], *Rana perezi* [15], *Bufo melanostictus* [3], and *Bufo marinus* [16], indicating variability among anuran species. Furthermore, different types of carbohydrates were identified in secretory cells in the stomach of *Bufo viridis* [17, 18] and *Rana esculenta* [19].

Regarding the specialized cells, studies of the stomach of adult frogs, as *Rana esculenta* [4] and *Rana aurora aurora* [13], as well as tadpoles of *Rana temporaria* [5]

TABLE 1: Lectins, their sources, and inhibitory sugars.

Source of lectin	Lectin abbreviation	Inhibitory sugar	Cat. vector no.	Dilution
Griffonia simplicifolia	GSL-1	α-Gal, α-GalNAc	B-1105	1 : 500
Arachis hypogaea	PNA	β-Gal>GalNAc	B-1075	1 : 1000
Artocarpus integrifolia	Jacalin	β-Gal>GalNAc	B-1155	1 : 500
Lens culinaris	LCA	α-Man, α-Glc	B-1045	1 : 500
Glycine max	SBA	$\alpha > \beta$-GalNAc	B-1015	1 : 1000
Solanum tuberosum	STL	α/β-GlcNAc	B-1165	1 : 1000

Gal = galactose, GalNAc = N-acetylgalactosamine, Man = mannose, Glc = glucose, and GlcNAc = N-acetylglucosamine.

demonstrated that oxynticopeptic cells occur in gastric glands, being responsible for hydrochloric acid production. Furthermore, oxynticopeptic cell has already been identified among glandular cells of the esophageal mucous layer of *Rana catesbeiana* [20] and *Rhinella icterica* [7]. In mammals, the production of pepsinogen as well as hydrochloric acid is performed by two distinct cell types [9]. On the other hand, in anurans, like *Bombina variegata* [4], *Bufo marinus* [21], *B. viridis* [14], *Rana perezi* [15], and *Triturus carnifex* [22], oxynticopeptic cells produce and release both hydrochloric acid and pepsinogen. Ruiz and coworkers [16] related the fact that, in *B. marinus*, oxynticopeptic cell is able to secrete pepsinogen concomitantly with hydrochloric acid.

The ontogeny and distribution of endocrine cell were studied in several mammalian species like humans [23], mice [24], and horses [25]. Santos and coworkers [26] indicate that in bats (*Desmodus rotundus, Diphylla ecaudata*, and *Diaemus youngii*) the relative frequency and distribution of these cells vary according to species and their feeding habits. In anurans, endocrine cells occur in the stomach of *R. perezi* [15], *R. catesbeiana* [27], and *R. temporaria* [28]. Nevertheless, information about the occurrence of enteroendocrine (EC) cells in the bufonid stomach is still limited.

It is well known that the function of the digestive tube is regulated by peptides, whose property makes some of them able to act as hormones. Moreover, peptides also exhibit other functions; for example, there are antimicrobial peptides involved in the innate immune response, being also called host defense peptides. In anurans, peptides with broad-spectrum antimicrobial activities have been described not only in integument cells [29, 30] but also in peptic cells of the stomach [31, 32]. Cho and coworkers [33] suggest that buforin I, after being secreted into the gastric lumen, remains adhered to mucous that lines the stomach, providing a protective antimicrobial coat. This peptide exhibits other biological effects such as inhibition of botulinum, neurotoxins, and tissue factor-initiated coagulation. In addition, the anuran stomach is a general model for physiological investigations, being used for comparative purpose of the gastrointestinal tube development in vertebrates [15, 16, 21].

The aim of this study is to characterize the histological structure of the stomach of *Rhinella icterica*, a common toad in Brazil, employing histochemical techniques at light microscopy level in order to improve the knowledge of the anuran digestive system. Moreover, this work also intends to detect different sugar secreting cell types using lectin histochemistry and identify 5-HT-, CCK-, G-, and STT-cells using immunohistochemistry.

2. Material and Methods

2.1. Animals. Nine adult male toads *Rhinella icterica* (former *Bufo ictericus* Spix, 1824) were collected according to Brazilian laws (collecting permit number 020222.003111/96-14 issued by IBAMA) in the District of Resende in Rio de Janeiro State.

2.2. Tissue Preparation and Staining. For light microscopic studies, stomach fragments were fixed in 10% buffered formaldehyde and/or in Bouin's liquid and were processed according to the standard histological techniques for paraffin embedding.

2.3. Light Microscopy. Five-micrometer-thick serial slices were stained with hematoxylin-eosin (HE) and Gomori's trichrome method [34]. Staining with 1% Alcian blue (AB) 8GX at pH 1.0 and at pH 2.5 [35] was employed for the demonstration of sulfated and sulfated and carboxylated acidic glycoconjugates, respectively. The periodic acid Schiff (PAS) staining was employed to detect neutral glycoconjugates [35].

2.4. Lectin Histochemistry. Lectin histochemistry was employed for carbohydrate detection, and the used lectins are listed in Table 1.

The biotinylated lectins GSL 1, STL1, PNA, Jacalin, LCA, and SBA were used, being purchased from Vector (Burlingame CA, USA).

Once dewaxed and hydrated, tissue slices were immersed in 3% H_2O_2 for 10 minutes to suppress endogenous peroxidase activity, rinsed in 0.05 M phosphate-buffered saline (PBS) at pH 7.4, and incubated in lectin solutions of appropriate dilutions (Table 1) for 1 hour at room temperature. After three times of being washed in phosphate buffered saline (PBS), peroxidase activity was detected by incubation in a solution containing 0.05% 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and 0.005% H_2O_2 in 0.05 M PBS (pH 7.4) for 1 minute at room temperature before dehydration and mounting. As for the controls for lectin reaction, the incubation medium with buffer but without lectin was used.

Table 2: Primary antibodies used and their respective dilutions (phosphate-buffered saline + albumin), supplier, and antigen recovery buffer.

Antibodies	Dilution	Supplier	Cat. no.
Anti-serotonin (5-hidroxytryptamine, 5-HT)	1 : 8000	Sigma-Aldrich	S 5545
Rabbit anti-human gastrin (G) releasing peptide	1 : 400	Sigma-Aldrich	A429
Rabbit anti-human somatostatin (STT)	1 : 300	Dako	A0566
Rabbit polyclonal anti-cholecystokinin (CCK)	1 : 8000	Sigma-Aldrich	C 2581

2.5. Immunohistochemistry. For immunohistochemistry, paraffin sections were dewaxed, hydrated, and washed in phosphate buffered saline (PBS). They were incubated with methanol containing 0,3% H_2O_2 for 15 minutes to block any endogenous peroxidase. Sections were incubated with a 1 : 100 dilution of bovine serum albumin (B4287; Sigma) in PBS in a humid chamber during 30 minutes at room temperature. They were then incubated overnight with different antibodies (Table 2) at 4°C. Afterwards, they were after that incubated with biotinylated secondary antibody diluted to 1 : 200 (PK 7200, Vector Laboratories, Inc., UK) for 30 minutes, then they were diluted at 1 : 200, for 30 minutes (both from PK 6200, Vector Lab. Inc.). Subsequently, the peroxidase label was revealed by reaction with DAB at room temperature before dehydration and mounted using Enthelan (Merck).

3. Results

The stomach of *R. icterica* showed two regions: the fundic (Figure 1) and the pyloric regions (Figure 2). The gastric wall of both regions was formed by four major layers: mucosa, submucosa, muscularis, and serosa (Figure 1).

The mucosa showed gastric folds, and it was made of the lining epithelium supported by the lamina propria of loose connective tissue (Figure 1). The mucosa of the fundic and pyloric regions was lined by a simple columnar mucous epithelium (Figures 3 and 6). Occupying the entire mucosa, gastric glands opened into the bottom of the funnel-shaped depressions, named gastric pits (Figure 6). At pyloric region, the gastric folds were more prominent, while the gastric pits were deeper when compared to the fundic region (Figure 2).

Apical domain of the lining epithelial cells was acidophilic and exhibited strong staining after PAS (Figure 4) and PAF methods, but only a very weak alcianophilic reaction was observed at their surface (Figure 5). These histochemical findings indicated that their intake was rich in neutral glycoconjugates. The mucosa was separated from submucosa through muscular mucosa (Figures 1 and 6), which was clearly subdivided into inner circular and external longitudinal layers. At pyloric region, the muscular mucosa was subdivided into longitudinal internal and circular external layers (Figures 1 and 6). Isolated nodular lymphoid tissue
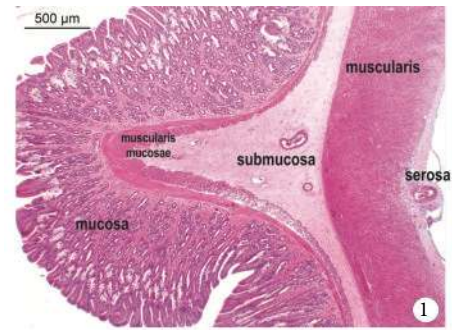

Figure 1: Light micrograph of the fundic region of the stomach of *R. icterica*. Note the mucosa, submucosa, muscular, and serosa layers. Gastric glands occupy the mucosa layer, being formed by mucous neck cells, predominantly at the upper portion, and oxynticopeptic cells, preferentially located at the lower portion. The muscular layer of smooth muscle is subdivided into longitudinal internal and circular external layers. HE staining.

Figure 2: Light micrograph of the pyloroduodenal junction of the stomach of *R. icterica*. At pyloric region, prominent gastric folds are evident. Isolated lymphoid nodes occur in mucosa at transition from the stomach to the intestine. HE staining.

occasionally occurred in the mucosa connective tissue and was frequently visualized in the mucosa layer at the pyloroduodenal junction (Figure 2).

The gastric glands were structured as simple tubular gland, frequently branched at the lower portion of the gland body (Figure 3). They were constituted by mucous neck cells, preferentially located at the neck (Figures 1, 3, 4, and 6), besides oxynticopeptic cells, which predominate at the gland body (Figure 3). Beyond these cells, EC cells occur scattered through the gland body but predominated at the neck among mucous neck cells (Figure 7).

The mucous neck cells were lightly stained in bright purple after PAS staining (Figure 4); however, no AB reaction was visualized (Figure 5), indicating that their content is rich in neutral glycoconjugates.

Oxynticopeptic cells showed finely granular acidophilic cytoplasm (Figures 3 and 6) and are numerous mainly in the lower portion of the gastric gland. These cells predominate at the fundic region, decreasing until the pyloric region, where the gastric glands were basically formed by mucous cells
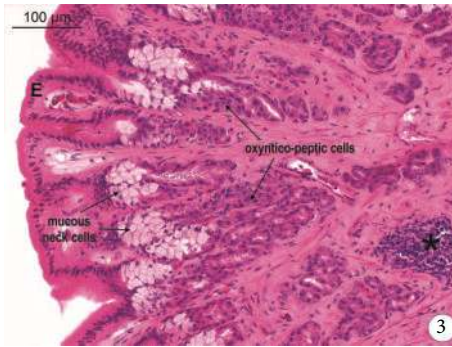
FIGURE 3: Light micrograph of the fundic region of the stomach of *R. icterica*. Mucosa is lined by simple columnar mucous epithelium (E), which forms simple tubular gastric glands composed of mucous neck cells at the upper portion of the gland and oxynticopeptic cells at lower portion. Isolated lymphoid nodules (⋆) occur in mucosa. HE staining.

FIGURE 4: Light micrograph of the fundic region of the stomach of *R. icterica*. Glycoconjugates are identified at apical domain of the lining epithelial cells and at mucous neck cells. PAS method.

FIGURE 5: Light micrograph of the fundic region showing alcianophilic reaction at apical domain of the oxyntic peptic cells of the stomach of *R. icterica*. Note the isolated alcianophilic cells in the connective tissue of the mucosa layer. AB method.

FIGURE 6: Light micrograph of the pyloric region of the stomach of *R. icterica*. The gastric pits are deeper when compared to the fundic region. Inset: detail of the gastric glands; the oxynticopeptic cells exhibit granular material scattered through their cytoplasm. Gomori's trichrome method.

(Figure 2). In addition, they showed no PAS staining; nevertheless, weak alcianophilic reaction at the apical cytoplasmic domain was detected (Figure 5).

5HT-IR cells occurred throughout the lining epithelium of both fundic and pyloric regions of the stomach at the base of the pit near the neck of the gastric gland and at the upper portion of the fundic glands among the mucous neck cells (Figure 7). 5-HT-IR cells displayed triangular shape, exhibiting projections that reach the lumen, being classified as open type (Figure 7, inset). Moreover, the close type of 5HT-IR cell was also visualized. On the other hand, STT-, CCK-, and G-IR cells were not detected in any region of the stomach.

Separating both connective tissues (of mucosa from that of submucosa) there was well-defined muscularis mucosa, which was formed by two layers: the inner circular and the outer most longitudinal, both composed of smooth muscular tissue (Figures 1 and 6). The submucosa was also formed by loose connective tissue, rich in blood vessels; however, no glands were visualized in this layer.

In both fundic and pyloric regions, the muscular layer also consisted of smooth muscle, subdivided into the circular and longitudinal layers (Figures 1 and 6).
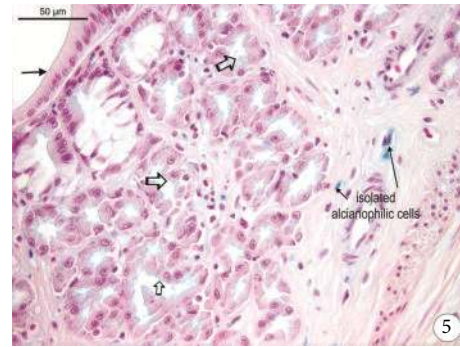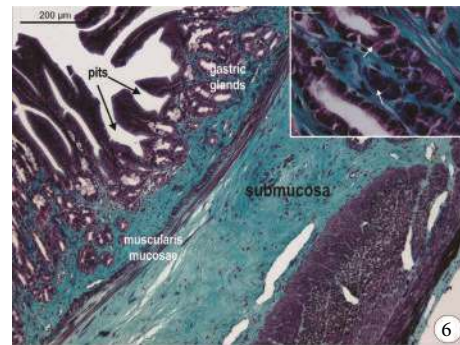
The serous layer was identified through a very thin sheet of loose connective tissue with some blood vessels and nerves, being recovered by the mesothelium (Figure 1).

Considering the lectin histochemistry, glycoconjugates residues exhibited preferential localization in the stomach (Figures 8(a)–8(d)).

The epithelial cells showed reaction to employed lectins, except to STL, indicating that their intake contained more $\alpha > \beta$ GalNAc residues than α-Gal, α-GalNAc, and β-Gal>GalNAc residues (see Table 3). However, $\alpha > \beta$-GalNAc residues predominated at epithelial cells of the gastric pits at the pyloric region (Figure 8(c)). The mucous neck cells from fundic and pyloric regions displayed positive reaction to GSL1, PNA, Jacalin, LCA, and SBA, except to STL, indicating α-Gal, β-Gal, α-Man, α-Glc, and α-GalNAc residues. The oxynticopeptic cells showed less α-Gal, β-Gal, and α,β-GalNAc, while α-Man, α-Glc residues were not detected. After lectin histochemistry, the extracellular matrix also revealed glycoconjugates as α-Man, α-Glc, and α-GalNAc residues, probably due to its glycosaminoglycans.

The lectin reactions are summarized in Table 3.

TABLE 3: Lectin histochemical labeling in the stomach of *R. icterica*.

Lectins	Inhibitory sugar	Epithelial cells	Mucous neck cells	Oxynticopeptic cells	MEC
GSL1	α-Gal, α-GalNAc	+++	++	+	+
PNA	β-Gal>GalNAc	++	++	+/−	−
Jacalin	β-Gal>GalNAc	++	++	+	+/−
LCA	α-Man, α-Glc	+	+	−	++
SBA	α>β-GalNAc	+++	++	+	−
STL	α/β-GlcNAc	−	−	+	−

(+) weak positive reaction; (++) moderate reaction; (+++) strong reaction; (−) negative reaction.
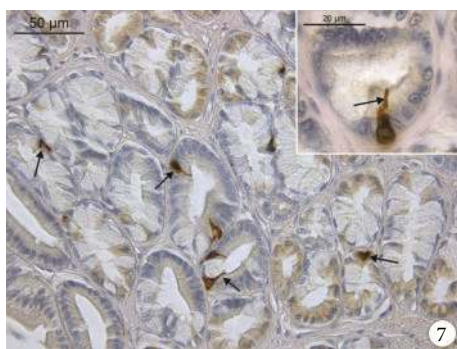

FIGURE 7: Immunohistochemistry for the 5-HT-IR cell identification. The 5-HT-IR cells (→) occur among the lining epithelium and glandular cells of gastric glands. Inset: detail of the open type of 5-HT cells, showing cytoplasmic processes (→) that arise glandular lumen.

4. Discussion

In *R. icterica*, the stomach presented four distinct layers, showing the same pattern for the digestive tube as described for its esophagus [7] and for other vertebrates [5, 36, 37]. Like in *R. pipiens* [38] (Norris, 1959) and *R. aurora aurora* [13], the stomach was characterized by two gastric regions: fundic and pyloric. Nevertheless, Gallego-Huidobro and Pastor [15] noticed that in *R. perezi* the fundic region is subdivided into upper and lower portions.

In adult *R. icterica*, the gastric mucosa is constituted by a simple epithelium of columnar mucous cells supported by well vascularized loose connective tissue. In addition, in fundic glands, mucous cells predominated at the neck, while oxynticopeptic cells were preferentially located at the body region. For other anurans, the lining epithelium is constituted by mucous ciliated cells, as in the larval stage of *Xenopus laevis* [39] and in *R. temporaria* [5].

After using histochemical methods, it could be seen that in *R. icterica* the mucous cells produce neutral glycoproteins, rich in galactose, galactosamine, and glucosamine residues.

As a family of heavily glycosylated proteins, mucins are the major organic components of mucous, representing the protective layer that recovers the epithelium in many organs of human and animals, including the gastrointestinal tube [40]. It is well known that gastrointestinal mucins are glycoconjugates that contain various amounts of different carbohydrate moieties [41], produced by epithelial and goblet cells [9].

Mucous cells have already been described in the stomach of mammals, as *Rhesus* monkey and baboon [11], as well as in other vertebrates as fishes [42] and lizards [10, 18].

According to Suganuma and coworkers [12], amphibians were the first vertebrates to develop true mucous neck cells, which are the precursors of chief cells via intermediate cells [31]. In anurans, mucins are produced by goblet cells found in the esophagus and the intestines, but also by epithelial cells and mucous neck cells in the stomach [43]. In *R. icterica*, mucous neck cells of fundic region produce neutral glycoprotein, rich in galactose residues, similar to those of *R. esculenta* [19], *Ceratophrys ornata* [44], *Ceratophrys chalcides* [10], and reptile *Natrix natrix* [45].

In *R. aurora aurora*, epithelial cells of the stomach exhibit both PAS and AB reaction. However, these findings are partly in contrast to our observations, since mucous cells of the fundic and pyloric glands of *R. icterica* displayed positivity only for PAS method. Yet, our results corroborated the reports by Loo and Wong [3], who described that the lining epithelial cells of the stomach of *B. viridis* and *B. melanostictus* produce only neutral mucins. Moreover, lectin histochemical studies revealed that in *R. aurora aurora* [13], *B. viridis* [14], and *R. esculenta* [19] mucous secreting superficial cells produce galactose, but not galactosamine, since in *B. viridis* [14] and *R. aurora aurora* [13] gastric secretion is rich in galactose and mannose.

In *R. icterica*, the epithelial superficial cells produced galactose > galactosamine, being similar to those cells of Reptilia *Chalcides chalcides* [10]. Furthermore, galactose and α-N-acetylgalactosamine were detected at apical domain of oxynticopeptic cells, that is, at the same localization as that of alcianophilic reaction. This result is probably peculiar to amphibians as *R. icterica*, *Triturus carnifex* [22], and other vertebrates, like seps (*Chalcides chalcides*) [10] and snake (*Natrix natrix*) [45], since epithelial cells exhibited very weak alcianophilic reaction. On the other hand, α-N-acetylgalactosamine residue has been reported to bind specifically not only in the intracellular canaliculi and vesicular membranes of mammalian parietal cells [46–48] but also in oxynticopeptic cells of some nonmammalians, such as the green toad *B. viridis* [14] and the ruin lizard *Podarcis sicula campestris* [49]. Moreover, in *R. aurora aurora*, oxynticopeptic cells showed no reaction to any of the used lectin kinds [13]. Liquori and coworker [22] suggest that differences
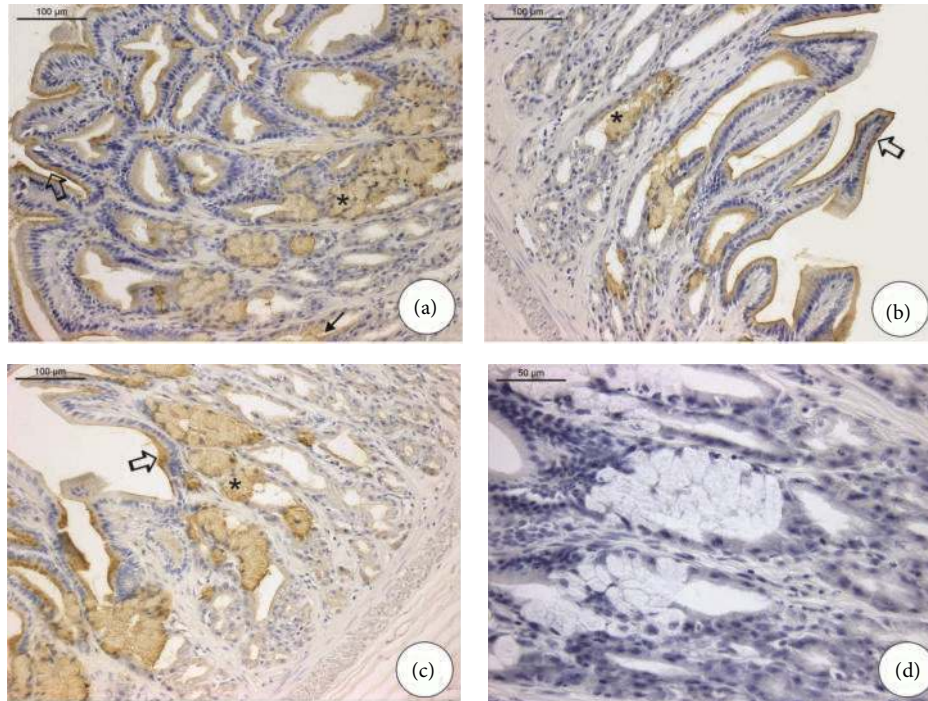
FIGURE 8: Lectin histochemistry. (a) Fundic region: the oxynticopeptic cells displayed a very weak positive reaction for GSL (→), in contrast to the epithelial cells (⇒) and mucous neck (⋆). (b) Pyloric region: note the moderate reaction in epithelial cells (⇒) and mucous neck cells (⋆) after PNA staining. (c) Pyloric region. Note the strong reaction at the apical domain of epithelial cells of the gastric pits as well as at mucous neck cells. (d) Negative control.

in nature of the oligosaccharide chains may occur between species of vertebrates.

In this study, oxynticopeptic cells revealed cytoplasmic acidophilic granules after staining with Gomori's trichrome. Norris [38] noticed that in *R. pipiens* the secretory granules of the oxynticopeptic cell are smaller and less numerous when compared to the pepsinogen granules of the frog esophageal glands.

Mannose-positive cells were also observed scattered throughout connective tissue of the mucosa and submucosal layers of the stomach of *R. icterica*, corresponding probably to mast cells, since they also exhibited alcianophilic reaction. Mast cells of lymph nodes as well as macrophage-like cells in the lung and in the liver exhibited mannose-specific binding sites [50].

Considering the gastrointestinal hormones, 5-HT, G, STT, glucagon, and CCK are the main hormones in mammals. 5-HT is an important neurotransmitter, able to stimulate contraction and vasodilatation of smooth muscle cells, displaying a strong regulatory role in digestive function [51]. These findings have established for the EC cells a promising research focus since EC cells are very sensitive to intestinal glucose, causing the 5-HT liberation. On the other hand, 5-HT activates neuronal receptors that stimulate gastric motility, inducing stomach emptying [52].

In anuran, different EC cell types were described in the stomach of larval phases, late metamorphosis, and adults. In tadpolesof *Rana temporaria*, EC cells were mainly located at the lining epithelium and occasionally found in glands [5, 53]. Villaro and coworkers [53] related the fact that in

Rana temporaria 5-HT and STT secreting cells are the most frequent endocrine cell types, occurring along the entire stomach, as it was observed for *R. icterica*. In addition, STT secreting cells were not detected. Previous report indicated that 5-HT secreting cells occurred in later phases of development (metamorphosis) of the stomach of *Alytes obstetricans* [54]. Although studies on bufonids are scarce, El-Salhy and coworkers [8] identified 5-HT secreting cells (5-HT cells), STT-secreting cells (D cells), G-secreting cells (G cells), glucagon secreting cells (A cells), and bombesin secreting cells (P cells) among the lining epithelium in gastric glands in *B. regularis*.

In *R. icterica*, the muscular layer showed similar pattern related to other amphibians as *R. catesbeiana* and *X. laevis* [51]. However, Norris [38] noticed that in *R. pipiens* the circular layer of muscularis is absent in lower portion of both fundic and pyloric regions.

This work showed that the stomach of *R. icterica* was similar to other anurans; moreover, 5-HT-IR cells occur among the lining epithelium and the mucous cells, occurring preferentially at the upper portion of the fundic glands. Considering glycoconjugates that contained α-, β-Gal, and α-GalNAc residues, they were also detected in the oxynticopeptic cells, what seems to be peculiar to this species.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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