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An immunohistochemical study of the gut neuroendocrine system in juvenile pejerrey *Odontesthes bonariensis* (Valenciennes)

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In this study, several neuropeptides were identified by immunohistochemistry in neuroendocrine cells (NEC) located in the gut epithelium and nerve cell bodies of the enteric nervous system of pejerrey *Odontesthes bonariensis*, a species that is a promising candidate for intensive aquaculture. The neuropeptides involved in orexigenic or anorexigenic action, *i.e.* gastrin, cholecystokinin-8, neuropeptide Y and calcitonin gene-related peptide (CGRP), displayed a significantly higher number of immunoreactive NECs in the anterior intestine, suggesting that this region of the gut plays an important role in the peripheral control of food intake. On the other hand, leu-enkephalin and vasoactive intestinal peptide (VIP), both associated with the modulation of the enteric immune system, showed no significant variations in the mean value of immunopositive NECs between the anterior and posterior intestine. This may indicate that their activity is required at a similar level along the entire gut. In addition, CGRP and VIP-immunoreactive neurons and nerve fibres were observed in the myenteric plexus, which might exert synergistic effects with the neuropeptides immunolocalized in NECs.

Key words: immunohistochemistry; intestine; myenteric nerve fibres; myenteric neurons; neuroendocrine cells; neuropeptides.

INTRODUCTION

In aquaculture, as in other forms of livestock rearing, nutrition is one of the most important factors for successful commercial production. Knowledge of gut morphology and physiology in fishes is, therefore, important in optimizing their nutrition and feeding in culture.

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The hypothalamus plays an important role in the regulation of food intake by integrating signals which come from two major peripheral systems, a short-term system and a long-term system (Jensen, 2001). The long-term system is modulated by leptin, a hormone that in fishes is synthesized mainly in the liver and, to a lesser extent, in the kidney, thymus and adipose tissue (Huising *et al*., 2006; Pfundt *et al*., 2009). Leptin reduces food intake by lowering the mRNA levels of neuropeptide Y (NPY) and increasing the expression of pro-opiomelanocortin A1 and A2 in the hypothalamus (Murashita *et al*., 2008). The short-term system is modulated in two ways, by the enteric nervous system and the diffuse neuroendocrine system (DNES). The DNES comprises various different cell types in the gut, which produce and secrete several neuropeptides with many effects on feeding habits (Jensen, 2001; Toni, 2004).

The control of food intake by gut DNES in mammals has been comprehensively reviewed (Valassi *et al*., 2008; Crespo *et al*., 2009; Hameed *et al*., 2009). Such information is not as available for fishes and, in many cases, it is difficult to extrapolate findings between species, in part due to high inter-species variation. Among the great diversity of neuropeptides described, gastrin (GAS), cholecystokinin (CCK-8), NPY, leu-enkephalin (leu-ENK), calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP), are especially important because they exert generalized actions which regulate digestive processes and feeding behaviour (López-Patiño *et al*., 1999; Jensen, 2001; Olsson & Holmgren, 2001; Martínez-Álvarez *et al*., 2009).

Gastrin and CCK-8 are anorexigenic neuropeptides located in neuroendocrine cells (NEC) in the gut of fishes (Ku *et al*., 2004; Volkoff *et al*., 2005; Bermúdez *et al*., 2007). Cholecystokinin is also expressed in the hypothalamus (Kurokawa *et al*., 2003) and exerts various gastrointestinal actions including gallbladder contraction, pancreatic enzyme secretion, stimulation of gastrointestinal motility and inhibition of gastric emptying (Jensen, 2001). Gastrin is secreted in response to food intake, regulating stomach secretion and motility. It could also be involved in growth of the gastric mucosal epithelium (Vigna, 2000).

Neuropeptide Y is a highly conserved orexigenic peptide that is mainly expressed by hypothalamic neurons (Jensen, 2001). López-Patiño *et al*. (1999) demonstrated that an increasing level of NPY enhances food intake in goldfish *Carassius auratus* (L. 1758), after short periods of fasting. The peptide is also found in NECs of the gut, although its presence in different regions varies among fishes (Cinar *et al*., 2006).

Leu-enkephalin is an opioid peptide derived from endorphins, it is also produced by NECs in fishes (Pan *et al*., 2000*a*) and plays a role in the regulation of inflammatory process (Radulovic *et al*., 1996; Dezfuli *et al*., 2004).

In the gut of teleosts, the peptide CGRP is found in NECs, as well as in neurons and nervous fibres in the submucosal and myenteric plexus (Cinar *et al*., 2006; Bermúdez *et al*., 2007). The CGRP has a profound inhibitory effect on intestinal motility (Shahbazi *et al*., 1998) and an anorexigenic effect (Martínez-Álvarez *et al*., 2009).

The VIP has been located in different regions of the gut, it is found in NECs as well as in nerve cell bodies and fibres in submucosal and muscular layers (Cinar *et al*., 2006). This peptide is involved in smooth muscle cell activity, both as an excitatory and an inhibitory neuropeptide (Olsson & Holmgren, 2001; Cinar & Diler, 2002). Olsson & Holmgren (2001) suggested that VIP regulates the secretions of the gut by inhibiting stomach secretion and stimulating secretions at the intestinal level. The

peptide also has an important function in the modulation of both innate and adaptive immune responses (Delgado *et al*., 2004).

Although the pejerrey *Odontesthes bonariensis* (Valenciennes 1835) is a promising species for intensive aquaculture in Argentina, no basic studies exist on its DNES, and phylogenetic distance makes it difficult to extrapolate knowledge on other species. In particular, *O. bonariensis*, in contrast to most other fishes, is a stomachless species.

Therefore, the aim of this study was to detect and assess the relative distribution of various neuropeptides in the gut of *O. bonariensis* by immunohistochemistry. These results contribute to a better understanding of the morphology and function of the *O. bonariensis* gut. The information gained will be applied in future studies into the development of artificial diets, to optimize the intensive culture of this species.

MATERIALS AND METHODS

FISH AND SAMPLING PROCEDURE

Ten healthy juvenile specimens of *O. bonariensis* [mass range 7·8–9·7 g; standard length (L_S) range 85–110 mm], of unknown sex and supplied by the Rosario Aquarium (Santa Fe, Argentina), were used. They were fed daily to satiation for 4 months with an artificial commercial diet (Truchina crumble, GEPSA Feeds; www.gepsa.com), composed of 47% total protein, 13% ethereal extract, 2% crude fibre, 10% humidity, 4–5% calcium and 1·7–2·7% phosphorus. After 1 day of fasting, the fish were sacrificed by anaesthetic overdose (100 mg l^{-1} benzocaine: Sigma; www.sigmaaldrich.com) and severance of the spinal cord. Thereafter, the anterior and posterior intestine were dissected out and cut into small pieces.

LIGHT MICROSCOPY AND IMMUNOHISTOCHEMISTRY

Intestine samples were fixed in Bouin's fluid for 12 h and embedded in paraffin wax. Sections (3–5 μm) were cut and placed on slides pretreated with Vectabond (Vector Laboratories; www.vectorlabs.com) and allowed to dry overnight, after which they were dewaxed and hydrated using standard histological techniques. To assess digestive structures by light microscopy, sections were then stained with haematoxylin and eosin (H&E).

For immunohistochemistry, unless otherwise stated, all incubations 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). Endogenous peroxidase activity was blocked by incubation in peroxidase blocking reagent (DakoCytomation; www.dako.com) for 30 min and, after a rinse in PBS, the sections were treated with 3% skimmed milk powder for 15 min to block non-specific antibody binding. Afterwards, the samples were briefly rinsed in PBS, incubated with the primary polyclonal antibody as indicated in Table I, washed with PBS and incubated for 30 min with anti-rabbit EnVision+ System Labelled Polymer-HRP (DakoCytomation; www.dakocytomation.com). After further rinsing, the sections were finally developed using 3,3 diaminobenzidine tetrahydrochloride (DakoCytomation) or Vector Vip Substrate (Vector Laboratories; www.vectorlabs.com), 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 swine gut were used as positive controls. Negative control slides were sections in which the primary antibody was replaced by PBS.

MEASUREMENTS AND STATISTICAL ANALYSIS

All samples were photographed under low-power magnification using a digital camera. In each section, the total number of immunopositive NECs for each antibody was determined in three sections for each neuropeptide. After this, the total epithelial area in each section

TABLE I. Antibodies employed in this study of *Odontesthes bonariensis*

Polyclonal antibodies against	Antibody working dilution	Incubation variables	Source (code)
GAS	1:600	3 h, RT	Peninsula Laboratory* (T-4347)
CCK-8	1:1000	3 h, RT	Peninsula Laboratory* (T-4254)
NPY	1:1500	ON, 4° C	Peninsula Laboratory* (T-4454)
leu-ENK	1:2000	3 h, RT	Peninsula Laboratory* (T-4290)
CGRP	1:800	ON, 4° C	Peninsula Laboratory* (T-4032)
VIP	1:400	ON, 4° C	Peninsula Laboratory* (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 (22–25° C); VIP, vasoactive intestinal peptide.
*www.penlabs.com.

was measured using the software Image J (1.42q in the public domain available from the National Institutes of Health; rsbweb.nih.gov/ij), and then the number of NECs mm^{-2} was calculated. Significant differences ($P < 0{\cdot}05$) between mean number of NECs in the anterior and posterior intestine for each antiserum and among different antibodies in each region were assessed by a *t*-test and one-way ANOVA with a Bonferroni *post hoc* test, respectively, using JMP software, Version 5.1.1 (SAS Institute Inc.; www.jmp.com).

RESULTS

All the primary antisera detected their specific neuropeptide in NECs, located in both the anterior and posterior intestine. The morphological features of NECs that were immunoreactive to each antiserum were similar [Fig. 1(a)–(f)]. NECs had their main axis perpendicular to the basement membrane and were slender in shape, with exception of the zone occupied by the nucleus. Some NECs had a triangular shape with their base situated over the basement membrane [Fig. 1(d), inset]. The nuclei of these cells were euchromatic, rounded or oval and were located in a middle or basal position. The entire cytoplasm of immunopositive NECs showed a diffuse and intense signal. Moreover, CGRP and VIP-immunoreactive structures were detected outside the epithelium, mainly in the muscle layer. Some neurons in the myenteric plexus exhibited a positive reaction to CGRP antiserum [Fig. 1(e), inset]; moreover, VIP-positive nerve fibres were observed at the same site surrounding nerve cell bodies [Fig. 1(f), inset].

Although all neuropeptides were identified throughout the gut of *O. bonariensis*, the mean number of NECs mm^{-2} varied between anterior and posterior regions. Significant differences in immunoreactive NECs were observed for GAS (*t*-test, $n = 10$, $P < 0{\cdot}001$), CCK-8 (*t*-test, $n = 10$, $P < 0{\cdot}001$), NPY (*t*-test, $n = 10$, $P < 0{\cdot}01$) and CGRP (*t*-test, $n = 10$, $P < 0{\cdot}001$), with higher values in the anterior than in the posterior intestine (Fig. 2). The leu-ENK- and VIP-immunopositive NEC numbers did not differ significantly between the two gut regions (Fig. 2).

As regards the main neuropeptides identified in the anterior intestine, GAS-immunoreactive NECs exhibited the highest number, significantly higher than CCK-8-,

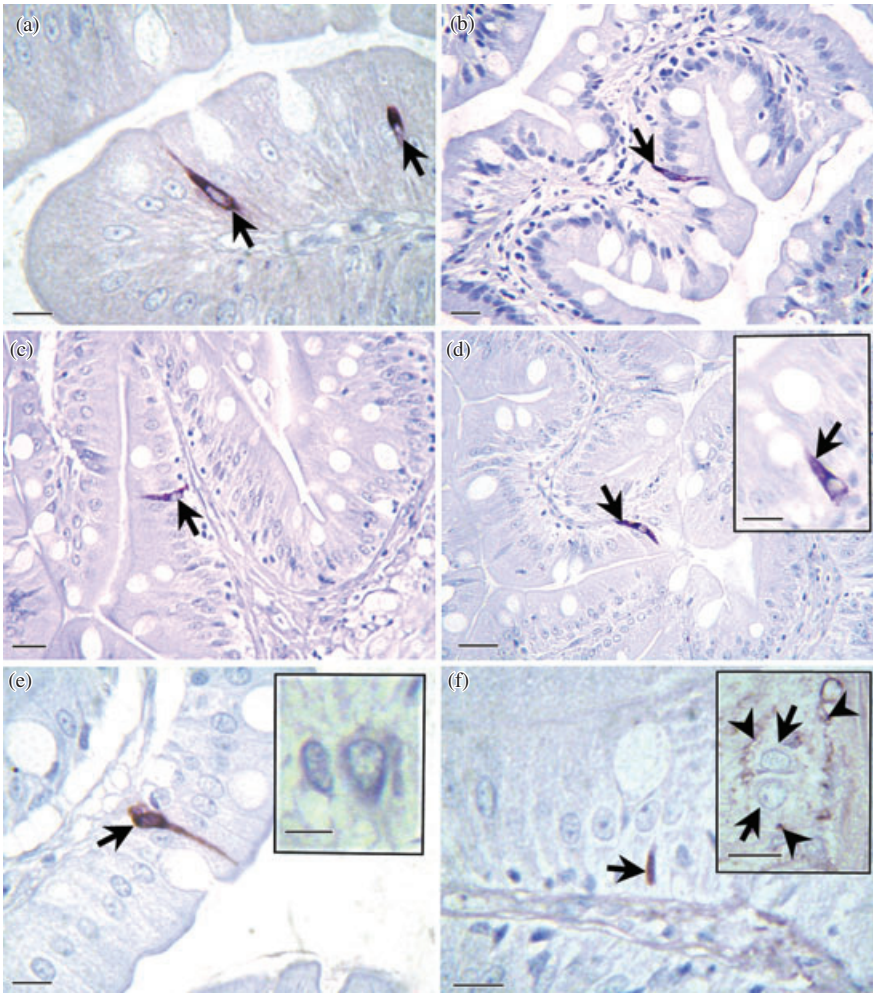

FIG. 1. (a) Two anti-gastrin immunoreactive neuroendocrine cells (⟶) in the anterior intestine of *Odontesthes bonariensis*. Scale bar = 10 μm. (b) A single neuroendocrine cell (⟶) positive to cholecystokinin-8 antiserum in posterior intestine. Scale bar = 10 μm. (c) Anti-neuropeptide Y immunopositive neuroendocrine cell (⟶) in the anterior intestine. Scale bar = 10 μm. (d) A neuroendocrine cell (⟶) in which a positive reaction to leu-enkephalin antibody is clearly seen in the anterior intestine. Scale bar = 20 μm. Inset: anti-leu-enkephalin immunoreactive neuroendocrine cell (⟶) showing its triangular shape in the epithelium of the posterior intestine. Scale bar = 10 μm. (e) A neuroendocrine cell (⟶) with a strong reaction to calcitonin gene-related peptide antiserum is observed in the anterior intestine. Scale bar = 10 μm. Inset: two nerve cell bodies immunopositive to calcitonin gene-related peptide in the myenteric plexus of the anterior intestine. Scale bar = 5 μm. (f) Single neuroendocrine cell (⟶) immunoreactive to anti-vasoactive intestinal peptide in the epithelium of the posterior intestine. Scale bar = 10 μm. Inset: nerve fibres (➤) immunopositive to vasoactive intestinal peptide antiserum surrounding two non-reactive neurons (⟶) in the myenteric plexus of the posterior intestine. Scale bar = 10 μm.

leu-ENK, CGRP and VIP-positive NECs (ANOVA, Bonferroni *post hoc* test, $n = 10$, $P < 0{\cdot}05$; Fig. 2). The mean number of NECs immunoreactive to NPY antibody in the anterior intestine was also high, significantly higher than leu-ENK-, CGRP- and

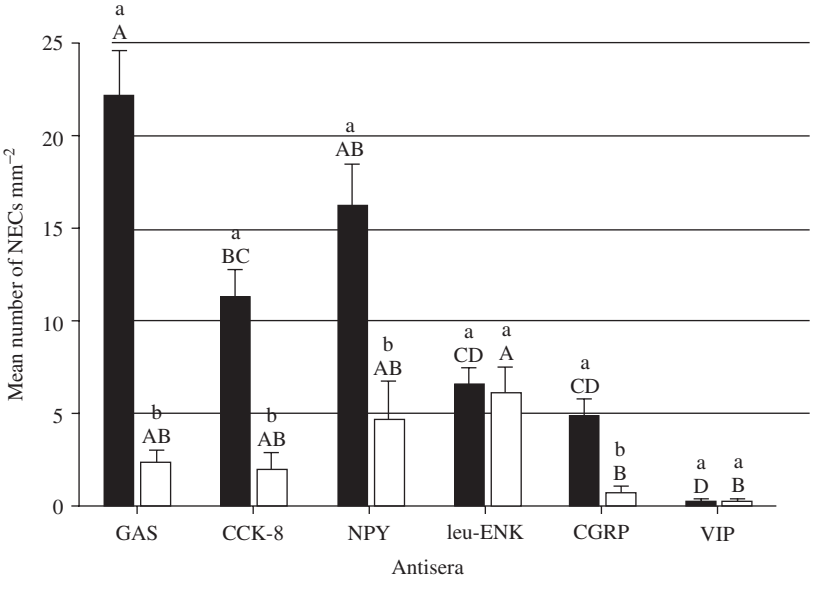

FIG. 2. Mean ± S.E. number of immunoreactive neuroendocrine cells mm^{-2} to each antiserum (see Table I) in the anterior (■) and posterior (□) intestine of *Odontesthes bonariensis*. Different lower case letters indicate significant differences between the anterior and posterior intestine for each antibody employed (*t*-test, $n = 10$, GAS and CCK-8, $P < 0{\cdot}001$; NPY, $P < 0{\cdot}01$; CGRP, $P < 0{\cdot}001$). Different upper case letters indicate significant differences among each antibody within the anterior or posterior intestine (ANOVA, Bonferroni *post hoc* test, $n = 10$, $P < 0{\cdot}05$).

VIP-positive NECs (ANOVA, Bonferroni *post hoc* test, $n = 10$, $P < 0{\cdot}05$; Fig. 2). Finally, the third most frequent neuropeptide observed was CCK-8, which had significantly higher numbers than VIP-immunopositive NECs (ANOVA, Bonferroni *post hoc* test, $n = 10$, $P < 0{\cdot}05$; Fig. 2). In the posterior region of the gut, the main neuropeptide in NECs was leu-ENK, which was significantly more numerous than CGRP- and VIP-immunoreactive NECs (ANOVA, Bonferroni *post hoc* test, $n = 10$, $P < 0{\cdot}05$; Fig. 2).

DISCUSSION

This study revealed the occurrence and distribution of six neuropeptides secreted by NECs in the gut of *O. bonariensis*. When these results are compared to existing studies on other species, they reveal that the presence, location and relative frequency of NECs immunoreactive to the neuropeptides differ greatly among fishes, presumably indicating marked interspecific variations in gastrointestinal physiology (al-Mahrouki & Youson, 1998; Domeneghini *et al*., 2000; Pan *et al*., 2000*a*, *b*; Lee *et al*., 2004; Cinar *et al*., 2006).

Gastrin and CCK are structurally related peptides with a common C-terminal sequence (Volkoff *et al*., 2005). In tetrapods, GAS is expressed in the antral mucosa of the stomach (Walsh, 1994) while, in fishes, the location of GAS-immunoreactive NECs varies among species. Some species such as northern snakehead *Channa*

argus (Cantor 1842) and yellow catfish *Pelteobagrus fulvidraco* (Richardson 1846) show GAS immunoreactivity only in the stomach (Pan *et al*., 2000*b*), whereas other species like Japanese flounder *Paralichthys olivaceus* (Temminck & Schlegel 1846) (Kurokawa *et al*., 2003) exhibit expression of GAS in the intestine. In addition, in the ricefield eel *Monopterus albus* (Zuiew 1793) a positive immunosignal in the oesophagal epithelium was reported (Pan *et al*., 2000*b*). This uncommon location for GAS seems to be frequent in cyprinids such as grass carp *Ctenopharyngodon idella* (Valenciennes 1844), black carp *Mylopharyngodon piceus* (Richardson 1846) and common carp *Cyprinus carpio* L. 1758, which display higher numbers of positive NECs in their foregut. In some cases, like silver carp *Hypophthalmichthys molitrix* (Valenciennes 1844), bighead carp *Hypophthalmichthys nobilis* (Richardson 1845), silver crucian carp *Carassius gibelio* (Bloch 1782) and bluntnose black bream *Megalobrama amblycephala* Yih 1955, the foregut is the only site of GAS immunoreactivity within the gut (Pan *et al*., 2000*a*). Despite the fact that *O. bonariensis* is a stomachless fish, GAS immunoreactivity was identified in NECs in both the anterior and posterior intestine, although the mean value of positive NECs was significantly higher in the anterior region. Gastrin is secreted in response to mechanical and chemical stimuli like the presence of proteins in the lumen of the gut after food intake, in mammals and fishes (Schubert & Makhlouf, 1992; Vigna, 2000). Bearing this in mind, the distribution of GAS immunoreactivity in *O. bonariensis* may indicate that the anterior intestine is the primary site for food digestion.

Cholecystokinin is an anorexigenic peptide that elicits a dose-dependent suppression of food intake (Himick & Peter, 1994; Volkoff *et al*., 2003) when secreted in response to a meal (Le Bail & Roeuf, 1997; Murashita *et al*., 2007). Cells immunoreactive to CCK antisera have been found in the nervous system, mainly in the hypothalamus and also in the gut (Jensen, 2001; Kurokawa *et al*., 2003). In the gut, species such as Korean aucha perch *Coreoperca herzi* Herzenstein 1896 (Lee *et al*., 2004) and Atlantic halibut *Hippoglossus hippoglossus* (L. 1758) (Kamisaka *et al*., 2001) show NECs immunopositive to CCK only in the anterior intestine. In brown trout *Salmo trutta* L. 1758 (Bosi *et al*., 2004), turbot *Psetta maxima* (L. 1758) (Bermúdez *et al*., 2007) and freshwater minnow *Zacco platypus* (Temminck & Schlegel 1846) (Ku *et al*., 2004), cells immunoreactive to CCK antisera were found throughout the gut but the highest number of positive cells was always in the anterior intestine, which is similar to the findings in *O. bonariensis*. Taking into account the biological activities of CCK-8 (Jensen, 2001), this pattern of distribution could indicate a primary role of the anterior intestine of *O. bonariensis* in modulation of gallbladder contraction and pancreatic enzyme secretion and in the stimulation of intestinal motility.

Neuropeptide Y exhibits a primary structure that is highly conserved from fishes to mammals, especially in its C-terminal dodecapeptide (Jensen, 2001). The peptide is widely expressed in the central nervous system of all vertebrates investigated (Cerdá-Reverter *et al*., 2000) and is mainly located in the hypothalamic neurons (López-Patiño *et al*., 1999). Like other neuropeptides, NPY is also found in the gut, with variations among species in its regional distribution. In the Australian bonytongue *Scleropages jardini* (Saville-Kent 1892), clown knifefish *Chitala chitala* (Hamilton 1822), elephantnose fish *Gnathonemus petersii* (Günther 1862) and longnose gar *Lepisosteus osseus* (L. 1758), NPY immunoreactivity was only found in NECs of the anterior intestine (Groff & Youson, 1997; al-Mahrouki & Youson, 1998) whereas in flower fish *Pseudophoxinus antalyae* Bogutskaya 1992 it was located in

the stomach and middle intestine (Cinar *et al*., 2006). By contrast, in the European eel *Anguilla anguilla* (L. 1758), reaction with NPY antiserum was only found in nerve fibres of both anterior and posterior intestine (Domeneghini *et al*., 2000) and, in the digestive tract of Atlantic salmon *Salmo salar* L. 1758, no expression of NPY was detected (Murashita *et al*., 2009). In *O. bonariensis*, NPY was immunolocalized in NECs of the anterior and posterior intestine, with a significantly higher relative number in the anterior. Since the main general effect of NPY is orexigenic, the higher number of NECs immunoreactive to NPY in the anterior intestine of *O. bonariensis* could indicate a role of this region as a primary source of signals to stimulate food intake in the absence of food at this site.

Leu-enkephalin is a pentapeptide which, in mammals, is mainly located in the central nervous system and in the gastrointestinal tract, with a presumptive role in the modulation of intestinal peristalsis (Lukiw, 2006). Another general function ascribed to leu-ENK is the modulation of inflammatory process (Radulovic *et al*., 1996). In the three-spined stickleback *Gasterosteus aculeatus* L. 1758 naturally infected with *Glugea anomala*, a significant variation in leu-ENK-immunoreactive nerve fibres was observed near to the parasites (Dezfuli *et al*., 2004). In *O. bonariensis*, no difference in leu-ENK immunoreactivity was observed between NECs of the anterior and posterior intestine, which could be related to the immunomodulatory action attributed to this peptide, which might be necessary at both locations.

The CGRP is widely distributed in both the central and peripheral nervous systems and in the gut of fishes and mammals (Van Rossum *et al*., 1997; Cinar *et al*., 2006; Bermúdez *et al*., 2007). The CGRP exerts a wide range of biological actions including neuromodulation, vasodilatation, reduction of food intake and gastrointestinal regulation (Van Rossum *et al*., 1997; Martínez-Álvarez *et al*., 2009). In relation to the last function, its inhibitory effect on intestinal muscle motility could be associated with its release *via* extrinsic or intrinsic sensory pathways, which mediate the peristaltic response to muscle stretch or mucosal stimulation, respectively (Grider, 1994; Shahbazi *et al*., 1998). As previously demonstrated in *P. maxima* (Bermúdez *et al*., 2007), CGRP immunoreactivity in *O. bonariensis* was found in both neurons and nerve fibres located mainly in muscle layers along the gut, plus in epithelial NECs, which were higher in number in the anterior intestine. On the contrary, in *A. anguilla* immunopositivity to CGRP was only observed in the myenteric plexus (Domeneghini *et al*., 2000) while in *P. antalyae* CGRP immunoreactivity was only detected in intestinal NECs (Cinar *et al*., 2006). The fact that CGRP and the other anorexigenic peptides analysed in this study, GAS and CCK-8, showed a significantly higher number of immunoreactive cells in the anterior intestine could indicate a synergistic action between them to inhibit food intake through satiety signals. Moreover, since the anterior intestine directly receives large prey items in this stomachless species, the higher number of NECs immunoreactive to CGRP antiserum at this location, in addition to the anti-CGRP immunoreactive nerve cell bodies and fibres along the entire gut, might also be associated with the modulation of muscle activity in response to mucosal stimulation and organ distension.

Vasoactive intestinal peptide has been detected by immunohistochemistry in several fish species although with great variations in immunoreactive cell types. Thus, in *P. antalyae*, positivity to VIP antiserum was found only in NECs and mainly those in the stomach (Cinar *et al*., 2006). Some species such as *Sander lucioperca* (L. 1758) and *P. maxima* exhibited VIP immunoreactivity in nerve cell bodies and fibres of

submucosal and muscular layers (Cinar & Diler, 2002; Bermúdez *et al*., 2007). In *Z. platypus* and *S. trutta*, no immunoreaction against VIP was observed (Bosi *et al*., 2004; Ku *et al*., 2004), although the absence of immunoreactivity reported by Bosi *et al*. (2004) in the latter species might be related to the physiological condition of the fish, because in parasitized *S. trutta* an increased positivity to VIP antibody has been reported (Dezfuli *et al*., 2000). In *O. bonariensis*, VIP-immunoreactive NECs were detected in both regions of the gut with no differences between them. Furthermore, immunoreactivity to VIP was also identified in nerve fibres in the myenteric plexus, surrounding nerve cell bodies that were immunonegative to VIP. This is similar to *A. anguilla*, suggesting that VIP-positive fibres are not of local origin, and thus they may play a regulatory role on intrinsic neurons of the gut (Domeneghini *et al*., 2000). In addition to its neuromodulatory function, VIP exerts a regulatory action on both innate and adaptive immune responses (Delgado *et al*., 2004). As was suggested in relation to leu-ENK distribution in the gut of *O. bonariensis*, the fact that NECs immunoreactive to VIP showed no differences between the anterior and posterior intestine could indicate that this peptide is also necessary at both locations for functions in local immunity.

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