Ghrelin and NUCB2/Nesfatin-1 Co-Localization With Digestive Enzymes in the Intestine of Pejerrey (*Odontesthes bonariensis*)

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ABSTRACT

Ghrelin (orexigenic) and nesfatin-1 (anorexigenic) are two peptides with opposing actions on food intake regulation and are mainly expressed in the hypothalamus and gut of mammals and fish. Both are involved in the regulation of a wide range of physiological processes in vertebrates, including metabolism, growth, and reproduction. However, the anatomical relationship between these peptides and the nutrient assimilation processes are not well understood. Thus, the aim of this work was to determine the localization of ghrelin, nesfatin-1, and several enzymes involved in the digestive process (lipoprotein lipase, aminopeptidase A, trypsin, and sucrase-isomaltase) in the intestine of pejerrey (*Odontesthes bonariensis*), a species with commercial importance in South America. We observed co-localization of ghrelin and nesfatin-1 in enteroendocrine cells, absorptive cells, and in cells of the lamina propia. Approximately half of the cells displaying ghrelin-like immunoreactivity co-localized the NUCB2/nesfatin-1-like signal. In addition, both peptides showed co-localization with lipoprotein lipase, aminopeptidase A, trypsin, or sucrase-isomaltase. All digestive enzymes except for aminopeptidase A and trypsin, showed high co-localization (68–88%) with both ghrelin-like and NUCB2/nesfatin-1-like signals in absorptive, enteroendocrine, and lamina propria cells. Together, our results provide immunohistochemical evidence supporting a role for both ghrelin and NUCB2/nesfatin-1 in the

Additional Supporting Information may be found in the online version of this article.

List of abbreviations: Ac = absorptive cell; EEc = enteroendocrine cell; Ep = Epithelium; Gc = Goblet cell; IHC = Immunohistochemistry; Lp = lamina propia; M = mucosa; SI = Sucrase-isomaltase

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Key words: digestive enzymes; ghrelin; intestine; nesfatin-1; pejerrey

Ghrelin and nesfatin-1 are two peptidyl regulators of metabolic processes in vertebrates. Ghrelin was discovered as the ligand for the growth hormone secretagogue receptor 1a (GHSR1a), capable of stimulating growth hormone release from the anterior pituitary (Kojima *et al.,* 1999). This hormone, which is mainly synthesized by the gut, is composed of 28 amino acids in mammals, and is released from its 117-amino acid precursor called preproghrelin. Ghrelin acts in the brain by stimulating food intake and regulating body weight and glucose metabolism, both in mammals and in fish (Cowley *et al.,* 2003; Cummings, 2006; Riley *et al.,* 2009; Jönsson, 2013). Besides, it has a role in some peripheral tissues, where it regulates, for instance, gut motility and gastric acid secretion, taste sensation, glucose metabolism (Müller *et al.,* 2015), brown fat thermogenesis, and stress and anxiety (Tschöp *et al.,* 2000; Kojima and Kangawa, 2005; Castañeda *et al.,* 2010; Al Massadi *et al.,* 2011; Sato *et al.,* 2012). In tilapia and goldfish (Unniappan *et al.,* 2002), ghrelin treatment increases food intake and stimulate lipogenesis and tissue fat deposition to promote a positive energy status (Kaiya *et al.,* 2008; Peddu *et al.,* 2009; Kang *et al.,* 2011). Moreover, ghrelin regulates carbohydrate metabolism (Jönsson, 2013) and glucose homeostasis, regulating the expression and translocation of glucose transporters in goldfish (Blanco *et al.,* 2017a).

The gut shows the highest expression of *preproghrelin* mRNA in both mammals (Menzies *et al.,* 2014) and fish (Sánchez-Bretaño *et al.,* 2015; Volkoff, 2015). Besides, the presence of the ghrelin peptide has been reported in the intestine of several fish species, including rainbow trout (Sakata *et al.,* 2004), Japanese eel (Kaiya *et al.,* 2006), goldfish (Kerbel and Unniappan, 2012), and zebrafish (Olsson *et al.,* 2008). In addition, ghrelin mRNA expression was detected in a wide range of organs and tissues, including muscle, liver, spleen, and white adipose tissue (Unniappan and Peter, 2005; Manning *et al.,* 2008; Feng *et al.,* 2013; Bertucci *et al.,* 2016; Blanco *et al.,* 2016).

Nesfatin-1 was discovered as an N-terminal cleavage product of the protein nucleobindin-2 (NUCB2), which is encoded by the *nucb2* gene (Oh-I *et al.,* 2006). When administered either centrally or peripherally, nesfatin-1 reduces feeding in mammals (Stengel *et al.,* 2009; Goebel *et al.,* 2011; Mortazavi *et al.,* 2015) as well as in fish (Gonzalez *et al.,* 2010; Kerbel and Unniappan, 2012; Lin *et al.,* 2014). Additionally, nesfatin-1 modulates cardiovascular function, glucose metabolism, and reproduction in mammals (Goebel *et al.,* 2009; García-Galiano *et al.,* 2010a, 2010b; Scotece *et al.,* 2014; Stengel, 2015). Tissue distribution of *nucb2/nesfatin-1* mRNAs has been reported both in mammals (Goebel-Stengel and Wang, 2013; Mohan and Unniappan, 2013; Kim *et al.,* 2014) and fish (Lin *et al.,* 2014; Hatef *et al.,* 2015). In both classes, the highest *nucb2/nesfatin-1* mRNA expression was found in the gastrointestinal tract, which is even higher compared to its expression found in the brain, where this peptide plays its anorexigenic role (Prinz and Stengel, 2016). While *nucb2/nesfatin*-1 distribution has been reported by PCR techniques (Lin *et al.,* 2014; Hatef *et al.,* 2015; Blanco *et al.,* 2016), only few reports are available on the anatomical distribution of this peptide in fish (Gonzalez *et al.,* 2012, 2010; Hatef *et al.,* 2015).

It is well known that many gastrointestinal peptides, including ghrelin (Peeters, 2005; Schubert, 2010), participate in the regulation of gastric functions and digestive processes in mammals. Ghrelin has been shown to modulate the expression of pepsin in the stomach and duodenum of rats (Warzecha *et al.,* 2006), the activity of pepsin in gastric mucosal cells of piglets (Du *et al.,* 2016) and the activity of lysosomal hydrolases in rabbits (Witek *et al.,* 2005). While ghrelin seems to be an important modulator of gastrointestinal functions in mammals, whether this function is conserved in fish is poorly understood (Blanco *et al.,* 2017b). Some studies have demonstrated that ghrelin contributes to gastric emptying and gastric acid secretory capacity in rats (Tian *et al.,* 2014; Yang *et al.,* 2017). To date, no data is available on gastric functions of nesfatin-1 in fish.

The pejerrey, (*Odontesthes bonariensis*) is an atherinid fish inhabiting the continental waters of South America (Gómez-Requeni *et al.,* 2012). Despite the high potential of pejerrey as an aquaculture species, its farming presents several difficulties related to low growth rates in captivity (Grosman and González Castelain, 1995; Miranda and Somoza, 2001). The main limitation to promoting rapid growth rates under rearing conditions arises from the lack of knowledge on the nutritional requirements of larvae and juveniles of pejerrey. The natural food for pejerrey is mainly zooplankton, although teleost, mollusks, crustaceans, and insects are also consumed. Besides, the digestive tract of pejerrey lacks a "true" well-developed stomach and has a length equal or shorter than the body length, suggesting an omnivorous feeding regime (Boschi and Fuster, 1959). The results obtained from studies investigating peptide and protein involvement in food intake regulation and digestive processes will improve the knowledge of pejerrey biology and physiology and could eventually help enhance yields from cultures. The aim of this work was to characterize the localization of ghrelin and nesfatin-1 in the intestine of pejerrey, in relation to several digestive enzymes (lipoprotein lipase, aminopeptidase A, trypsin, and sucrase-isomaltase).

MATERIALS AND METHODS

Fish

Experiments were carried out on adult pejerrey (3 years) that were bred in the INTECH facilities. After hatching, pejerrey fry were kept in 140 L open flow-

through water tanks (3,000 fry/tank), under a 12:12 hr light-dark photoperiod, at a controlled temperature of 18°C and a dissolved oxygen concentration of 8 ± 1 ppm. Fry were fed with nauplii of Artemia sp. four times per day. After 30 days, pejerrey fry were transferred to 300 L open flow-through water system tanks (300 fry/tank) under the same environmental conditions and fed with a commercial starter feed (Shulet bebe®, Shulet, Argentina). After 6 months, juveniles of pejerrey were transferred and kept in 20,000 L open flow-through water system tanks (100 fish/tank) under a natural photoperiod. The water temperature was 18 ± 2°C, the dissolved oxygen concentration was 8 ± 1 ppm and the water salinity was 15 g/L. Fish were fed two times per day with a commercial diet (Shulet Adulto®, Shulet, Argentina). All experimental procedures involving fish were strictly in accordance with the UFAW Handbook on the Care and Management of Laboratory Animals and the IIB-INTECH internal regulations.

Immunohistochemistry

Pejerrey, fasted for 24 hr, were sacrificed by spinal transection, and then perfused in 4% paraformaldehyde (PFA) diluted in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Since pejerrey is a stomach-less species, sections of 3 cm from the middle gut (located caudal to the esophagus and the anterior intestine engrossment) were removed and post-fixed for 3 hr in a fresh 4% PFA solution. Tissues were processed (dehydrated and embedded in paraffin) at the INTECH histological processing service, CONICET-UNSAM. Paraffin blocks were then sectioned at 7-μm thickness using a rotary microtome, and transverse sections were mounted onto Superfrost™ slides (Thermo Fisher Scientific, Waltham, MA). The protocol for immunohistochemistry (IHC) was performed as previously described with minor modifications (Diotel *et al.,* 2011). Briefly, sections were deparaffinized with xylene (twice, for 5 min each) and rehydrated through a series of graded ethanol (100% for 2 min twice, and once in 95%, 85%, 70%, 50%, and 30%, for 30 sec each step). Then, sections were washed with 0.85% NaCl (twice for 10 min) and 0.1 M PBS (once for 5 min) and blocked in 0.1 M PBS containing 0.5% of bovine serum albumin (45 min). Afterwards, sections were incubated for 16 hr with a primary antibody against ghrelin, or a primary antibody against nesfatin-1, or with a mixture of primary antibody against ghrelin and primary antibody against nesfatin-1 (rabbit anti-mouse/rat ghrelin antibody, Catalog # H-031-31, Phoenix Pharmaceuticals, Burlingame, CA; mouse anti-rat nesfatin-1, Catalog # ALX-804-854-C100, Enzo Life Sciences, Brockville, ON, Canada) both diluted to 1:200, at room temperature. The following day, sections were washed twice in PBS and once in 0.2% Triton PBS for 10 min each and subsequently incubated with the following secondary antibodies: Texas Red anti-rabbit IgG (Vector Laboratories, Burlington, ON, Canada) and FITC anti-mouse IgG (Abcam, Toronto, ON, Canada) for ghrelin and nesfatin-1 detection respectively, both in a 1:2,000 dilution, for 1 hr at room temperature. To determine if ghrelin and nesfatin-1 co-localize in intestinal cells, a mixture of both secondary antibodies was applied. Ghrelin and nesfatin-1 antibodies detect both precursor (preproghrelin or NUCB2, respectively) and processed ghrelin or nesfatin-1. Therefore, positive immunostaining or immunoreactivity (IR) is referred to as ghrelin-like IR and NUCB2/nesfatin-1-like IR. We used two different approaches for the evaluation of specific staining. In the first approach, a separate set of negative control slides was incubated with the secondary antibodies only (no primary antibody control). In the second approach, preabsorption controls were carried out. Briefly, mixtures of either goldfish synthetic ghrelin or nesfatin-1 and the corresponding primary antibodies at a 1:10 ratio were incubated overnight (Ramesh *et al.,* 2015; Bertucci *et al.,* 2017b). Slides were then incubated with this preabsorption mixture. All primary and secondary antibodies were diluted in antibody diluent reagent (Dako, Mississauga, ON, Canada). After incubation with secondary antibodies, slides were washed twice in PBS and once in 0.2% Triton PBS for 5 min. Sections were mounted using VECTASHIELD Mounting Medium containing 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlington, ON, Canada). Slides were then assessed using a Nikon Eclipse Ti-Inverted fluorescence microscope (Nikon Instruments, Melville, NY), and images were captured using a Nikon DS-Qi1 MC camera. Images were analyzed using the NiS Elements Basic Research Imaging Software on a Lenovo ThinkPad workstation. The studies were done using sections from 3 fish (15–20 sections per fish).

Relative Distribution of Ghrelin, Nesfatin-1, and Digestive Enzymes

To determine whether ghrelin and/or nesfatin-1 co-localize with the digestive enzymes (sucrase-isomaltose; aminopeptidase A; trypsin and lipoprotein lipase), sections of intestine were incubated as described above with a mixture of primary antibody against ghrelin (mouse anti-rat ghrelin, Catalog # ab57222, Abcam, Toronto, ON, Canada) or nesfatin-1 (mouse anti-rat nesfatin-1, Catalog # ALX-804-854-C100, Enzo Life Sciences, Brockville, ON, Canada) and primary antibody against one of the four studied digestive enzymes (rabbit anti-human sucrase-isomaltase, Catalog # ab98872; rabbit anti-human aminopeptidase A, Catalog # ab109775; rabbit anti-human trypsin, Catalog # ab200997; rabbit anti-human lipoprotein lipase, Catalog # ab137821; all from Abcam, Toronto, ON, Canada). Each digestive enzyme antibody was diluted 1:200, at room temperature. Since heterologous antibodies were used in this work, positive immunostaining or immunoreactivity (IR) is referred to as sucrase-isomaltase-like IR, aminopeptidase A-like IR, trypsin-like IR and lipoprotein lipase-like IR. All these antibodies were previously used in goldfish (Bertucci *et al.,* 2017b; Blanco *et al.,* 2017b). Western blot analysis using total proteins of pejerrey intestine was conducted to confirm the specificity of each antibody (Supporting Information Fig. S1). All antibodies show bands at the expected protein mass.

Quantification of Immunoreactive Cells

Quantification of immunopositive cells in the intestine for each peptide (ghrelin-like IR or NUCB2/nesfatin-1-like IR) and each digestive enzyme (sucrase-isomaltase IR, aminopeptidase A-like IR, trypsin-like IR or lipoprotein lipase-like IR) was performed in three independent sections (each from three different fish). The average number of cells showing signal in an area of 200 μm × 150 μm

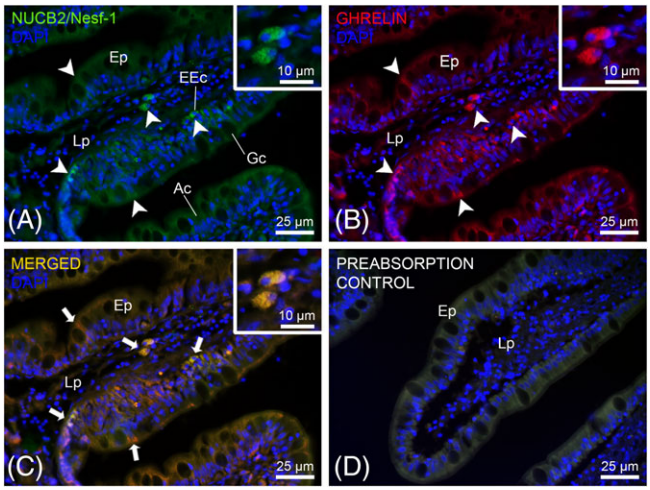

Fig. 1. Ghrelin-like and NUCB2/nesfatin-1-like immunoreactivity in pejerrey intestine. NUCB2/nesfatin-1-like signal (green, A); ghrelin-like signal (red, B); co-localization of both peptides (yellow, C); and preabsorption control (D). Blue staining corresponds to the cellular nucleus (DAPI). Arrowheads indicate cells with immunoreactivity signal from NUCB2/nesfatin-1-like **(A)** or ghrelin-like **(B)**. Arrows indicate cells in which both peptides co-localize **(C)**. Ep: Epithelium. Lp: lamina propia. EEc: enteroendocrine cell. Gc: Goblet cell. Ac: absorptive cell. Scale bars (µm) are indicated in each panel.

from each intestinal section was calculated, and this number is expressed in the corresponding figure legend. The set of values were plotted in pie charts to show the relative abundance of either ghrelin or NUCB2/nestfatin-1 positive cells, and immunoreactive cells for each digestive enzyme.

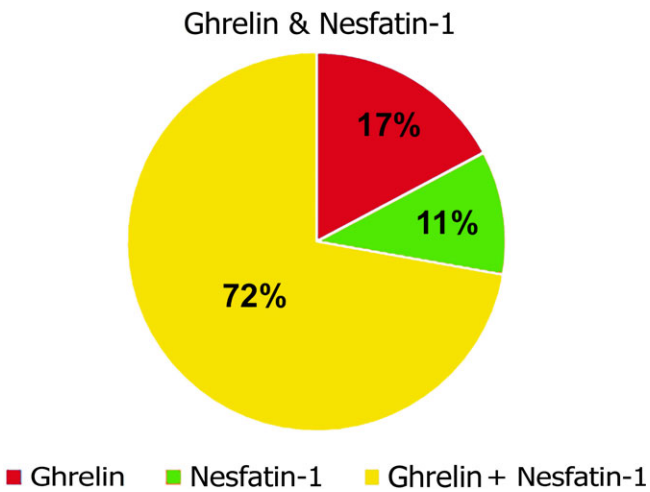

Fig. 2. Percentage of ghrelin-like and NUCB2/nesfatin-1-like expressing cells in the pejerrey intestine. The percentage of cells with ghrelin-like (red, average number of cells showing signal = 35 ± 8) or NUCB2/nesfatin-1-like (green, average number of cells showing signal = 21 ± 3) or both immunoreactive signals (yellow, average number of cells showing signal = 145 ± 16) was calculated relative to the total number of labeled cells in an area of 200 µM × 150 µM from an intestine section. N = 3 intestine areas from three different fish.

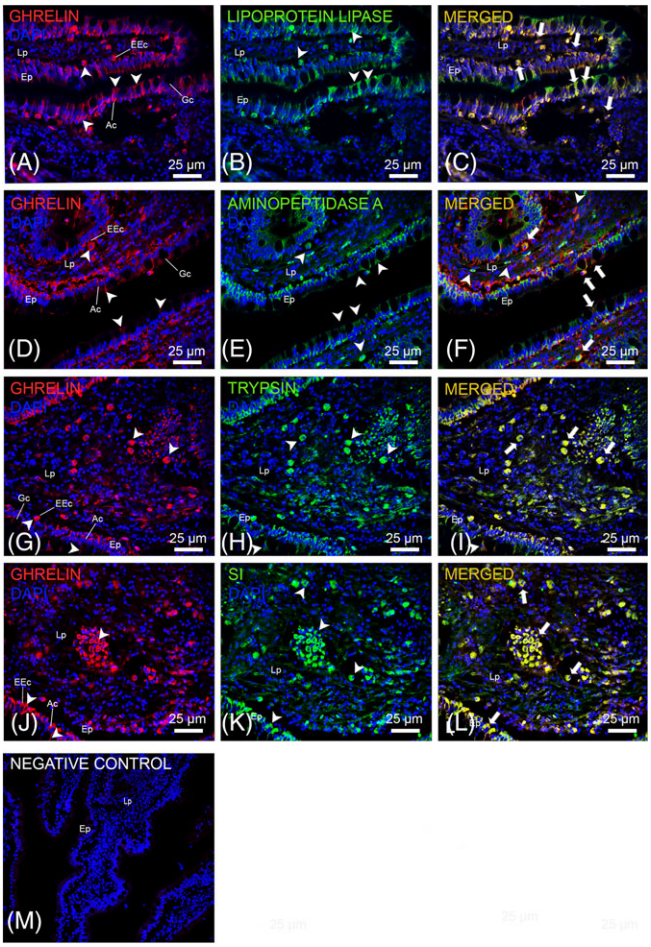

Fig. 3. Immunostaining for ghrelin and digestive enzymes in the pejerrey intestine. Ghrelin-like signal is shown in red (**A**, **D**, **G**, **J**), while digestive enzymes are shown in green: lipoprotein lipase-like (**B**); aminopeptidase A-like (**E**); trypsin-like (**H**); sucrose-isomaltase-like (**K**). Cells co-expressing ghrelin and lipoprotein lipase-like (**C**); aminopeptidase A-like (**F**); trypsin-like (**I**); or sucrose-isomaltase-like (**L**) are shown in yellow. Negative control carried out without the incubation step of anti-nesfatin-1 antibody and anti-ghrelin antibody (**M**). Blue staining corresponds to the cellular nucleus (DAPI). Arrowheads indicate cells with immunoreactivity signal from ghrelin-like or each digestive enzyme. Arrows indicate cells in which both targets co-localize. Ep: Epithelium. Lp: lamina propia. EEc: enteroendocrine cell. Gc: Goblet cell. Ac: absorptive cell. Scale bars (µm) are indicated in each panel.

RESULTS

Ghrelin and Nesfatin-1 Co-Localize in Pejerrey Intestine

As indicated in Figures 1, 3, and 5, epithelial cells are located close to the intestinal lumen, forming a monolayer where their nuclei are compactly grouped in the inner side of the epithelium, while cells located deeper in the intestinal villi belong to the lamina propia. Enteroendocrine cells (EEc) that produce enteric hormones are found in the basal part of the intestinal epithelium. NUCB2/nesfatin-1-like IR was mainly observed in EEc and absorptive cells (Ac) from the epithelium, and in some cells from the lamina propia

TABLE 1. Percent of cells with ghrelin; nesfatin-1; and ghrelin + nesfatin-1 signal

	Percent	SE
Ghrelin	17	4
Nesfatin-1	11	2
Ghrelin + Nesfatin-1	72	8

SE: standard error. N = 3 sections from three different fish.

TABLE 2. Percent of cells with ghrelin; aminopeptidase; and ghrelin + aminopeptidase signal

	Percent	SE
Ghrelin	28	3
Aminopeptidase	44	15
Ghrelin + Aminopeptidase	29	12

SE: standard error. N = 3 sections from three different fish.

TABLE 3. Percent of cells with ghrelin; isomaltase; and ghrelin + isomaltase signal

	Percent	SE
Ghrelin	11	2
Isomaltase	9	1
Ghrelin + Isomaltase	79	4

SE: standard error. N = 3 sections from three different fish.

TABLE 4. Percent of cells with ghrelin; lipase; and ghrelin + lipase signal

	Percent	SE
Ghrelin	8	1
Lipase	9	1
Ghrelin + Lipase	83	12

SE: standard error. N = 3 sections from three different fish.

(Fig. 1A). On the other hand, ghrelin-like signal was mainly observed in Ac and in some EEc from the epithelium and in some cells from the lamina propia (Fig. 1B). Several of these intestinal cells from either the epithelium or lamina propia co-expressed both ghrelin-like and NUCB2/nesfatin-1-like signals in their cytoplasms (Fig. 1C). Preabsorption controls did not result in IR signal (Fig. 1D). Quantification of the two cell populations shows that ghrelin-like- and NUCB2/nesfatin-1-like -positive cells are almost equally abundant within the pejerrey intestine (Fig. 2). Furthermore, 72% of cells expressing either ghrelin-like signal or nesfatin-1-like signal co-express both substances (Fig. 2, Table 1).

TABLE 5. Percent of cells with ghrelin; trypsin; and ghrelin + trypsin signal

	Percent	SE
Ghrelin	6	1
Trypsin	8	1
Ghrelin + Trypsin	86	5

SE: standard error. N = 3 sections from three different fish.

Ghrelin and Nesfatin-1 Co-Localize with Digestive Enzymes in the Intestine of Pejerrey

Ghrelin-like and digestive enzymes-like IR within the pejerrey intestine is shown in Figure 3 and quantitative data are presented in Figure 4 and Tables 2–5. Ghrelin-like IR was found to abundantly co-localize with lipoprotein lipase-like IR in cells located both in the epithelium and in the lamina propia (Fig. 3C). The percentage of cells expressing either ghrelin-like or lipoprotein-lipase-like signals and show co-localization of both substances was around 85% (Fig. 4A, Table 4). Intestinal cells containing aminopeptidase A-like IR were mainly observed in the epithelium, although some cells from the lamina propia yielded positive signal (Fig. 3E). Co-localization of this enzyme-like IR with ghrelin-like IR was restricted to few EEc in the epithelium (Fig. 3F). Quantification shows that only one third (29%) of cells expressing aminopeptidase A-like or ghrelin-like signal also presents co-localization of both substances (Fig. 4B, Table 2). Trypsin-like IR was widely distributed in the epithelium and lamina propia from the pejerrey intestine (Fig. 3H). A high percent of cells expressing ghrelin-like or trypsin-like IR also show co-localization of both substances (86%) in lamina propia and in EEc from the epithelium (Figs. 3I and 4C and Table 5). Finally, a strong signal for sucrase-isomaltase-like IR was

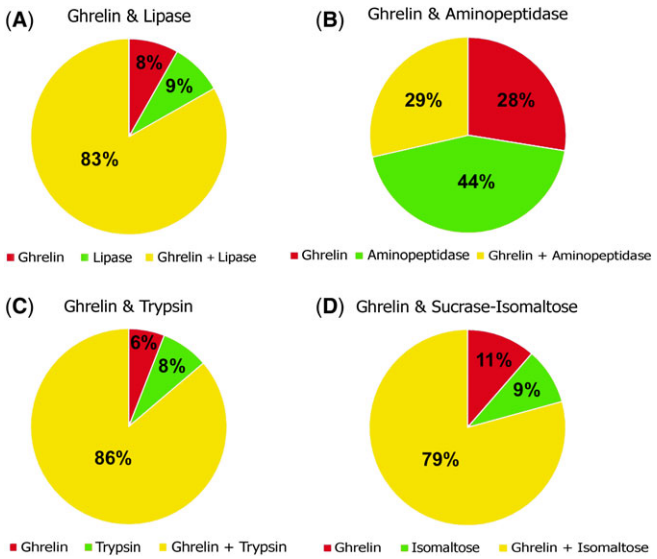

Fig. 4. Percentages of co-localization of ghrelin-like with the digestive enzymes lipase-like **(A)**; aminopeptidase A-like **(B)**; trypsin-like **(C)**; and sucrose-isomaltase-like **(D)**. The red portion of the graph represents the percentage of cells with ghrelin-like signal relative to the total number of cells with signal in an area of 200 μM × 150 μM from an intestine section. Average numbers of cells showing ghrelin signal = 15 ± 2 **(A)**; 27 ± 3 **(B)**; 10 ± 2 **(C)**; 18 ± 3 **(D)**. The green portion of the graph represents the percentage of cells with digestive enzyme signal relative to the total number of cells with signal in an area of 200 μM × 150 μM from an intestine section. Average numbers of cells showing digestive enzyme signal = 16 ± 1 **(A)**; 43 ± 14 **(B)**; 13 ± 2 **(C)**; 15 ± 2 **(D)**. The yellow portion of the graph represents the percentage of cells with both signals (ghrelin-like + digestive enzyme-like) relative to the total number of cells with signal in an area of 200 μM × 150 μM from an intestine section. Average number of cells showing co-localization signal = 145 ± 22 **(A)**; 28 ± 12 **(B)**; 146 ± 8 **(C)**; 130 ± 5 **(D)**. N = 3 intestine areas from three different fish.

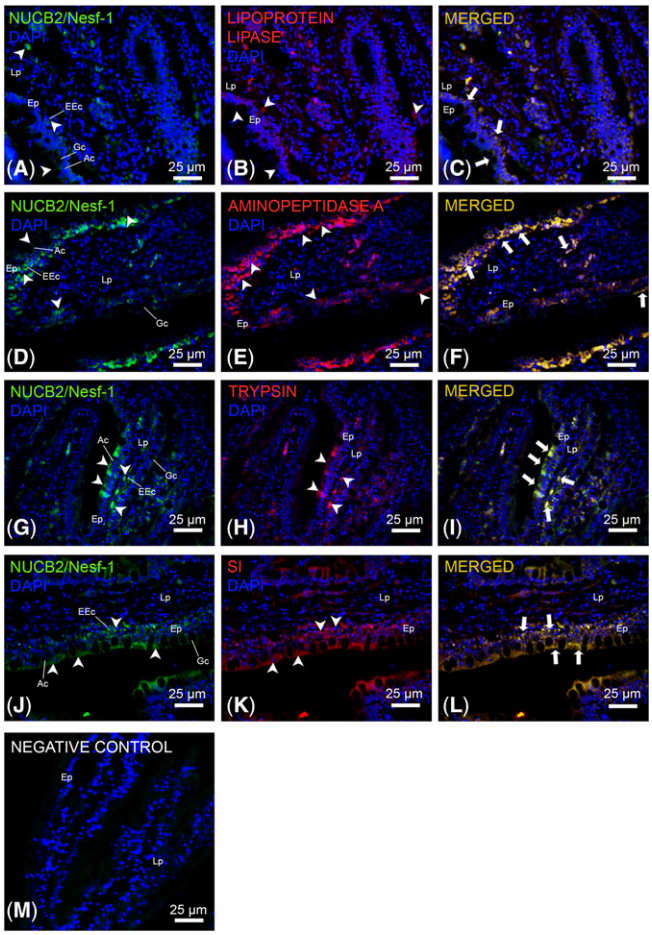

Fig. 5. Co-localization of NUCB2/nesfatin-1-like with digestive enzymes in the pejerrey intestine. Lipoprotein lipase-like (**A**, **B**, **C**); aminopeptidase A-like (**D**, **E**, **F**); trypsin-like (**G**, **H**, **I**); sucrose-isomaltase-like (**J**, **K**, **L**). Negative controls were carried out without the incubation step of anti-nesfatin-1 antibody and without anti-ghrelin antibody (**M**). The NUCB2/nesfatin-1-like signal is shown in green, the signal of the digestive enzymes is shown in red color, while the yellow signal corresponds to the presence of both peptides. Blue staining corresponds to the cellular nucleus (DAPI). Arrowheads indicate cells with an immunoreactivity signal from NUCB2/nesfatin-1-like. Complete arrows indicate cells in which both proteins co-localize. Ep: Epithelium. Lp: lamina propia. EEc: enteroendocrine cell. Gc: Goblet cell. Ac: absorptive cell. Scale bars (µm) are indicated in each image.

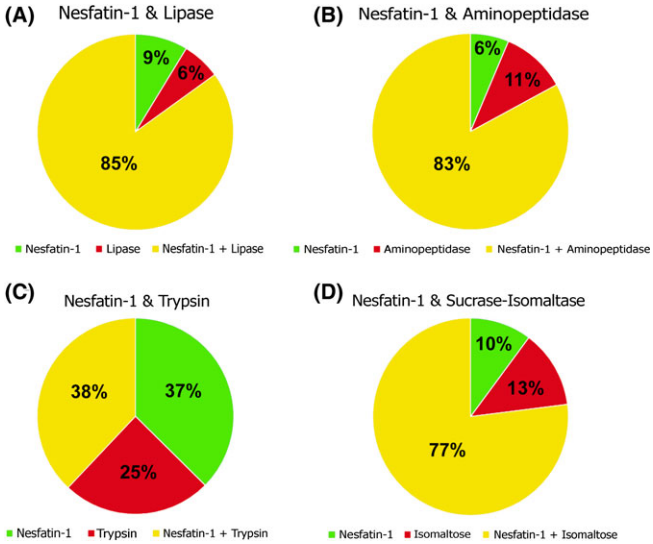

Fig. 6. Percentages of co-localization of NUCB2/nesfatin-1-like with the digestive enzymes lipoprotein lipase-like **(A)**; aminopeptidase A-like **(B)**; trypsin-like **(C)**; and sucrose-isomaltase-like **(D)**. The green portion of the graph represents the percentage of cells with NUCB2/nesfatin-1-like signal relative to the total number of cells with signal in an area of 200 µM × 150 µM from an intestine section. Average numbers of cells showing nucb2/nesfatin-1 signal = 14 ± 8 **(A)**; 8 ± 4 **(B)**; 35 ± 18 **(C)**; 18 ± 4 **(D)**. The red portion of the graph represents the percentage of cells with digestive enzyme signal relative to the total number of cells with signal in an area of 200 µM × 150 µM from an intestine section. Average numbers of cells showing digestive enzyme signal = 10 ± 4 **(A)**; 14 ± 6 **(B)**; 23 ± 18 **(C)**; 22 ± 11 **(D)**. The yellow portion of the graph represents the percentage of cells with both signals (NUCB2/nesfatin-1-like + digestive enzyme-like) relative to the total number of cells with signal in an area of 200 µM × 150 µM from an intestine section. Average number of cells showing co-localization = 142 ± 12 **(A)**; 113 ± 28 **(B)**; 35 ± 0.5 **(C)**; 136 ± 30 **(D)**. N = 3 intestine areas from three different fish.

TABLE 6. Percent of cells with nesfatin-1; aminopeptidase; and nesfatin-1 + aminopeptidase signal

	Percent	SE
Nesfatin-1	6	3
Aminopeptidase	11	4
Nesfatin-1 + Aminopeptidase	83	21

SE: standard error. N = 3 sections from three different fish.

detected in the pejerrey intestine, especially in Ac from the epithelium (Fig. 3K). Sucrase-isomaltase-like signal was also observed in clusters of cells from the lamina propia. Around 80% of cells with ghrelin-like IR or sucrase-isomaltase-like IR showed co-localization of both substances (Figs. 3L and 4D and Table 3).

NUCB2/nesfatin-1-like IR was found to co-localize with lipoprotein lipase-like IR, aminopeptidase A-like IR, sucrase-isomaltase-like IR and trypsin-like IR in the pejerrey intestine (Fig. 5). Co-localization of NUCB2/nesfatin-1-like IR with lipoprotein lipase-like IR, aminopeptidase A-like IR or trypsin-like IR was mainly restricted to EEc and some cells from the lamina propia (Fig. 5C,F,I). The percentage of cells showing NUCB2/nesfatin-1-like IR or IR from each digestive enzyme and co-localization of both substances was: 85%, 83%, and 38%, respectively (Fig. 6A–C, Tables 6, 8, 9). The co-localization of NUCB2/nesfatin-1-like IR with sucrase-isomaltase-like IR was found in EEc as well as in Ac from the epithelium (Fig. 5L). The percent of cells with NUCB2/nesfatin-1-like

TABLE 7. Percent of cells with nesfatin-1; isomaltase; and nesfatin-1 + isomaltase signal

	Percent	SE
Nesfatin-1	10	2
Isomaltase	13	6
Nesfatin-1 + Isomaltase	77	17

SE: standard error. N = 3 sections from three different fish.

TABLE 8. Percent of cells with nesfatin-1; lipase; and nesfatin-1 + lipase signal

	Percent	SE
Nesfatin-1	9	5
Lipase	6	3
Nesfatin-1 + Lipase	85	7

SE: standard error. N = 3 sections from three different fish.

TABLE 9. Percent of cells with nesfatin-1; trypsin; and nesfatin-1 + trypsin signal

	Percent	SE
Nesfatin-1	37	19
Trypsin	25	19
Nesfatin-1 + Trypsin	38	1

SE: standard error. N = 3 sections from three different fish.

or sucrase-isomaltase-like IR that also showed co-localization of both peptides was 77% (Fig. 6D, Table 7).

DISCUSSION

In this study, we found that a high percentage of cells co-localize both ghrelin and NUCB2/nesfatin-1 in the intestine of pejerrey. In mammals, it was demonstrated that gastric X/A-like cells release both peptides (Stengel *et al.,* 2010; Kerbel and Unniappan, 2012; Mohan *et al.,* 2014), which are located in different pools of vesicles (Stengel *et al.,* 2010). The presence of both orexigenic and anorexigenic peptides in the same cell highlights the importance of that kind of cell as a food intake regulator, able to enhance or inhibit the food intake depending on external signals (such as nutrients and other food intake-regulating peptides). Following this idea, our data suggest a role of intestinal cells in the regulation of food intake and energy expenditure in pejerrey (McGowan and Bloom, 2007; Parker *et al.,* 2014). Furthermore, it was previously demonstrated that dietary macronutrients modulate the mRNA and protein expression of ghrelin and nesfatin-1 *in vivo* (Blanco *et al.,* 2016) and *in vitro* (Bertucci *et al.,* 2017a) in fish. The presence of both peptides in EEc reported here suggests that macronutrients could regulate the release of ghrelin and nesfatin-1 from intestinal cells in pejerrey. Nevertheless, this hypothesis should be tested in future research. Additionally, the presence and co-localization of ghrelin and nesfatin-1 in EEc could be related to important functions that both peptides play in mammals, such as regulation of gastrointestinal motility (Atsuchi *et al.,* 2010) and/or acid secretion (Masuda *et al.,* 2000), working in an endocrine and/or paracrine manner.

Ghrelin-like and nesfatin-1-like signals are also abundant in epithelial Ac. These cells play a major role in nutrient absorption and release of enzymes that contribute to nutrient degradation. Therefore, to gain a better understanding about the range of physiologic functions attributed to both peptides, their co-localization with several digestive enzymes in the intestine was explored. Digestive enzymes were found predominantly located in Ac and EEc, which is in accordance with the fact that these enzymes are attached to the brush border membrane or secreted into the lumen. However, it was also observed in the lamina propia of the pejerrey intestine. The presence of digestive enzymes in deep portions of the mucosa has been reported previously in the porcine small intestine (Gonzalez *et al.,* 2013), in the goldfish intestine (Blanco *et al.,* 2017b) and in the snow trout (Mir and Channa, 2010). The localization of lipoprotein lipase in the lamina propia could be due to its presence in lymph spaces, blood vessels and blood capillaries (Mir and Channa, 2010), which could be associated with the biological function of this enzyme in blood. Trypsin presence in mesenteric veins and its transport from the lumen into the circulation have been reported in mammals (Borges *et al*., 1995, 2003). The trypsin-like signal found in the lamina propia of pejerrey could point to a similar situation in fish. The nature of the presence of sucrase-isomaltase and aminopeptidase in the lamina propia should be addressed in future research.

Lipoprotein lipase is a glycoprotein with a key role in lipid metabolism. This enzyme hydrolyzes triglycerides from circulating chylomicrons and lipoproteins releasing free fatty acids to be utilized by peripheral tissues (Auwerx *et al.,* 1992). To perform this task, lipoprotein lipase is secreted to the interstitial space and transferred to the luminal surface of endothelial cells (Auwerx *et al.,* 1992). In fish, the expression of lipoprotein lipase was found in several tissues (Liang *et al.,* 2002; Lindberg and Olivecrona, 2002), while this enzyme activity was determined in adipose tissue, red and white muscles, heart, brain, and vitellogenic ovaries of rainbow trout (Black *et al.,* 1983; Lindberg and Olivecrona, 2002). In addition, several studies have suggested that ghrelin is involved in the regulation of lipid metabolism (Barazzoni *et al.,* 2005; Kaiya *et al.,* 2008; Stengel *et al.,* 2010; Kang *et al.,* 2011; Müller *et al.,* 2015). Therefore, its co-localization with lipoprotein lipase could be considered as another evidence supporting this role, perhaps acting in an autocrine manner. On the other hand, the role of nesfatin-1 in regulating hepatic lipid metabolism and peripheral lipid accumulation in mammals has been demonstrated, since chronic infusion of nesfatin-1 reduced plasma triglycerides in mice fed a normal or high fat diet, respectively (Yin *et al.,* 2015). The co-localization of nesfatin-1 with lipoprotein lipase in intestinal cells suggests an autocrine role of this peptide in lipid metabolism in fish as well, although this hypothesis should be further investigated.

Aminopeptidase A is a 109 kDa homodimeric zinc-metallopeptidase that catalyzes the cleavage of glutamatic and aspartatic amino acid residues from the N-terminus of polypeptides (Georgopoulou *et al.,* 1985). Trypsin is a serine protease found in the digestive system of many vertebrates (Georgopoulou *et al.,* 1985). In the intestine, trypsin catalyzes protein hydrolysis, breaking them down into smaller peptides. In a previous work from our group, it was demonstrated that ghrelin co-localizes and modifies the expression of aminopeptidase A and trypsin in goldfish (Blanco *et al.,* 2017b). To the best of our knowledge, this is the first report showing co-localization of nesfatin-1 with either trypsin or aminopeptidase A. Considering the ghrelin effect on trypsin and aminopeptidase A gene and protein expression (Blanco *et al.,* 2017b) and the tight relation between ghrelin and nesfatin-1 activities (Kerbel and Unniappan, 2012; Stengel *et al.,* 2013, 2010), we hypothesize that nesfatin-1 could be involved in the regulation of protein degradation. Moreover, we demonstrated that the essential amino acid

for fish L-tryptophan, can modify ghrelin and NUCB2/nesfatin-1 gene and protein expression *in vitro* (Bertucci *et al.,* 2017b). These results, together with the co-localization found in the present work, support the notion that both ghrelin and nesfatin-1 have a role in controlling protein catabolism. Further research must be done to confirm this idea. The percentage of co-localization of aminopeptidase A with ghrelin was around 25%, that is, 1 out of 4 cells expressing ghrelin or aminopeptidase A also co-express both. This percent is lower than the values for co-localization of ghrelin with the other digestive enzymes, or than the percentage of co-localization of nesfatin-1 with aminopeptidase A, which was around 85%. This could point to a major role for nesfatin-1 in aminopeptidase A regulation.

The enzyme sucrase-isomaltase is a glucosidase located on the brush border of the apical membranes of Ac in the small intestine of mammals (Brunner *et al.,* 1979). It is a type II transmembrane glycoprotein that digests dietary carbohydrates, such as starch and isomaltose (Sjöström *et al.,* 1980). The role of carbohydrates in fish nutrition is not completely clear, but in omnivorous fish they are more relevant (Wilson, 1994). It has been shown that ghrelin and nesfatin-1 play a key role in the carbohydrate metabolism in fish (Riley *et al.,* 2009; Delporte, 2013; Mortazavi *et al.,* 2015; Ramesh *et al.,* 2015; Stengel, 2015; Blanco *et al.,* 2017a). Therefore, the high percentage of co-localization of ghrelin and nesfatin-1 with the digestive enzyme sucrase-isomaltase found in this work suggest another level of involvement of both peptides in carbohydrate metabolism.

In conclusion, we present evidence for the presence and co-localization of ghrelin and nesfatin-1 in pejerrey intestine. Moreover, the co-localization of ghrelin or nesfatin-1 with digestive enzymes in the pejerrey intestine reported here could expand our knowledge on the role of both peptides in nutrient absorption in fish.

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