# Orexins and the receptor OX2R in the gastroenteric apparatus of two teleostean species: Dicentrarchus labrax and Carassius auratus

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# Orexins and Receptor OX2R in the Gastroenteric Apparatus of Two Teleostean Species: *Dicentrarchus Labrax* and *Carassius Auratus*

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ABSTRACT

Orexin A and B peptides and the receptor OX2R were studied in sea bass and goldfish gastroenteric tract by immunoblotting combined with densitometric analysis using NIH Image J software and immunohistochemical techniques. These teleost species present a different gut organization and diverse feeding habits. Immunoblotting experiments showed one band of 16 kDa corresponding to prepro-orexin, and one band of 38 kDa corresponding to the OX2R receptor. Immunohistochemical localization of OXA and OXB was observed in the enteric nervous system throughout the gastroenteric tract of both species. OXA and OXB immunoreactive cells were found in the gastric and intestinal regions of sea bass, and were mainly found in the basal region of folds in intestinal bulb, and in the midgut and hindgut of goldfish. The distribution of OX2R was mainly detected in the mucosa of the gastroenteric tract of sea bass and goldfish. This distribution suggests an endocrine action of OXA and OXB in the gastrointestinal tract as well as involvement in the peripheral control of food intake and digestive processes in both species. This study might also serve to determine the productive factors in breeding and as a baseline for future experimental studies on the regulation of the gastroenteric functions in non-mammalian vertebrates. Anat Rec, 00:000–000, 2016. © 2016 Wiley Periodicals, Inc.

**Key words: Orexins; sea bass; goldfish; gastroenteric apparatus**

## INTRODUCTION

Orexin A (OXA) and orexin B (OXB) are two neuropeptides widely localized in the hypothalamic feeding centers and in the peripheral organs of the gastroenteric apparatus of vertebrates (Sakurai et al., 1998; Kirchgessner, 2002; Lòpez et al., 2009; Matsuda et al., 2009). Like in mammals, in fish orexins are generated from a

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common precursor prepro-peptide (Sakurai et al., 1998; Kaslin et al., 2004). Orexin sequences ranging from mammals (human, mouse, rat, pig, dog; Dyer et al., 1999; Hungs et al., 2001; Sakurai et al., 1998), amphibians (*Xenopus laevis*, Shibahara et al., 1999), birds (chicken, zebrafinch; Ohkubo et al., 2002) to teleosts (goldfish, zebrafish, cod, stickleback, medaka, pufferfish, Alvarez and Sutcliffe, 2002; Kaslin et al., 2004; Xu and Volkoff, 2007) have been identified through either molecular cloning or bioinformatic studies. Among teleosts, the structure of the OXB sequence is more conserved than OXA (Kaslin et al., 2004; Xu and Volkoff, 2007; Wong et al., 2011). Genes encoding pre-pro-orexins have been identified in fish species, such as zebrafish (Kaslin et al., 2004), goldfish (Wong et al., 2011), atlantic cod (Xu and Volkoff, 2007), winter flouder (Buckley et al., 2010), orange-spotted grouper (Yan et al., 2011), *Nile tilapia* (Chen et al., 2011), pupperfish (Alvarez and Sutcliffe, 2002) and stickleback (Wong et al., 2011). Sequence conservation of orexins between fish, human and mouse ranges from 34 to 52% (Kaslin, et al. 2004; Xu and Volkoff, 2007; Buckley et al., 2010).

The G protein-coupled receptors OXR1 and OXR2 bind orexins with different selectivity: OXR1 binds only to OXA, whereas OXR2 has affinity to both OXA and OXB (Sakurai et al., 1998). Vertebrate OX1R and OX2R are also highly conserved, both show 64% identity with the human forms (Wong et al., 2011). Only OX2R gene has been identified and isolated in chicken, xenopus and zebrafish genomes (Ohkubo et al., 2003; Wong et al., 2011).

In terrestrial and aquatic vertebrates, orexins regulate energy homeostasis and circadian functions interacting with a variety of neurotransmitters/hormones in the brain and peripheral tissues (Kirchgessner, 2002; Volkoff et al., 2010). In particular, the existence of a brain-gut network of orexin-containing cells appears to promote the sleep-wake cycle and locomotor behaviour, energy metabolism, drinking and feeding-associated processes (Kirchgessner, 2002; Kukkonen et al., 2002; Matsuda et al., 2011).

In fish, the role of an active orexin system has been discussed in various and sometimes conflicting physiological and pharmacological studies performed in goldfish and zebrafish (Volkoff et al., 1999; Faraco et al., 2006; Nakamachi et al., 2006; Miura et al., 2007). In goldfish central injection of both OXA and OXB stimulates appetite and food consumption, with a more potent action for OXA (Volkoff et al., 1999). In zebrafish, the neuronal population expressing orexins regulates sleep and wake (Prober et al., 2006; Yokogawa et al., 2007; Elbaz et al., 2012).

Orexins and their receptors are extensively expressed in the gastroenteric apparatus of mammals as described by immunohistochemical, PCR and *in situ* hybridization studies (Kirchgessner, 2002; Nakabayashi et al., 2003; Korczynsky et al., 2006; Heinonen et al., 2008; Romański and Goździewska-Harłajczuk, 2009). In contrast, little is known about orexin mRNA and protein expression in the gastroenteric tract of fish. In particular, some studies showed low but indicative RT-PCR signals of orexin mRNA expression in the stomach and gut of atlantic cod, winter flounder and orange-spotted grouper (Xu and Volkoff, 2007; Buckley et al., 2010; Yan et al., 2011). The present survey aims to study the expression and localization of prepro-orexin (PPO), orexin A (OXA), orexin B (OXB) and orexin receptor (OX2R) in the gastroenteric tract of two teleostean species, sea bass and goldfish. These teleost species have developed different strategies for food intake, a different adaptative morphological organization of the digestive apparatus, and belong to different aquatic environments: *Carassius auratus* (goldfish), a phyto omnivorous and stomachless fresh water teleost, and *Dicentrarchus labrax* (sea bass), a predator and carnivorous saltwater stomach-containing teleost. In addition, data on the localization of other neuropeptides involved in the regulation of food intake are already available in the digestive tract of the two fish species (Arcamone et al., 2009; Russo et al., 2011).

## MATERIALS AND METHODS

### Animals

The study was carried out on 8 fasted goldfish (*Carassius auratus*), a freshwater teleost purchased from a fish import company dealer (Carmars. a.s.), and on 8 fasted sea bass (*Dicentrarchus labrax*), a marine teleost, purchased from a local hatchery (Acquacoltura Acquamarina - Villa Literno, Italy). Animals were anesthetized and euthanized in a 0.1% solution of ethyl 3-aminobenzoatemethane sulfonate (MS 222; Sigma, St. Louis, MO; A-5040). Their gastroenteric apparatus was entirely and quickly dissected in segments along the cranial/caudal axes and internal blood coaguli and debris were washed off by a physiological solution. The sea bass stomach was subdivided into cardial, blind sac, pyloric and caecal regions and the intestine into midgut and hindgut. From the goldfish intestinal bulb (proximal portion of midgut homologous to the gastric region of sea bass), midgut and hindgut (for anatomical remarks, see Arcamone et al., 2009) were dissected. Of each species, 4 samples were frozen at −80°C for biochemical analysis, and 4 were fixed in Bouin's fluid (12-18 h at room temperature, RT), for histological and immunohistochemical analyses.

### Protein Extraction and Western Blotting

Frozen samples were homogenized in buffer (consisting of 50 mMTris–HCl, pH 7.0, 150 mMNaCl, 2% Triton, 5 mM EDTA, 10mg/ml leupeptin, 0.1 U/ml aprotinin, 1 mM PMSF) using an Ultra-Turrax homogenizer and centrifuged at 16,000g for 20 min at 4°C. Aliquots of supernatant were subjected to SDS–PAGE analysis using 4% to 12% Bis-Trisgels (NuPAGE, Invitrogen). After separation, the proteins were electrophoretically transferred to nitrocellulose membrane with the iBlot transfer system (Invitrogen). Then the membranes were blocked in TBS-T buffer (150 mM NaCl, 20 mM TrisHCl pH 7.4, 0.1% Tween-20) containing 5 g 100 mL-1 milk for 1 h at RT. The blots were then incubated overnight with primary antibody anti-prepro-orexin (code AB3096, Millipore Corporation, Billerica, MA, USA), produced in rabbit, and diluted 1:1000 in TBS-T and containing 2.5% milk. After three washes in TBS-T, the membranes were incubated with secondary antibody, horseradish peroxidase conjugated anti-rabbit (code sc-2004; 1:10000; Santa Cruz Biotechnology Inc., CA USA) in TBS-T for 1 h and visualized by ECL. After a stripping procedure, the membranes were reprobed with the orexin receptor R-2 (code sc-8074; 1:1000, Santa Cruz Biotechnology Inc., CA USA) or anti-actin antibodies (code A4700; 1:1000; Sigma; St. Louis, MO). Secondary antibodies for the visualization of the

orexin receptor R 2 and β-actin were horseradish peroxidase conjugated anti-goat (code 305-035-003; 1:1000, Immuno Research) and anti-mouse (code sc-2005; 1:1000, Santa Cruz Biotechnology Inc., CA USA), respectively. Proteins were visualized with the ECL Advanced Western blotting detection kit (Amersham) on C-Digit System. To monitor loading of gel lanes, the same blots were stripped and reprobed using an anti-actin monoclonal antibody (code A4700; 1:1000, Sigma; St. Louis, MO) as primary antibody. Homogenate of rat brain was employed as positive control. The intensities of the protein bands were quantified by densitometry analysis using NIH Image J software (NIH, National Institute of Health, Bethesda, MD, USA) (http://imagej.nih.gov/ij/). In particular, the relative signals from each sample were determined by comparing the intensities of the protein (prepro-orexin and orexin receptor R2) bands with those obtained from β-actin, considering that the same blot membrane was stripped and reprobed against the orexin receptor R 2 and β-actin, respectively. All experiments were repeated in triplicate, and mean intensities ± standard deviations were calculated.

### Immunohistochemistry

The samples were taken quickly, stretched and fixed by immersion in Bouin's fixative (12-24h) at RT. After washing, the samples were processed for paraffin embedding, and transverse sections of 7 μm were cut. Then they were immunohistochemically stained using the avidin-biotin (ABC) method. The sections were treated with citrate buffer 0.01 M, pH 6.0, heated in a microwave oven by two cycles of 5 min at maximal power to retrieve the antigen. Endogenous peroxidase activity was blocked with 0.3% $H_2O_2$ for 20 min at RT. The sections were incubated with primary antisera, diluted with normal rabbit serum (NRS; code S-1000; Vector Lab. Inc.; 1:5 in PBS containing 0.2% Triton X-100™, code 9002-93-1; Sigma, St. Louis, MO, pH 7.4) in a moist dark room overnight at 4°C. Orexin A and B primary antibodies (orexin-A(C-19): sc8070; orexin-B (C19): sc-8071; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:1000; the receptor OX2R (sc-8074, Santa Cruz Biotech.) was diluted 1:1500. All the primary antibodies employed recognized the C-terminal of the protein. In particular, the epitope for orexin A and orexin B was mapped between the aa 50–100 of the orexin A of human origin (O43612), and the antibody for the orexin receptor R 2 recognized the region C20, mapping the epitope among the last 50 aa at the C-terminal of the orexin receptor R 2 of human origin. A biotinylated RAG IgG (code 111-000-120; Jackson Imm. Lab.; West Grove, Pa, USA; 1:200) was utilized as secondary antiserum, diluted with PBS containing 0.2% Triton X-100™ (code 9002-93-1; Sigma, St. Louis, MO) and 4,5% NRS. Sections were rinsed three times in PBS, and Vectastainavidin-biotin reagent (code PK-6100; Vector Lab. Inc., CA, USA; 30 min at RT) was employed. Finally, after three rinses in PBS, sections were incubated in a solution of 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (code D5905; Sigma, St. Louis, MO) in 15 ml Tris buffer (0.5 M; pH 7.6), containing 0.03% $H_2O_2$ to reveal the immunostaining. The images were acquired using a Nikon Eclipse 90*i* microscope equipped with a Nikon DS F1c digital camera.

### Controls

The specificity of immunohistochemical staining was tested in repeated trials as follows: (1) previous absorption of each primary antiserum with an excess of the relative peptide (100 mg of peptide/ml of diluted antiserum; OXA/sc-8070p; OXB/sc8071p; OX2R/sc-8074p; Santa Cruz); (2) substitution of primary antibody or anti-rabbit IgG by PBS or non immune serum (negative controls); (3) use of rat duodenum, as positive controls.

## RESULTS

### Western Blot

The expression of both prepro-orexin (PPO) and OX2R in sea bass and goldfish along the gastrointestinal tract (GI) was evaluated by Western blotting (Fig. 1a, a'). In both species, the polyclonal antibody against PPO showed one band of approximately 16 kDa, and that against OX2R of about 38 kDa (OX2R), according to the range previously reported (Johren et al., 2001) in rat brain (used as a positive control). These results were normalized against β-actin and analysed by densitometry (Fig. 1b, b'). In sea bass, PPO and OX2R were widely distributed in all examined tracts, albeit with slight differences among the tissues (Fig. 1a). Fig. 1b illustrates results from densitometry, showing lower levels of OXR2 than those observed from PPO (medium between tissues PPO = 0.846 vs OXR2 = 0.224). As for sea bass, PPO and OX2R in goldfish were expressed widely in all tissues, although the levels of expression of the proteins calculated by densitometry remained similar (PPO = 0.769 vs OXR2 = 0.585).

### Immunohistochemistry

***OXA and OXB - sea bass.*** OXA and OXB immunoreactive cells were found in gastric and intestinal regions, either in the epithelium or in the nervous plexuses. In the cardial mucosal folds, round or oval OXA and more elongated OXB immunoreactive cells (Fig. 2a) were sparsely detected. Intense OXA immunoreactive myenteric nerve cell bodies (Fig. 2b) and thin bundles of immunoreactive nerve fibers in the cardial muscular layers (Fig. 2b) were also observed. In the blind sac, OXA (Fig. 2c) and OXB immunoreactive cells, showing basal and apical cytoplasmic processes, were frequently found in the epithelium at the base of the folds. Weak OXA and OXB immunoreactive myenteric nerve cell bodies of blind sac were seen. Moreover, in this tract, some OXA and OXB immunoreactive nerve fibers were identified in the submucosa and around the vessels of the muscular layers. In the pyloric region, OXA (Fig. 2d) and OXB immunoreactive cells, showing intense immunoreactive apical and/or basal processes, were observed in the epithelial folds. A discrete presence of OXA and OXB (Fig. 2e) immunoreactive nerve fibers was detected in the muscular layers and in the pyloric submucous plexus. In the caeca and in the midgut, many OXA (Fig. 2f) and OXB immunoreactive cells were found in the epithelium of the intestinal folds. Sometimes, epithelial elongated cells (Fig. 2f) were characterized by a very thin apical positive cytoplasmic process, and an intensely positive short basal process. In the mucosa of hindgut OXA and OXB immunoreactive cells were rare

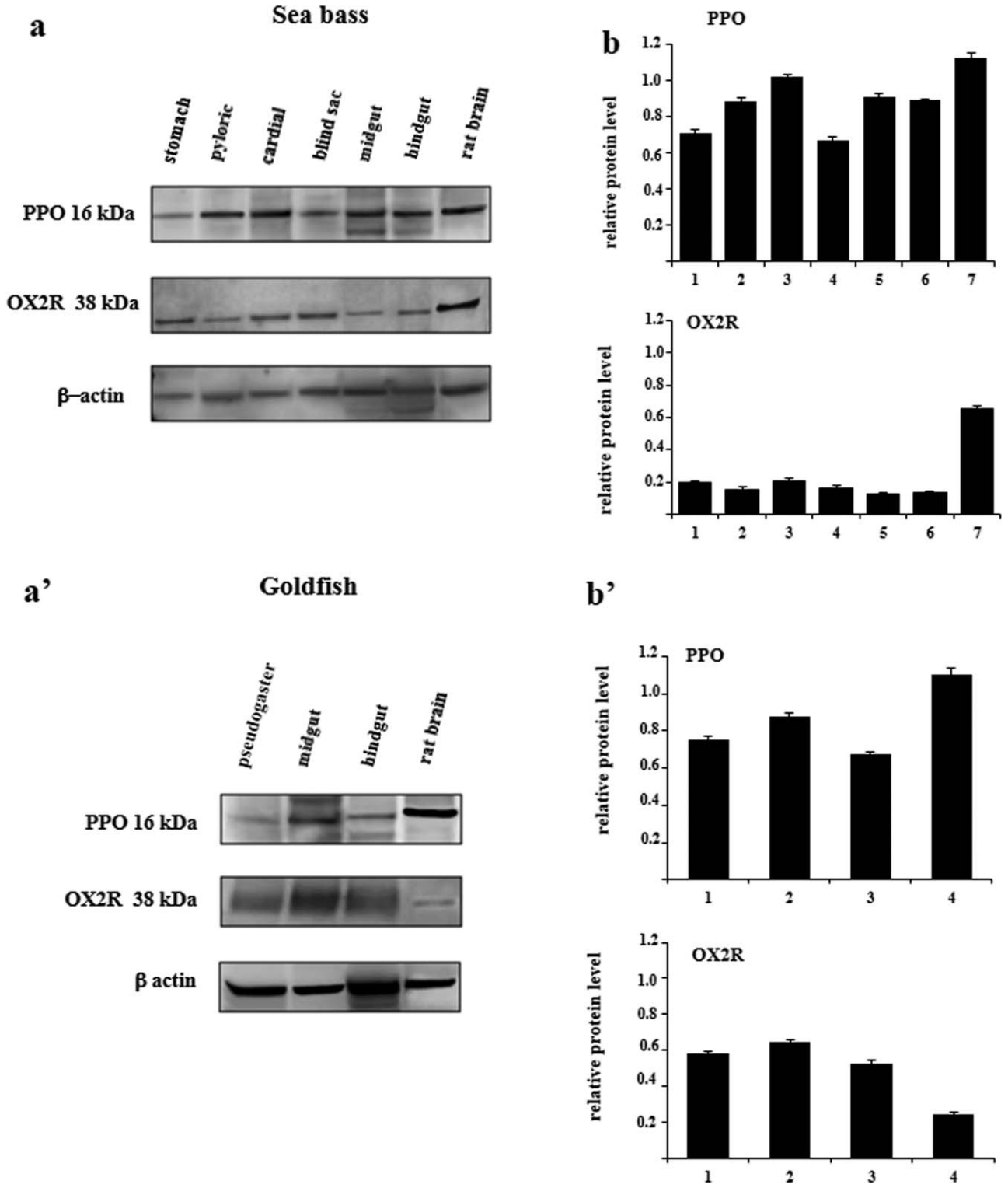


Fig. 1. Levels of PPO and OXR2 in different gut regions in sea bass (a), goldfish (a') determined by Western blotting. Rat brain was used as a positive control. β-actin expression from the same samples served as an internal control to verify equal protein loading. (b, b') Densitometric analysis (mean ±SD) of the immunoblot reported in sea bass and goldfish, respectively. Relative expression levels were obtained in each sample by normalization of PPO and OXR2 to that of β-actin. In b: 1 stomach; 2 pyloric stomach; 3 cardial stomach; 4 blind sac; 5 midgut; 6 hindgut; 7 rat brain. In b': 1 intestinal bulb; 2 midgut; 3 hindgut; 4 rat brain.

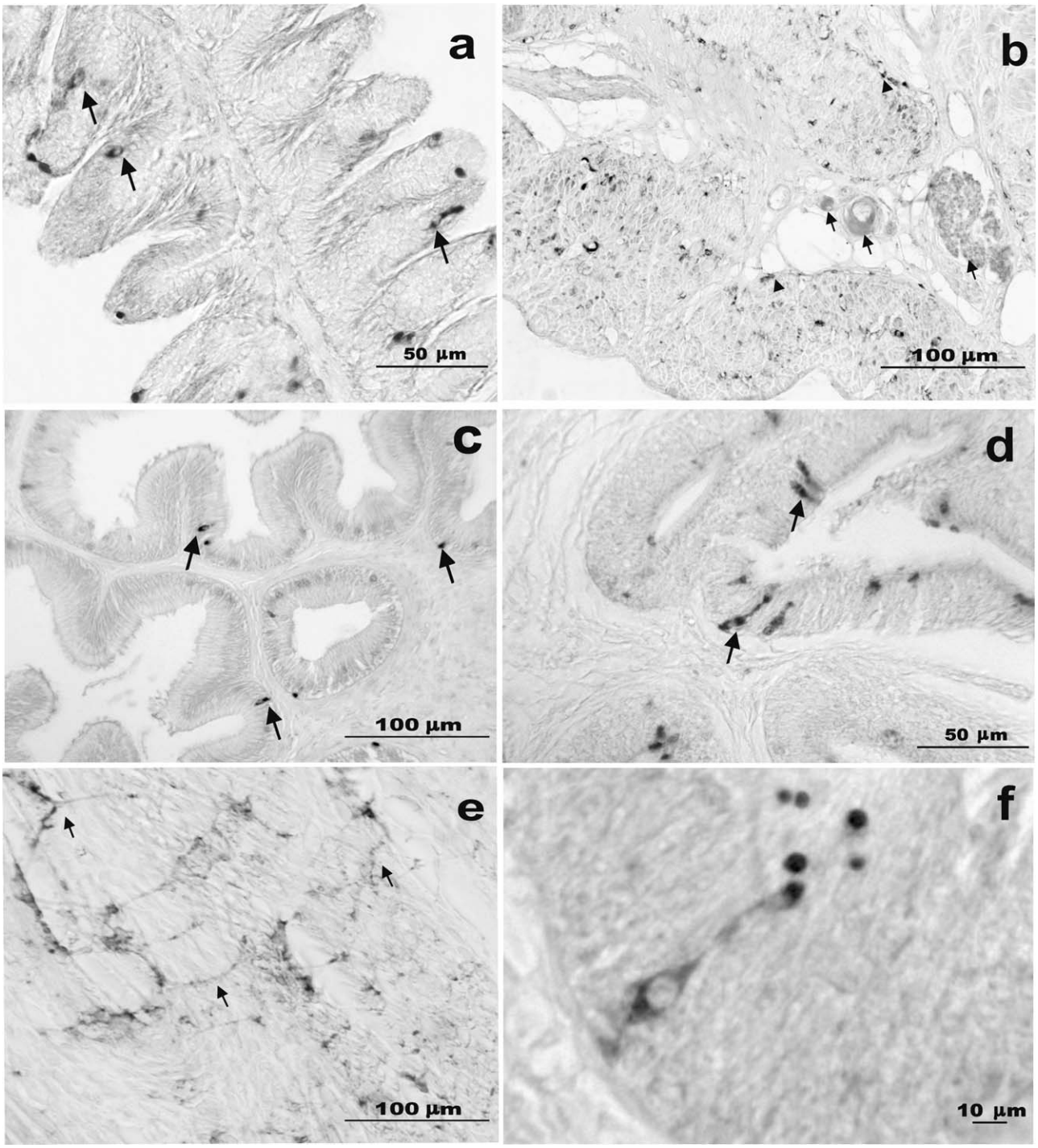


Fig. 2. Sea bass. OXA and OXB in the stomach and intestine. a. OXB in immunoreactive elongated cells (arrows) with apical process in the epithelium of cardial mucosa. b. OXA in immunoreactive nerve cell bodies (arrows) of the myenteric cardial plexus and in nerve fibers (arrowheads) of the muscle layers. c. OXA immunoreactive cells in the epithelium of blind sac fold (arrows). d. OXA in immunoreactive elongated cells (arrows) of the pyloric epithelium. e. OXB immunoreactive nerve fiber network in the circular muscular sheet of the pyloric region (arrows). f. OXA immunoreactive cells with basal and apical processes in the midgut.

and scattered. Few OXA and OXB immunoreactive myenteric nerve cell bodies were detected both in the midgut and hindgut. In addition, rare immunoreactive thin nerve fibers were found in the myenteric plexus, the circular sheet and the submucous plexus of the midgut and hindgut.

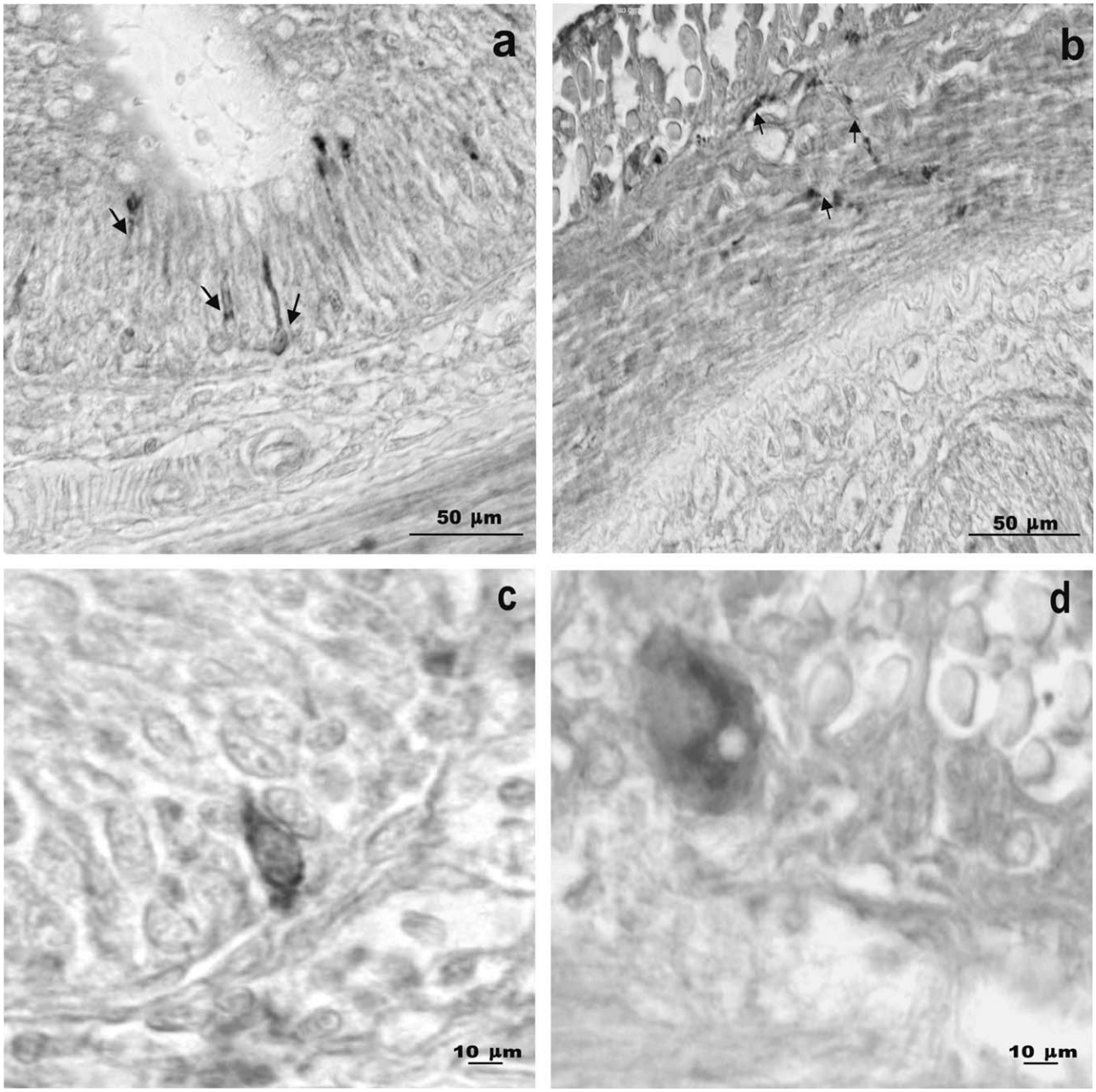


Fig. 3. Goldfish. OXA and OXB in the intestine. a. OXA immunoreactive cells in the intestinal bulb (arrows). b. OXA immunoreactive nerve fibers in the circular muscular sheet of the midgut (arrows). c. OXB immunoreactive cells at the base of intestinal folds. d. OXB immunoreactive myenteric neuron in the hindgut.

***OXA and OXB - goldfish.*** OXA (Fig. 3a) and OXB (Fig. 3c) immunoreactive cells were mainly found in the basal region of folds in intestinal bulb, midgut and hindgut. OXA (Fig. 3a) and OXB immunoreactive cells were very thin and showed long apical processes. In the midgut and hindgut OXA (Fig. 3b) and OXB (Fig. 3d) immunoreactive myenteric nerve cell bodies and nerve fibers in the muscular sheets were often detected.

***OX2R - sea bass.*** In the cardial region, blind sac and pyloric regions, OX2R immunoreactive cells were recognized in the epihelium (Fig. 4a). They were more rare at the base of the folds and sparse in the gastric glands. Perivascular OX2R immunoreactive nerve fibers were observed in the muscular sheets. In the midgut (Fig. 4b) and hindgut, various oval or almost elongated OX2R immunoreactive cells were observed in the

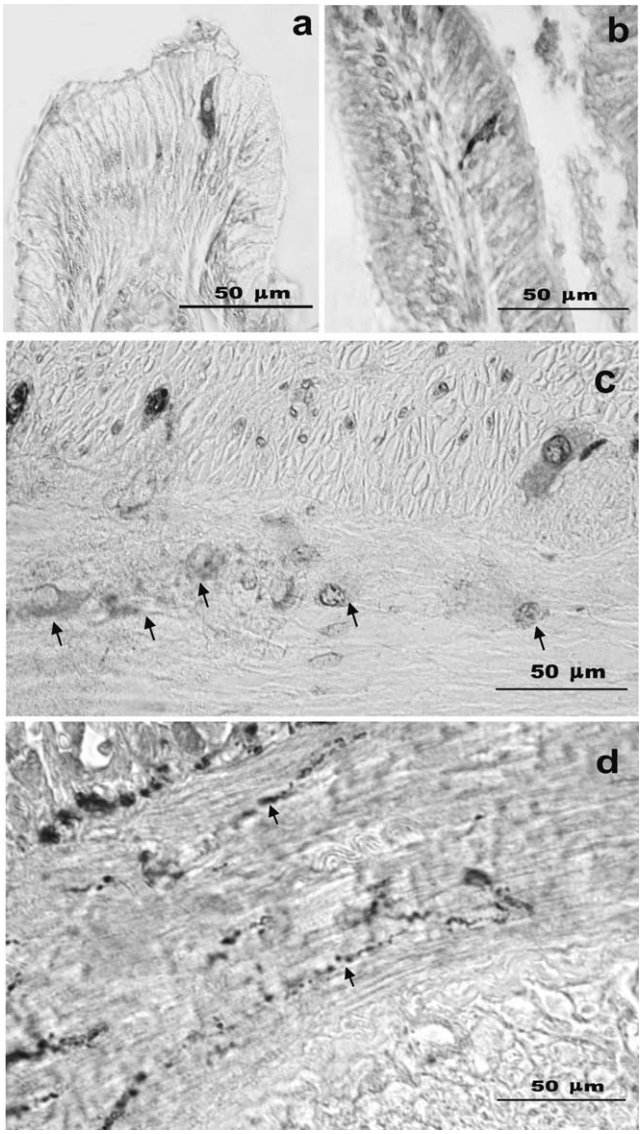


Fig. 4. Sea bass (a-c) and goldfish (d). OX2R in the gut. a. OX2R immunoreactive cells in the epithelium of cardial gastric folds. b. OX2R immunoreactive cells in the epithelium of the midgut. c. OX2R immunoreactive nerve cell bodies (arrows) in the myenteric plexus and nerve fibers in the circular muscular sheet of the midgut. d. OXR2 immunoreactive neuron and nerve fibers (arrows) in the myenteric plexus and circular muscular sheet.

epithelium. Moreover, some weakly positive nerve cell bodies were detected in the myenteric plexus of the midgut (Fig. 4c) and hindgut.

***OX2R - goldfish.*** In all examined intestinal tracts, OX2R immunoreactive cells were widespread in the epithelium, with the staining mainly observed in the apical portion. In addition, OX2R positivity was detected in nerve cell bodies and nerve fibers of the myenteric plexus and circular muscular sheets (Fig. 4d). Some OX2R immunoreactive nerve fibers were found in the submucous and mucous plexuses.

## DISCUSSION

This study is the first report on the expression and immunolocalization of PPO, orexins and the orexin receptor in the gastroenteric tract of saltwater (sea bass) and freshwater fishes (goldfish). These two teleost species show a different morphological organization of the digestive tract and feeding habits and they are a suitable model for investigating food intake regulation (D'Angelo et al., 2016; Arcamone et. al, 2009).

The Western blot analysis demonstrates the presence of PPO and OX2R in the sea bass and goldfish gastroenteric tract by identifying a band of molecular weight similar to what has been reported in mammalian brain by Johren et al., 2001. Densitometric analysis revealed higher expression level of OX2R in the intestinal tract of goldfish compared to sea bass. This observation is also in accordance with the immunohistochemical results. The higher expression of OX2R in goldfish could be ascribed to its specific role in the orexin regulatory system of the intestine in relation to the metabolic state of the organism (Karteris et al., 2005).

The immunohistochemical results report the presence of OXA and OXB in cells, nerve cell bodies and nerve fibers. The polyclonal antisera employed are raised against human OXA and OXB peptides, and recognize the C-terminal region. In teleosts, this region shows substantial aminoacidic conservation compared to mammals, and it is of essential importance in orexin receptor binding (Lang et al., 2006; Huesa et al., 2005; Faraco et al., 2006). Strongly immunoreactive cells to both orexin peptides are markedly present in the cardial region, blind sac and pyloric stomach of sea bass; mild immunoreactive cells are observed in the intestinal bulb of goldfish. OX2R-like immunoreactive cells are moderately positive in the gastroenteric tract of both species. Neurons and nerve fibers innervating the myenteric and submucous plexuses and muscular sheets of the sea bass stomach and goldfish intestinal bulb show discrete positivity to OXA and OXB, and lower immunoreactivity to the orexin receptor. In spite of differences in the structural organization of the digestive system among vertebrates, the observations regarding orexins in sea bass and goldfish are in agreement with the distribution and morphology of orexins reported in human, mouse, rat, guinea pig and chicken (Kirchgessner and Liu, 1999; Kirchgessner, 2002; Nakabayashi et al., 2003; Arcamone et al., 2014; Gatta et al., 2014). Immunoreactivity to OX2R is less abundant in nerve cell bodies of the ENS of seabass and goldfish compared to mammals (Kirchgessner, 2002).

Orexins are predominantly known as neuromodulators displaying neurocrine or endocrine activities. OXA acts as a peripheral mediator of energetic homeostasis, and fasting activates intestinal OXA secretomotor nerve cell bodies, leading to increased motility (Kirchgessner, 2002). It could be possible that the occurrence of OXA and OXB in the enteric nervous system of sea bass and goldfish implicates a role in the regulation of gut motility. However, further experiments are needed to demonstrate this hypothesis. The immunoreactivity to OX2R has been detected in the circular musculature and in the submucous and myenteric plexuses, similarly to what is reported in mammals (Naslund et al., 2002). These observations suggest a possible involvement of orexins

in the modulation of enteric motility and secretion. Interestingly, the immunopositivity to OXA and OXB has been observed in nerve fibers innervating blood vessels and muscular sheets, reinforcing the hypothesis of involvement of orexins in the visceral regulation of blood flow in sea bass and goldfish.

In addition, the presence of orexins in enteroendocrine cells in both species, stronger in sea bass, might suggest that, in these species, gut activity may also be influenced by orexins, as postulated for mammals (Näslund et al., 2001; Kirchgessner, 2002). Indeed, OXA and OXB could regulate absorptive processes of aminoacid, glucose and fatty acid, as well as the iono-osmotic regulation of fluids in the gut of these teleosts.

Furthermore, the occurrence of orexins and the receptor OX2R in enteroendocrine cells provides intuitive evidence for neuroendocrine and/or paracrine functions in the GI tract of fish, as hypothesized for other peptides involved in food control (for review, see Volkoff et al., 2005).

The slight difference in the expression and distribution of immunoreactivity between sea bass and goldfish orexins and the orexin receptor could be ascribed to different physiological processes of the various gastroenteric tracts in relation to their feeding habits. In sea bass the immunostaining observed reveals the presence of both OXA and OXB in the cell bodies of the mucosa and mainly in fibers of the muscular layer. Interestingly, some fibers containing orexin are also found in the mucosal epithelium, where nutrients are absorbed from the lumen of the intestine. These observations are highly interesting. Indeed, when compared to other stomach-containing fish species, i.e. trout, both peptides and the receptor are absent (Varricchio et al., 2015). In goldfish, digestive processes are carried out only by enzymes of pancreatic juice and trypsin secreted by pancreatic endocrine cells and released into the intestine (Hoar, 1979; Olsson, 2009).

In fish, like in higher vertebrates, orexin increases food consumption as an orexigenic factor and enhances locomotor activity, involving also other peptides, with synergic or antagonist actions (Volkoff et al., 2010). In goldfish, it has been suggested that the orexigenic actions of orexin-A and NPY are mutually mediated by their signaling pathways, and mutually play crucial roles in the regulation of feeding behavior as orexigenic neuropeptides (Kojima et al., 2009). In addition, in goldfish, ghrelin increases feeding through the activation of the orexin system (Miura et al., 2007).

The orexin system has been widely described in the central nervous system (CNS) of several fish species (Kaslin et al., 2004; Huesa et al., 2005; Amiya et al., 2007; Xu and Volkoff, 2007; Kojima et al., 2009; Yan et al., 2011). The present survey demonstrates that orexin immunoreactive nerve cell bodies are found also outside the CNS, in sea bass and goldfish. Although the food intake regulation is under the control of the hypothalamic centers (Huesa et al., 2005), the presence of the two neuropetides and receptor in the gut suggests that signals and mechanisms originating in the gut could affect the feeding behaviour by the use of the gut-brain axis.

In conclusion, the cell-type localizations of the orexins and OX2R in the GI tract of a stomach and stomachless species, suggest that these peptides do not only act as neurotransmitters in the local control of the GI tract, but may also be involved as endocrine modulators in the regulation of feeding and metabolic homeostasis. Further functional investigations may help to better explain the role of the orexin system in the energy balance of fish and the interaction between feeding and digestive physiology, and the possible functional diversifications in these fish species.

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