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Neuroendocrine system of the digestive tract in *Rhamdia quelen* juvenile: An immunohistochemical study

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ABSTRACT

In this work, an immunohistochemical study was performed to determine the distribution and relative frequencies of some neuromodulators of the digestive tract of silver catfish (*Rhamdia quelen*). The digestive tract of silver catfish was divided into six portions; the oesophagus, stomach, intestine (ascendant, descendant and convoluted segments), and rectum. Immunohistochemical method using a pool of specific antisera against-gastrin, -cholecystokinin-8, -leu-enkephalin, -neuropeptide Y, -calcitonin gene-related peptide (CGRP), and -vasoactive intestinal peptide (VIP) was employed. Immunoreactivity to all antisera was identified in neuroendocrine cells (NECs) localized in the gut epithelium, although no reaction was observed in the oesophagus or stomach. The morphology of NECs immunopositive to each antibody was similar. They were slender in shape, with basally located nucleus, and their main axis perpendicular to the basement membrane. The number of NECs immunoreactive to all antisera was higher in the ascendant and descendant intestine, exhibiting a decreasing trend toward distal segments of the gut. In addition, immunoreactivity to CGRP and VIP was observed in the myenteric plexus and nerve fibers distributed in the mucosal, submucosal and muscular layers. The higher number of immunopositive NECs in the ascendant and descendant intestine may indicate the primary role of these segments in the control of food intake by means of orexigenic and anorexigenic peripheral signals.

1. Introduction

The silver catfish or South American catfish (*Rhamdia quelen*, Quoy and Gaimard, 1824), is a freshwater fish widely distributed in America from Mexico to Argentina (Silfvergrip, 1996). This species has omnivorous feeding habits, showing preference for fish, crustaceans, insects, plant remains and organic detritus (Gomes et al., 2000). *R. quelen* is considered a promising species for semi-intensive or intensive aquaculture in Brazil and Argentina since their larvae accept dry diets from early days of life with high survival rates and quick growth (Piaia and Radünz Neto, 1997; Cardoso et al., 2004; Hernández et al., 2009a).

In fishes, like in other vertebrates, digestive processes such as motility, secretion, absorption and immunity are modulated by the neuroendocrine system. The hypothalamus plays an important role in the regulation of several gastrointestinal functions, producing factors that stimulate (orexigenic) or inhibit (anorexigenic) food intake by integrating diverse peripheral signals (Lin et al., 2000; Volkoff et al., 2005).

These peripheral signals may come from: (1) the autonomic nervous system (sympathetic and parasympathetic), (2) the enteric nervous system, and (3) the diffuse neuroendocrine system (DNES) (Le Bail and Bœuf, 1997; Jensen, 2001; Buddington and Krogdahl, 2004).

The DNES of fishes shows similarities to its mammalian counterpart with regard to the regulatory processes. However, the DNES presents unique functional characteristics related to habitat (freshwater or saltwater), season, reproductive period or developmental stage (Buddington and Krogdahl, 2004).

Several studies have described the distribution and relative frequency of cells belonging to DNES located in the gastrointestinal tract (GIT) of fishes using immunohistochemical techniques (Domeneghini et al., 1999; Cinar and Diler, 2002; Bosi et al., 2004; Lee et al., 2004; Bermúdez et al., 2007; Vigliano et al., 2011). In catfishes, Bosi et al. (2006) analyzed the distribution of immunoreactive cells to only one neuropeptide (galanin) in the GIT of three species: *Ameiurus melas*, *Silurus glanis* and *Clarias gariepinus*.

Up to date, despite the potential of this species for aquaculture, no basic studies on the DNES of *R. quelen* have been performed. Therefore, the aim of this study was to detect the distribution and

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Table 1
List of primary antisera used in this study.

Polyclonal antibodies against	Working dilution	Incubation parameters	Source (code)
GAS	1:600	3 h at RT	Peninsula Labs (T-4347)
CCK-8	1:1000	3 h at RT	Peninsula Labs (T-4254)
leu-ENK	1:2000	3 h at RT	Peninsula Labs (T-4290)
NPY	1:1500	ON at 4 °C	Peninsula Labs (T-4454)
CGRP	1:800	ON at 4 °C	Peninsula Labs (T-4032)
VIP	1:400	ON at 4 °C	Peninsula Labs (T-4246)

CCK-8, cholecystokinin-8; CGRP, calcitonin gene-related peptide; GAS, gastrin; leu-ENK, leu-enkephalin; NPY, neuropeptide Y; ON, over night; RT, room temperature; VIP, vasoactive intestinal peptide.

relative frequency of some neuromodulators of the GIT of *R. quelen* by means of immunohistochemistry. These results contribute to a better understanding of the morphology and function of the gastrointestinal tract in *R. quelen*. The information obtained will be applied in future studies for the development of artificial diets, to optimize the intensive culture of this species.

2. Materials and methods

The immunohistochemical study was performed at Department of Veterinary Clinical Sciences, School of Veterinary, University of Santiago de Compostela, Lugo, Spain.

2.1. Fish

In this study, five healthy juvenile specimens of silver catfish without sex distinction (mean weight and standard length: 152 ± 18.60 g; 195 ± 11.25 mm, respectively) collected from a stream in the proximity of the town of Herlitzka (Corrientes, Argentina) were used. The fish were sacrificed by chilling on ice and severing the spinal cord. Thereafter, the oesophagus, stomach, intestine (ascendant, descendant and convoluted segments), and rectum were dissected out and cut into small pieces as was described by Hernández et al. (2009b).

2.2. Light microscopy and immunohistochemistry

Samples were fixed in Bouin's fluid (12 h) and embedded in paraffin wax after dehydration in a graded ethanol series. Microtome sections (1–3 μm thick) were collected on slides pre-treated with Vectabond (Vector Laboratories, CA), allowed to dry overnight and then dewaxed and hydrated. To assess digestive structures by light microscopy, sections were then stained with haematoxylin and eosin (H&E). For immunohistochemistry, unless otherwise stated, all incubations described here were performed at room temperature (22–25 °C) in a humid chamber, and all washing procedures consisted of three successive 5 min immersions in 0.1 M phosphate-buffered saline (PBS; 8 mM Na_2HPO_4, 3 mM NaH_2PO_4, 150 mM NaCl, 0.5% [v/v] Tween-20). Endogenous peroxidase activity was blocked by incubation in Peroxidase Blocking Reagent (DakoCytomation, Denmark) for 30 min, and after a rinse in PBS, the sections were treated with 3% skim milk powder for 15 min to block non-specific antibody binding. Subsequently, the samples were incubated with the primary polyclonal antibodies listed in Table 1, washed with PBS, and incubated for 30 min with anti-rabbit EnVision + System Labelled Polymer-HRP (DakoCytomation, CA). After further rinsing, the sections were finally developed using 3,3 diaminobenzidine tetrahydrochloride (DakoCytomation, CA) or Vector Vip (Vector Laboratories, CA), immersed in deionized water to stop the reaction, counterstained with haematoxylin, dehydrated, and coverslipped. In each series of stained sections, positive and negative controls were included to assess the specificity of the assay. Sections of pig intestine were used as positive controls. Negative control slides were sections in which the primary antibody was replaced by PBS.

Table 2
Regional distribution and intensity of immunoreaction in neuroendocrine cells in the digestive tract of juvenile *R. quelen*.

Polyclonal antibodies against	Segments of the gut			
	AI	DI	CI	R
GAS	+++	++	+	–
CCK-8	+++	++	+	–
leu-ENK	+++	+++	++	+
NPY	+++	++	+	–
CGRP	+	+	–	–
VIP	+	–	–	–

AI, ascendant intestine; DI, descendant intestine; CI, convoluted intestine; R, rectum. Abbreviations of antisera as in Table 1. Intensity of immunoreactions: (–), absent; (+), low; (++), medium; (+++), strong.

2.3. Quantitative analysis

Images were obtained using an Olympus BX-50 photomicroscope equipped with an Olympus DP-12 camera. Three digitized images were collected from each studied organ using a 200× enlargement and sections were examined. The number of immunoreactive neuroendocrine cells to each antiserum per analyzed segment was recorded and the intensity of immunoreaction was classified into the following categories: absent (–) or low (+), medium (++) and strong (+++) immunoreactivity.

3. Results

In this study, we determined the immunolocalization of gastrin (GAS-), cholecystokinin-8 (CCK-8-), leucine-enkephalin (leu-ENK-), neuropeptide Y (NPY-), CGRP- and VIP-like immunoreactivity in different cell types from the TGI of *R. quelen*.

3.1. Diffuse neuroendocrine system

In the epithelium, immunoreactivity to each antisera employed was observed in neuroendocrine cells (NECs) (Figs. 1 and 10). NECs were located among enterocytes in the epithelial layer through the gut. They were slender in shape, although wider in the zone occupied by the nucleus, and exhibited granular content in the supranuclear cytoplasm (Fig. 1, insets). In some cases, NECs showed a pyramidal outline with the base adjacent to the epithelial basement membrane. The nuclei of these cells were euchromatic, rounded or oval and located in a middle or basal position (Fig. 1, insets). NECs were observed in the ascendant, descendant and convoluted intestine as well as in the rectum, but never in the oesophagus or stomach. However, the type of neuropeptides identified in each segment of the gut differed greatly. In the ascendant intestine all neuropeptides assessed were identified in NECs. On the contrary, NECs situated in the rectal epithelium only immunoreacted with the antiserum against leu-ENK (Fig. 2). The absence of immunoreaction to CGRP and VIP antisera observed in the rectum was also seen in the convoluted intestine (Fig. 2). The intensity of immunoreaction and the number of immunoreactive-NECs to each antiserum showed wide variation in the alimentary canal. Both parameters exhibited a clear decreasing trend from cranial to caudal segments of the gut (Table 2 and Fig. 2). In addition, in each region, the number of leu-ENK-like immunoreactive NECs was always higher than those immunopositive for the other peptides. CGRP- and VIP-like NECs were observed in low numbers and mainly restricted to the ascendant intestine (Table 2 and Fig. 2).