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# Neurotransmitters, neuromodulators, and neurotrophin receptors in the gut of pantex, a hybrid sparid fish (*Pagrus major* x *Dentex dentex*). Localizations in the enteric nervous and endocrine systems

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**Summary.** The gut of Pantex, a sparid hybrid fish (*Pagrus major* x *Dentex dentex*) with a great potential importance for the Italian aquaculture, was histochemically and immunohistochemically investigated in order to evidence components of the intramural nervous and diffuse endocrine systems. The general structural aspects of the intramural nervous system were shown by the Nissl-thionin staining. As in most other fish, it was only organized in the myenteric plexus. Acetylcholinesterase (AChE) activity was observed in both nerve cell bodies and terminals all along the gut. The NADPH-diaphorase reactivity too, possibly linked to the synthesis and release of nitric oxide, was present in nerve cell bodies and nerve terminals of the oesophagus, stomach and intestine. In addition, the intramural nervous system was shown to contain Trk (tyrosine-kinase) receptors for neurotrophin, as evidenced by Trk A-, Trk B- and Trk C-like immunoreactivities, thus suggesting an involvement of neurotrophin in the function of this system. Trk B- and Trk C-like immunoreactivities were detected in epithelial endocrine cells, too. The additional presence of serotonin- and met-enkephalin-like immunoreactivities in numerous endocrine cells in the epithelial layers of the stomach and intestine was showed.

**Key words:** Fish hybrids, Fish gut, Neuro-endocrine system, Acetylcholinesterase, NADPH-diaphorase, Trks

## Introduction

In aquaculture hybrids are usually produced in order to obtain a peculiar combination of favourable characteristics (Shiraishi et al., 1993) or to create desirable new characteristics (McKay et al., 1992), or to exploit hybrid vigour (Noga et al., 1994).

In the *Sparidae*, a family in which several species of a high commercial value are present, a hybrid fish called Pantex has been recently created between female *Pagrus major* and male *Dentex dentex* (Colombo et al., 1998). Pantex has the potential to be a good candidate for commercial aquaculture in Italy, because it is easily reared and has a fast growth rate (it grows ten times faster than *D. dentex* at early stages). Last but not least, it is morphologically similar to *D. dentex*, and thus it will be possibly favourably accepted by the consumers. Our preliminary observations show that Pantex, like many other hybrids of *Sparidae* (Diskin, 1993; Colombo et al., 1998), has reduced or absent development of gonads. This possibly further enhances the commercial value of it through a resulted increase in edible muscle mass, as well as the security of its management in farming conditions owing to its sterility.

Taking into account that nothing is known about structural aspects of the gut of the Pantex, the aim of this work was to examine components which coordinate secretory and motor gastrointestinal functions, such as the intramural nervous and the diffuse endocrine systems. Structural details of the neuroendocrine system will be described in this paper as regards the occurrence and localization of the classical neurotransmitter acetylcholine, and of putative neuromodulators (neuropeptides, serotonin and nitric oxide).

Neuropeptides, from both neuronal and endocrine origin, have been above all in recent years thoroughly described in the fish gut (Bjenning and Holmgren, 1988; Kiliaan et al., 1992, 1993; Reinecke et al., 1997; Domeneghini et al., 1999, 2000; Defzuli et al., 2000).

In addition, Trk-like receptors of neurotrophins will be detailed. Neurotrophins are a family of polypeptide growth factors acting on development, differentiation and maintenance of many neuronal populations (Barbacid, 1995; Fariñas and Reichardt, 1996; Reichardt

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and Fariñas, 1997). Recently a neurotrophin influence on non-neuronal cells, like epithelial endocrine cells of the gut (Esteban et al., 1995; Shibayama and Koizumi, 1996; Lucini et al., 1999) and pancreas (Kanaka-Gantenbein et al., 1995; Miralles et al., 1998) has been hypothesized. In teleosts, neurotrophins like Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin 3 (NT3) are similar to mammalian ones. Another two neurotrophins, NT6 and NT7, have been detected in fish species only (Lai et al., 1998; Nilsson et al., 1998; Heinrich and Lum, 2000). Some of us have recently found NGF- and NT3-like immunoreactivities in the gut of other fish (unpublished data).

Neurotrophins act on cells by means of high affinity transmembrane Tyrosine-kinase (trk) proteins (Meakin and Shooter, 1992; Bothwell, 1995) encoded by the family of *trk* proto-oncogenes (Barbacid, 1993). In the central nervous system of the zebrafish, five Trk receptors (TrkA, TrkB1, TrkB2, TrkC1, TrkC2) were sequenced (Martin et al., 1995, 1998). They are structurally homologous to the three known mammalian Trk proteins (TrkA, TrkB and TrkC), especially in the intracellular kinase regions. In mammals TrkA binds to NGF, TrkB to BDNF and NT 4/5, and TrkC to NT3. Preliminary experiments on the interaction of fish NTs with fish Trks indicate that BDNF prefers TrkB1 and NT3 prefers TrkC1 (Heinrich and Lum, 2000).

## Materials and methods

Five adult Pantex fish (Fig. 1), obtained from "Ittica Mediterranea" fish hatchery (Petrosino, TP, Italy), were used for this study. Body weight was about 500g. Fish were killed by an overdose of MS222 (Sandoz, Italy) anaesthesia at 10 a.m. and gut specimens were collected immediately after sacrifice. Several samples of oesophagus, stomach and intestine (pyloric caeca, proximal, medium and distal intestine) were fixed in 4% paraformaldehyde in 0.01M phosphate buffered saline (PBS) (NaCl 138mM, KCl 2.7 mM) pH 7.4 for 4-5 h at 4 °C, rinsed overnight in PBS, then in 20% sucrose in the same buffer for 24 h at 4 °C, and finally snap-frozen in liquid nitrogen-cooled isopentane. Other specimens of the same organs were snap-frozen as above without previous fixation. Finally, other small fragments of the same organs were dehydrated after fixation and paraffin embedded. Serial sections of the specimens were processed as follows.

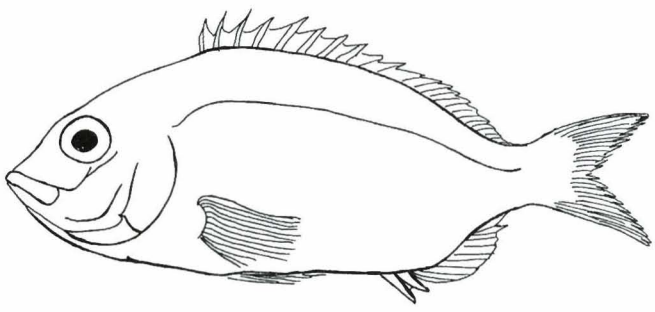

**Fig. 1.** A silhouette is shown of an adult pantex.

### *Histochemistry*

#### NISSL-thionin

Paraffin embedded sections (4 $\mu$m) were picked up on gelatin-coated glass slides, dewaxed, rehydrated to water and stained for the general morphology of neurons by Nissl-thionin staining (Lillie, 1965).

#### AChEase

Cryostat sections (10 $\mu$m) from unfixed specimens were stained for acetylcholinesterase (AChEase) according to Karnowsky and Roots (1964) and Filipe and Lake (1983). AChEase may be considered a marker of cholinergic neurons of fish (Radaelli et al., 1998; Domeneghini et al., 1999, 2000) where, in contrast to other vertebrates, unspecific cholinesterases are not present (Pecot-Dechavassine, 1961). The specificity of the stain was verified by excluding acetylthiocholine iodide from the incubating medium, which abolished all reactivity. Positive controls included mammalian (bovine and horse) gut and skeletal muscle samples (rat), as well as specimens from gut of other fish (sea bream, eel, sturgeon).

#### NADPH-diaphorase

Cryostat sections (20 $\mu$m) from both fixed and unfixed specimens were picked up on gelatin-coated glass slides and incubated for 1 h at 37 °C in 0.1M PBS pH 7.4, containing 0.15 mg/ml nitroblue tetrazolium (Sigma, Italy), 0.1% Triton X-100 and 1 mg/ml NADPH (Sigma), according to Scherer-Singler et al. (1983). The sections were then rinsed in PBS, dehydrated and mounted in Eukitt. Utilizing fixed sections the results were clearer and sharper. The specificity of this stain was verified by excluding NADPH from the incubating medium, which abolished all reactivity. Positive controls included mammalian and fish gut samples.

### *Immunohistochemistry*

Dewaxed sections (4 $\mu$m) as well as cryostat sections (10 $\mu$m) from both fixed and unfixed specimens were used. The results obtained with the different procedures were similar, except for the neurotrophin receptor immunohistochemistry which gave results only on fixed and dewaxed sections. The immunohistochemical staining was performed using the peroxidase-antiperoxidase (PAP) method (Sternberger, 1979) and the peroxidase-linked avidin-biotin complex (ABC) method (Hsu et al., 1981). After rinsing in distilled water, the sections were treated with 3% $H_2O_2$ (20 min)

**Table 1.** Antisera against tyrosine-kinase proteins.

| ANTISERA* | ANTIGEN | SOURCE | DILUTION |
|---|---|---|---|
| Rabbit anti-TrkA | human COO-domain 763-777 | Santa Cruz Biotechnology, USA sc-118 | 1:500 |
| Rabbit anti-TrkB | human COO-domain 794-808 | Santa Cruz Biotechnology, USA sc-12 | 1:500 |
| Rabbit anti-TrkC | human COO-domain 798-812 | Santa Cruz Biotechnology, USA sc-117 | 1:500 |

*: these antibodies react with the following aminoacid sequences in the tyrosine-kinase domain: 763-777 of the deduced human 140 kDa TrkA; 794-808 of the predicted mouse 145 kDa TrkB; 798-812 of the predicted mouse 145 kDa TrkC.

**Table 2.** Antisera against neurotransmitters (and related proteins) and neuromodulators.

| ANTISERA | SOURCE | DILUTION |
|---|---|---|
| rabbit anti-human calcitonin gene-related peptide (CGRP) | Peninsula, UK, IHC 6009 | 1:500 |
| rabbit anti-rat calcitonin gene-related peptide (CGRP) | Peninsula, UK, IHC 6006 | 1:500 |
| rabbit anti-Substance P | Chemicon, USA, AB1566 | 1:600 |
| rabbit anti-porcine vasoactive intestinal peptide (VIP) | Genosys, UK, CA-08-340 | 1:600 |
| rabbit anti-somatostatin | Peninsula, UK, IHC 8004 | 1:400 |
| rabbit anti-methionine-enkephalin | Peninsula, UK, IHC 8602 | 1:800 |
| rabbit anti-serotonin | Peninsula, UK, IHC 61066 | 1:3000 |
| goat anti-choline acetyltransferase | Chemicon, USA, AB144 | 1:500 |
| goat anti-vesicular acetylcholine transporter | Chemicon, USA, AB1578 | 1:1000 |
| rabbit anti-nitric oxide synthase I | Chemicon, USA, AB1552 | 1:500 |
| rabbit anti-tyrosine-hydroxylase | Chemicon, USA, AB151 | 1:1000 |

to block the endogenous peroxidase activity and rinsed in phosphate- buffered saline solution (PBS) (pH 7.4) containing 0.2% Triton X-100 and 0.1% bovine serum albumin. Background was prevented by incubating the sections with 1:5 normal goat (Vector, USA) or swine (Dako, Italy) or donkey serum (Chemicon, USA) for 30 min prior to the incubation with primary antibodies. The primary antisera (Table 1, 2) were applied overnight at 4 °C in a humid chamber.

After rinsing in PBS buffer, the sections were incubated for 30 min at room temperature in goat anti-rabbit IgG (Vector) for the PAP technique, and in biotinylated swine anti-rabbit IgG (Dako) or donkey anti-goat IgG (Chemicon) for the ABC technique. After rinses in PBS, the sections were treated with PAP complex (1:100, UCB, Belgium) for 30 min at room temperature, or with the labelling complex (avidin conjugated to horseradish peroxidase, Dako). After washing in PBS, the immunoreactive sites were visualized using a freshly prepared solution of 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 15 ml of a 0.5M Tris buffer at pH 7.6 and containing 1.5 ml of 0.03% $H_2O_2$. Finally, sections were slightly counterstained with Mayer's hematoxylin in order to ascertain structural details, mounted using Eukitt and examined under an Olympus BX50 photomicroscope.

### Controls

The specificity of immunostaining was verified: 1) by incubating sections with PBS instead of the specific antisera (see above); 2) by incubating sections with normal rabbit or goat serum instead of primary antisera; 3) by incubating sections with PBS instead of secondary antibodies; and 4) by incubating sections with preabsorbed antisera with the respective antigens (10-100 $\mu$g/ml). The preabsorption procedures were carried out overnight at 4 °C. The results of these controls were negative. As positive controls fish (sturgeon, eel and sea bream) and mammalian (rat, dog) gut samples were used, as well as rat skeletal muscle. For neurotrophin receptor immunohistochemistry, a further specificity control was performed in order to ascertain possible cross-reactivities, by incubating sections with inappropriate antigens. This did not modify the immunostaining.

## Results

### *Histochemistry*

Neurons were evidenced in the myenteric plexus all along the gut by the Nissl-thionin staining (Fig. 2a). In the intestine Nissl-thionin positive nerve cell bodies were found in both the myenteric plexus and inner musculature (Fig. 2b).

AChE activity was evidenced in oesophageal nerve terminals which were seen in contact with striated muscle fibres, in form of motor end plates (Figs. 2c, d), as well as in nerve bundles variously running in the tunica propria-submucosa (Fig. 2d). In the stomach AChE-reactive nerve cell bodies were especially numerous in sub-serous ganglia (Fig. 2e) whereas in the intestine AChE-positive neurons were detected in the myenteric plexus (Fig. 2f).

In the oesophagus, NADPH-diaphorase reactivity

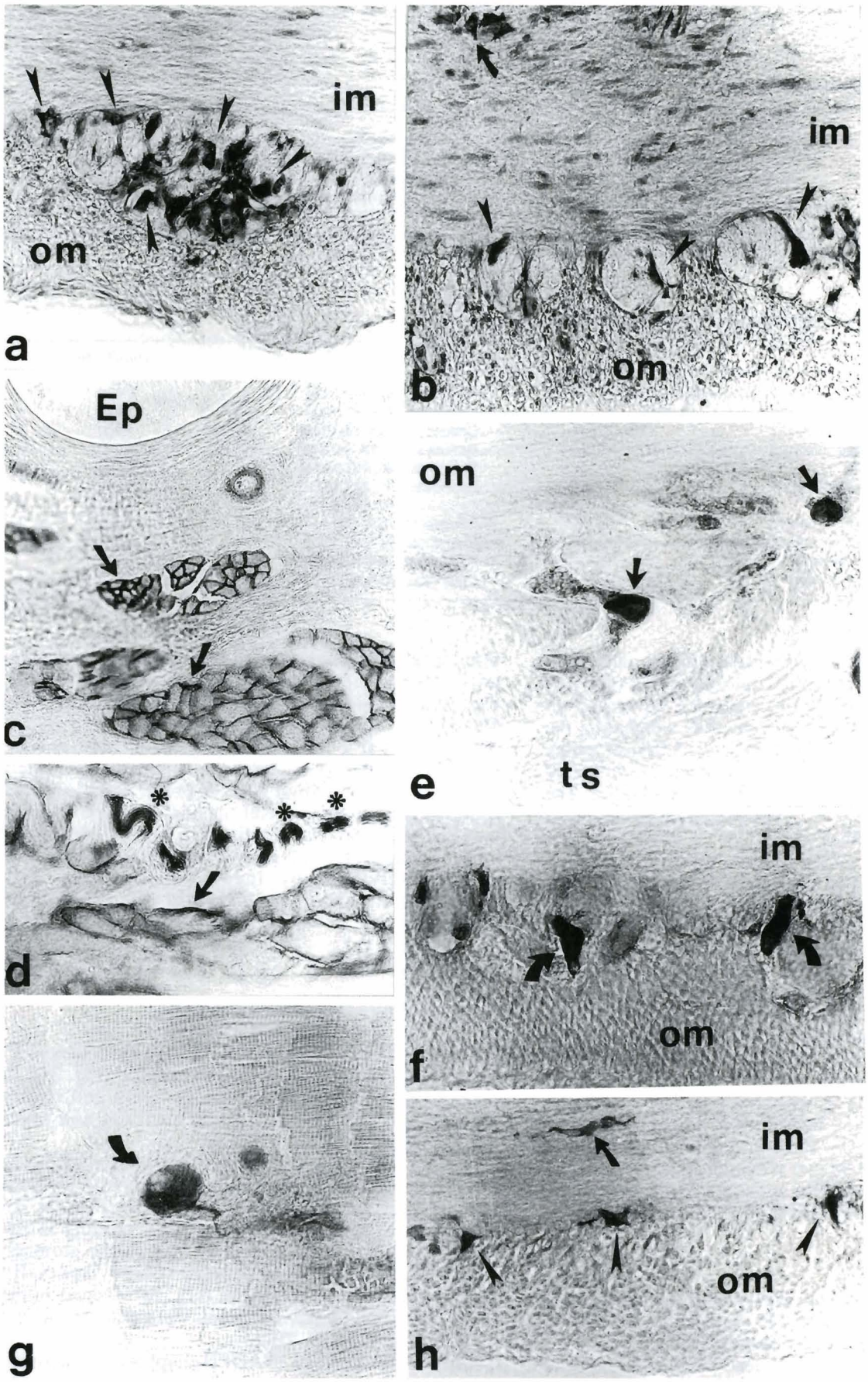


**Fig. 2. a.** Nissl-thionin staining in the stomach. The reaction is present in nerve cell bodies of the myenteric plexus (arrowheads). im: inner musculature; om: outer musculature. x 380. **b.** Nissl-thionin staining in the proximal intestine. The reaction is present in nerve cell bodies of the myenteric plexus (arrowheads) and inner musculature (arrows). im: inner musculature; om: outer musculature. x 380. **c.** AChEase reactivity in the striated musculature of the oesophagus, transversally sectioned. Reactive nerve terminals (arrows) contact muscle fibres. Ep: mucosal epithelium. x 190. **d.** AChEase reactivity in the striated musculature of the oesophagus is evident in bundles of reactive nerves (asterisks) and in single nerve terminals (arrow) contacting muscle fibres. x 260. **e.** AChEase histochemistry in the stomach. Reactivity is present in nerve cell bodies (arrows) located in a sub-serous ganglion. om: outer musculature; ts: tunica serosa. x 400. **f.** AChEase histochemistry in the distal intestine. A strong reactivity is present in nerve cell bodies (arrows) of the myenteric plexus. im: inner musculature; om: outer musculature. x 380. **g.** NADPH-diaphorase histochemistry in the oesophagus. Reactivity is present in one roundish nerve cell body (arrow) located among the striated muscle fibres. x 300. **h.** NADPH-diaphorase histochemistry in the pyloric caeca. Reactivity is present in nerve cell bodies of the myenteric plexus (arrowheads) and of the inner musculature (im) (arrows). om: outer musculature. x 300

was detectable in roundish nerve cell bodies located among the striated muscle fibres (Fig. 2g) and in both nerve cell bodies and subtle nerve terminals of the tunica propria-submucosa. In the stomach, NADPH-diaphorase reactivity was present in nerve cell bodies of the myenteric plexus and in both nerve cell bodies and terminals of the tunica propria-submucosa. In the intestine NADPH-diaphorase reactivity was seen in nerve cell bodies located in both the myenteric plexus and inner musculature (Fig. 2h). These neurons were small and polygonal.

*Immunohistochemistry*

Trk proteins

Trk A-like immunoreactivity (IR) was detected in both nerve cell bodies of the myenteric plexus and nerve terminals located in the propria-submucosa of all the intestinal segments. Trk B-like IR was observed in nerve cell bodies of the myenteric plexus of the stomach and intestine (Fig. 3a). Trk C-like IR was seen in nerve cell bodies of the myenteric plexus as well as in subtle nerve terminals running in the propria-submucosa of the intestinal tracts (Fig. 3b). In addition, Trk B- and Trk C-like IRs were detected in numerous endocrine cells. Trk B-like IR was present in gastric endocrine cells which were located in the deep gastric pits and in the luminal end of the glands, as well as in the intestinal folds where they appeared elongated and slender in shape. Trk C-like immunoreactive endocrine cells were detected in the intestinal folds only (Fig. 3c).

Neurotransmitters and neuromediators

Serotonin-immunoreactive epithelial endocrine cells were detected in the proper gastric glands of the stomach (Fig. 3d). They were usually localized in the deep gastric pits and in the luminal end of the glands.

Numerous met-enkephalin-like immunoreactive endocrine cells were observed in the intestinal mucosal folds (Fig. 3e). Especially the medium intestine was rich in this endocrine cell type, which were usually slender and elongated.

Somatostatin-, CGRP-, substance P, and VIP-like immunoreactivities were never detected in any localization.

Immunohistochemistry for choline acetyltransferase and vesicular acetylcholine transporter, as well as for nitric oxide synthase-I failed to give any reaction in fish tissues, although repeated experiments with more prolonged incubation times were performed and even if these antisera stained mammalian tissues. Tyrosine-hydroxylase immunohistochemistry failed to give reaction on gut samples of Pantex although it stained the gut from both mammals and other fish species.

## Discussion

In the present study the intramural nervous and the diffuse endocrine systems of the gut of Pantex have been examined in order to give a general anatomical evaluation of them and to understand, where possible, the functional correlations of these systems, also comparing them with what is known for other fish . The enteric nervous and the diffuse endocrine systems share a key role in the control of multiple functions of the gut, and this in turn has a pivotal role in the relationships between the inner and the outer environments.

As in most fish species, the enteric nervous system shows the prominent presence of the myenteric plexus, in which nerve cell bodies as well as nerve terminals are present. Neurons are in addition detectable in a subserous localization in the stomach, whereas only nerve terminals are usually described in the tunica propria-submucosa, probably coming from the neurons localized in the ganglia of the tunica muscularis or serosa. Finally, isolated neurons may be sometimes evidenced among the fibres of the tunica muscularis.

Some enteric neurons are possibly cholinergic, as evidenced by the histochemical reaction for AChEase. Upon the same histochemical grounds we recently described (Domeneghini et al., 1999, 2000) the presence of possible cholinergic neurons in other fish too, with a similar distribution along the gut. Also in the Pantex, like in previously studied fishes, the esophageal striated muscle fibres receive a prominent (if not exclusive) cholinergic innervation. As immunohistochemistry for choline acetyltransferase and vesicular acetylcholine transporter failed to give reaction on gut and striated muscle samples of Pantex as well as of several other fish (Radaelli et al., 1998; Domeneghini et al., 1999, 2000; other our unpublished observations), our choice of the histochemical method for acetylcholinesterase is, at present, the most valid to identify fish cholinergic neurons in the gut intramural innervation. Unfortunately, a tyrosine hydroxylase-immunoreactivity was never observed in the gut intramural innervation of the Pantex, and thus we cannot at present offer a morphological picture of the possible antagonistic adrenergic component of it. On the contrary, we have recently immunohistochemically described the presence of tyrosine hydroxylase-immunoreactive nerve terminals in the gut of *Acipenser transmontanus* (Domeneghini et al., 1999) and *Anguilla anguilla* (Domeneghini et al., 2000), and Read and Burnstock (1968, 1969) previously histochemically described the adrenergic innervation of the gut in other fish species.

It is generally accepted that NADPH-diaphorase activity is a selective marking tool for neuronal nitric oxide (NO) synthase (Hope et al., 1991). NO is a gaseous mediator which is reputed to be an inhibitory nonadrenergic-noncholinergic (NANC) neurotransmitter in the gut of mammalian species (Sanders and Ward, 1992). The Pantex shows in its enteric nervous system the presence of possibly nitrergic, NADPH-d-reactive neurons, and this is in agreement with what has been observed in other fish (Li and Furness, 1993; Olsson and Karila, 1995; Olsson and Holmgren, 1997; Schleiffer and Raul, 1997; Domeneghini et al., 1999). Upon histo-

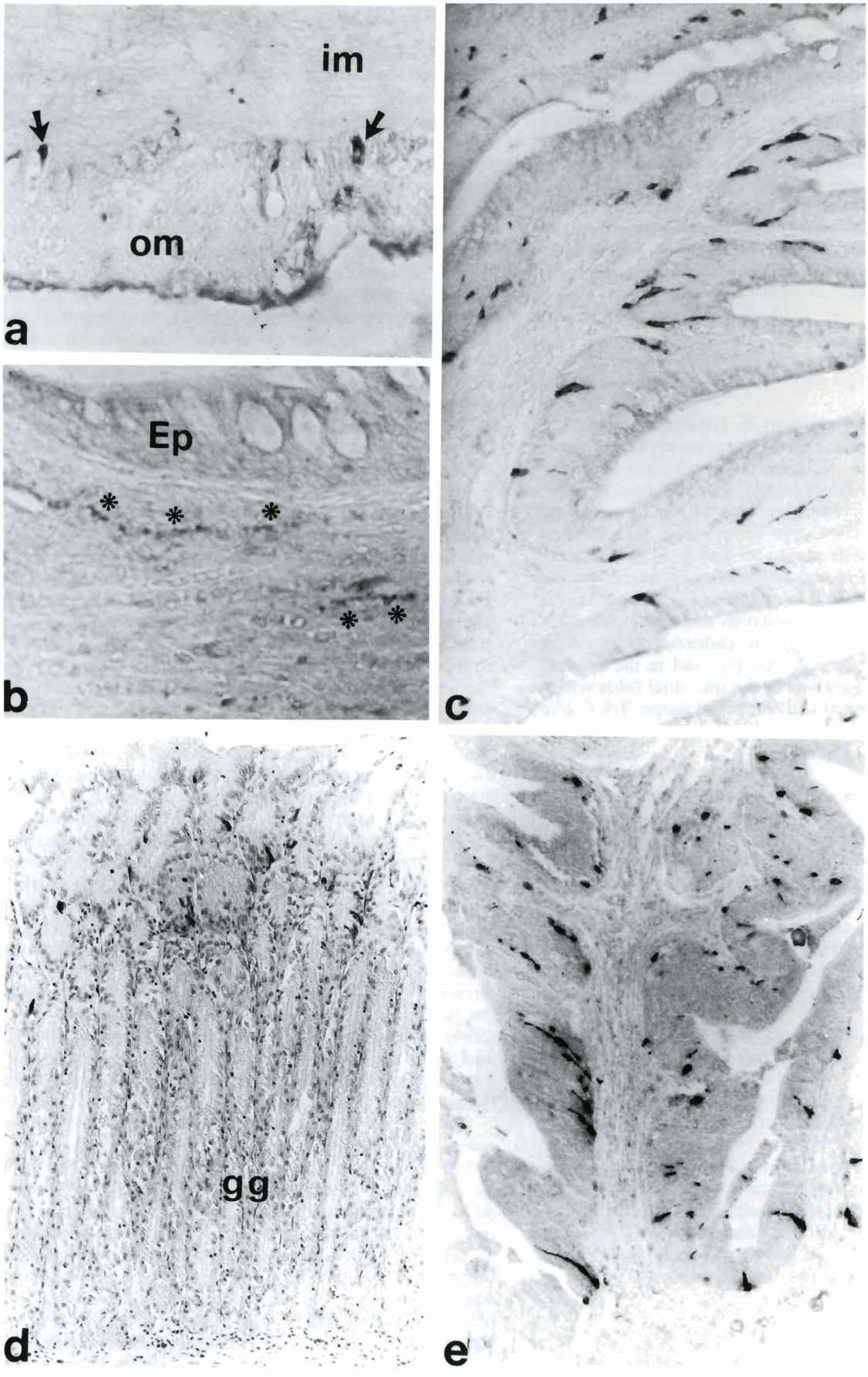


**Fig. 3. a.** Trk B-like immunohistochemistry in the stomach. Reactivity is present in nerve cell bodies (arrows) of the myenteric plexus. im: inner musculature; om: outer musculature. x 380. **b.** Trk C-like-immunoreactivity in the proximal intestine. Reactivity is present in subtle nerve terminals running in the propria-submucosa (asterisks). Ep: mucosal epithelium. x 420. **c.** Trk C-like-immunoreactivity in the distal intestine. Reactivity is evident in numerous epithelial endocrine cells. x 400. **d.** Serotonin-immunoreactivity in the stomach. Reactivity is present in rather numerous endocrine cells. gg: gastric glands. x 200. **e.** Met-enkephalin-like-immunoreactivity is present in numerous endocrine cells. x 200

and immunohistochemical grounds, we have quite recently hypothesized that in the silver eel gut nitrergic neurons may be in a functional relationship with both cholinergic neurons and adrenergic nerves (Domeneghini et al., 2000). According to Karila and Holmgren (1995), NO plays functional roles in fish gut at least towards muscle tone regulation and peristaltic reflexes. In this respect, it is noteworthy that small and polygonal NADPH-d-reactive neurons are present within the smooth circular musculature of the intestine, and that roundish reactive neurons are present among the striated oesophageal muscle fibres. This notation may at present offer more than one explanation. It may be that in some organs of the Pantex gut nitrergic neurons are directly related to target structures with the aim to elicit peristalsis, whose reflexes are known to be entirely enteric (Furness and Costa, 1987).

It is noteworthy that the enteric nervous system of the Pantex lacks any reactivity to some putative accessory neuromediators (CGRP-, substance P-, VIP-, somatostatin-, met-enkephalin-like peptides, as well as serotonin), whereas it shows the presence of neurotrophin-receptors. Both the absence of the former substances and the presence of the latter ones possibly characterize the intramural innervation of the Pantex gut, and possibly make it different if compared with other fish (Bjenning and Holmgren, 1988; Kiliaan et al., 1993; Visus et al., 1996; Karila et al., 1998; Domeneghini et al., 1999, 2000; Lucini et al., 1999; Defzuli et al., 2000).

Trk A-, Trk B- and Trk C-like immunoreactivities were all detected in the Pantex enteric nervous system, above all in the myenteric plexus. The antisera against Trk A, Trk B and Trk C employed in this study react with the tyrosine-kinase catalytic domain of the specific Trk mammalian proteins. Since the amino acid sequences in fish neurotrophin receptors are highly homologous to those of mammals (Martin et al., 1995), we can assume that the Trk proteins evidenced in Pantex are equivalent to functional isoforms of mammalian proteins.

Trk B- and Trk C-like immunoreactivities were detected in epithelial endocrine cells of the pantex gut, too. In Trk-immunoreactive endocrine cells of the stomach of teleost fish, somatostatin- and CGRP-like-immunoreactivities were demonstrated by De Girolamo et al. (1999). The diffuse endocrine system of the Pantex gut not only contains epithelial cells immunoreactive towards Trk proteins, but also endocrine cells in which serotonin and a met-enkephalin-like peptide are immunohistochemically identifiable. Serotonin-immunoreactive endocrine cells are gastric, whereas met-enkephalin-like immunoreactivity is present in numerous epithelial endocrine cells of the intestine. Even if present together with other sometimes numerous endocrine cell types, and even if some different distributive patterns are described, these endocrine cell types are immunohistochemically detectable in several other fish (Elbal and Agulleiro, 1986; Abad et al., 1987; Elbal et al., 1988; Pan and Fang, 1993; Barrenechea et al., 1994; Reinecke et al., 1997; Domeneghini et al., 1999, 2000; Defzuli et al., 2000).

In conclusion, the present study has demonstrated that the gut neuroendocrine system of the Pantex possesses both similarities to that of other fish and some unique characteristics. Within the latter ones, it is noteworthy the presence of Trk proteins which, despite the paucity of neurotransmitters and putative neuromediators identified in this in comparison with other fish, possibly enlarges the functional roles of the gut neuroendocrine system.

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