Occurrence of two NOS isoforms in the developing gut of sea bass *Dicentrarchus labrax* (L.)

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Summary. In this work we have examined the appearance and distribution of nitric oxide synthase (NOS), with histochemical, immunohistochemical and biochemical methods, during development of the sea bass (*Dicentrarchus labrax*) gut.

The data showed that both the calcium-calmodulin dependent neuronal isoform (nNOS) and calcium-independent inducible isoform (iNOS) are present in the larval gut of sea bass. The nNOS-immunoreactivity was present in the epithelial cells and enteric nerve cells of gut both in the 8-day-old specimens and in the 24-day-old-larvae. In the adult nNOS-immunoreactivity disappeared from epithelial cells, remaining in the wall intramural neurons and fibers. The iNOS-immunoreactivity was present in the epithelial cells of 24-day-old-larvae and was not detectable in the adult gut. Western blot analysis and determination of NOS activity also demonstrated the presence of the two NOS isoforms, nNOS and iNOS, in the gut of 24-day-old specimens. The presumably different roles played by the two isoforms of enzyme are discussed. The presence of nNOS isoform in the gut enteric neurons of the same larval stages of *D. labrax* in which we previously demonstrated the presence of substance P and Vasoactive Intestinal Polypeptide (VIP), may suggest that all these three components of the motility control system are already present in the larval phase. Nitric oxide (NO) may be also involved in the early immune response. The present results on the occurrence of iNOS isoform in epithelial gut cells of the same regions in which the gut-associated lymphoid tissue (GALT) will differentiate, may suggest for NO a role in early defence mechanisms, before the establishment of immune responses in GALT. Finally, the developmental and regional differences in nNOS and iNOS expession also suggest a regulatory role in development and differentiation of the sea bass gut.

Introduction

The sea bass (*Dicentrarchus labrax*) is a species of great commercial interest. It is very important in aquaculture and thus highly studied. We are carrying out research set on appearance and localization of regulatory molecules during larval development of this fish (Mola et al., 2004, 2005; Pederzoli et al., 2004). We demonstrated the presence of substance P (SP) and vasoactive intestinal polypeptide (VIP) from the early stages of the gut development of *D. labrax*, hypothesising that these peptides play an important role as neuromodulators influencing the motility of the digestive tract from the early stages of development (Pederzoli et al., 2004). Indeed, it is known that the distension of the gut by a passing bolus generates two separate reflexes to the circular muscle layer; an ascending orally directed excitatory reflex and a descending anally directed inhibitory reflex. In most vertebrates, including teleosts, nitric oxide (NO) and/or VIP/PACAP often act as neuromodulators in the descending inhibitory reflex pathway, whereas the excitatory ascending way uses SP or related tachykinins (see Olsson and Holmgren, 2001).

NO also plays an important role in the immune-neuroendocrine communications by means of complex relationships with other molecules, including adrenocorticotropic hormone (ACTH), within the same cells or between different cells (for a review see Ottaviani et al., 1997). We showed that ACTH is linked to the early immune phenomena both in gut and in other organs of *D. labrax* (Mola et al., 2004, 2005).

NO produced in nerve cells is synthesised by the constitutive calcium-calmodulin dependent isoform of nitric oxide neuronal synthase (nNOS) and is released as picomoles for short periods in response to physiological stimulations. NO produced in immune cells is

synthesised by the inducible calcium independent isoform of nitric oxide synthase (iNOS) and is released as nanomoles over long periods. The conditions able to induce NOS, whose mechanisms are largely unknown, differ among cell types and several agents regulate the expression of iNOS (for a review see Ottaviani et al., 1997).

In this issue, we have examined the appearance and distribution of the two isoforms of NOS (nNOS and iNOS), with histochemical, immunohistochemical and biochemical methods, during development of the sea bass gut to verify the presence of nNOS in nerve cells of gut and/or of iNOS in gut cells with a possible immune role. Our aim was to better define the pattern of neuromodulators influencing the gut motility (among which NO, VIP and SP are known) and of immune-neuroendocrine communication molecules (among which NO and ACTH are known) in larval gut of *D. labrax.*

Material and methods

Animals

In our previous works (Mola et al., 2004, 2005; Pederzoli et al., 2004) we studied several larval stages of development of sea bass (from 2 to 24 day after hatching) and these research demonstrated that 8 and 24 days are "key" stages for developing gut, in particular for immune response (see Mola et al., 2005). For these reasons we chose these two stages for the present work.

Larvae of *Dicentrarchus labrax* (L.) of 8 and 24 days after hatching (respectively about 6 and 12 mm in length) and young adults of 5 months (8-10 cm in length) were obtained from the aquaculture fish-farm "Valle Figheri", Lova (Venice, Italy).

In this fish-farm the standard rearing conditions for larvae are: 24 h light photoperiod at 16-18°C temperature and 40‰ salinity for the first 12 days. Subsequently the salinity is lowered to 30‰. The diet consists of rotifers supplied from the 5th day to the 16th and newly hatched *Artemia* starting from the 10th. At the standard rearing conditions adopted from many years in the fish-farm "Valle Figheri" all the observations on living animals indicate that they start to feed exogenously at 4 days after hatching (moment of opening of the mouth). Thus 4-17 days represents the lecitoexotrophic phase (18 days is the moment of complete reabsorption of yolk). When the animals are 3 months-old (5-6 cm in length) they are transferred to large fishponds and the diet consists of pellets of plankton and powdered fish.

Before sacrifice, the fish were anaesthetized in benzocaine in cold tap water to avoid animal pain according to the guide lines of the Council for International Organization of Medical Sciences.

Larval specimens *in toto* (ten for each stage) and fragments of ten adult middle gut were fixed in freshly depolymerized 4% paraformaldehyde in 0.1M phosphate buffer saline (PBS) pH 7.4 for 24 h at 4°C. Then the specimens were cryoprotected in sucrose (25%) overnight at 4°C, cut in a 14 µm transversal section with a cryostat (Reichert-Jung) and mounted on chrome-alum coated slides. All slides were air-dried and processed for the following methods.

Histochemistry

The histochemical demonstration of NADPH-diaphorase was performed according to the method of Li and Furness (1993). After a brief washing in 0.1M Tris buffer (pH 7.6) the slides were incubated at room temperature in a medium containing 0.25 mg/ml Nitroblue Tetrazolium (NBT), 1 mg/ml ß-NADPH and 0.2% Triton X-100 in the same Tris buffer. The incubation was performed for 4h, renewing the mixture after 2h. The specificity of the staining was tested by omission of the substrate reaction. All reagents were obtained from Sigma (St. Louis, MO., USA). The reaction was stopped by immersion in the same buffer and the slides were mounted with gelatinated coverslips.

Immunohistochemistry

For Biotin Avidin System (BAS) technique the slides were rinsed three times in PBS 0.01M, pH 7.4 and then incubated for 30 min in 0.3% H_2O_2 to block endogenous peroxidase activity. They were then placed in PBS containing 0.3% Triton X-100 and blocked with 5% normal goat serum (DAKO, Denmark) for 30 min. The slides were incubated with the primary antibody in a humid chamber at 4°C overnight. Following rinsing with PBS, an incubation for 30 min at room temperature with the secondary antibody goat anti-rabbit (titer 1:300) (DAKO, Denmark) was performed. After rinsing in PBS and Tris 0.1M pH 7.6, an incubation with Avidin Biotin Complex (ABC, Vector, Vectastain, Burlingame, CA) in Tris for 45 min at room temperature followed. The reaction was visualized with 3,3'-diaminobenzidine tetrachloride (10 mg each 15 ml Tris) (Sigma). Immunostaining was allowed to develop in 10 min with 8 µl 30% H_2O_2. Negative control slides were prepared by omitting the primary antibody, substituting this with normal serum or by preadsorbing them in liquid phase with the homologous antigens (50 µg/ml diluted antiserum). All sections were rinsed in Tris and *aqua fontis*, dehydrated and mounted in Eukitt, and observed with a Zeiss Axioscop microscope.

The following rabbit polyclonal antibodies (Santa Cruz Biotecnology, Inc., U.S.A.) were used:
- antibody raised against a recombinant protein corresponding to aminoacid 2-300 mapping at the aminoterminus of NOS1 (H-299) of human origin (titer 1:150) for nNOS detection;
- antibody raised against a recombinant protein corresponding to aminoacid 2-175 mapping at the amino terminus of NOS2 (H-174) of human origin (titer 1:300) for iNOS detection.

Some slides were observed by Nomarski interference.

Western Blot analysis

Fifty larvae (24-days-old) and fifteen young adults (5-months-old) sea bass were quickly killed by decapitation. Decapited larvae and adult gut, after three washings in cold tap water, were homogenized with Ultra-Turrax (Heidolph DIAX 900, Germany) in three vol of ice-cold 50mM Tris-HCl buffer pH 7.5, containing 320mM sucrose, 1mM EDTA, 1mM dithiothreitol and a protease inhibitor cocktail. The homogenate was centrifuged at 20,000xg for 30 min at 4°C. The pellet was re-suspended twice with PBS, centrifuged and re-suspended with the homogenisation buffer. Western blots were conducted both on homogenized larvae and adult guts of *D. labrax*. Proteins (30 µg lane) were loaded on 7.5% SDS-polyacrylamide gel and the resolved proteins were electrophoretically transferred to immun-blot polyvinylidene difluoride membrane (Bio-Rad, Hercules CA, USA). Membranes were blocked with 0.5% fat-free dry milk in TBST (10mM Tris pH 7.5, 100mM NaCl and 0.1% Tween 20). Immunodetection of nNOS and iNOS proteins was performed using the same two rabbit policlonal antibodies utilised for immunohistochemistry and before cited, diluted 1:1000 with TBST. Membranes were washed in TBST, incubated with biotinylated goat anti-rabbit IgG (Calbiochem, Merck Biosciences, Germany) diluted 1:2000 in the same buffer and incubated with streptavidin-horseradish peroxidase conjugate (Calbiochem) diluted 1:2000 in TBST. Proteins recognized by the antibody were revealed by reaction with 4-chloro-1-naphtol (Calbiochem). Molecular weight markers were simultaneously electrophoresed and stained with Coomassie blue. Rat cerebellum lysate was used as positive control for nNOS immunodetection and LPS pretreated rat liver lysate (Transduction Laboratories, Lexington, KY) was used as positive control for iNOS immunodetection. All the other reagents were obtained from Bio-Rad (Hercules CA, U.S.A.). Specific bands were scanned and quantified using the NIH image 1.59 software (National Institute of Health, USA). Protein concentrations were determined according to Bradford using bovine serum albumin as a standard.

Determination of NOS activity

NOS activity in soluble and particulate fractions was assayed following the conversion of radiolabeled arginine to citrulline according to Conte and Ottaviani (1995). Standard reaction mixtures contained 50 mM HEPES pH 7.4, 0.5 mM EDTA, 1 mM dithiothreitol, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NADPH, 10 µM FAD, 10 µM FMN, 100 µM BH_4, 1 µM calmodulin, 12 mM L-valine, 1 mM citrulline, a variable amount of L-arginine, 80,000-100,000 cpm of purified L-[2,3,4,5-^{3}H]

arginine monohydrochloride (Amersham, Buckingham-shire, England) and 2-10 µl of the same lysate used for western blot analysis in a final volume of 100 µl. After 10 min incubation at 37°C, [^{3}H]citrulline was quantified by liquid scintillation counting after separation from [^{3}H]arginine by cation exchange (AG50WX-8). The Na^+ form of AG50WX-8 was prepared by washing the H^+ form of the resin with 1M NaOH four times and then washing with H_2O until the pH was less than 7.5. The total NOS (Ca^{2+}-dependent and Ca^{2+}-independent activity) was determined by calculating the difference between the [^{3}H]citrulline produced in the presence and in the absence of 10 mM NG-monomethyl-L-arginine (L-NMMA, an inhibitor of mammalian NOS) in standard reaction mixture. To evaluate the Ca^{2+}-independence activity, 6 mM EGTA was added to the standard assay mixture (free calcium concentration 20 nM). In some experiments, 6 mM EGTA was added to the standard mixture which contained 0.25 mM $CaCl_2$ instead of 1.4 mM $CaCl_2$ for 3 nM free Ca^{2+} concentration. The free calcium concentration was calculated following Fabiato (1988). Enzyme activity is given as nmol of [^{3}H]citrulline formed per min per g of lysate. All reagents were obtained from Sigma (St. Louis. MO., USA). The results obtained are expressed as means ± SD of four independent experiments performed in triplicate.

Results

Histochemistry

In the 8-day-old specimens, with both trophic and exotrophic feeding, the intestinal mucosa consisted only of epithelial cells, and specialised cells, i.e. mucocytes, were not yet recognizable. Moreover, longitudinal and circular muscular layers were not distinguished. All epithelial cells and many nervous fibers of the intestinal wall, at all levels of the gut, showed strong NADPH-diaphorase activity (Fig. 1A). In the 24-day-old specimens, completely passed to the exotrophic feeding, the morphology of the mucosa and muscolar layers were similar to those of the previous stage. The NADPH-diaphorase activity was abundant in all epithelial cells (Fig. 1B,C). Many intramural neurons and fibers of the wall also showed NADPH-diaphorase activity (Fig. 1B,C). In the middle gut of 5 month-old specimens the NADPH-diaphorase activity disappeared from the epithelial cells and was localized only in neurons and fibers of the wall at all levels of the middle gut (Fig. 1D).

Immunohistochemistry

In the 8-day-old specimens immunoreactivity (IR) to antibodies anti-nNOS was present in the majority of epithelial cells (about 80%) and in numerous nervous fibers of the intestinal wall, at all gut levels (Fig. 2A), without regional differences. No IR to antibodies anti-

iNOS was detected. In the 24-day-old larvae about 30-40% of epithelial cells showed IR-nNOS-like (Fig. 2B). Many intramural neurons and fibers of the wall were immunoreactive to nNOS antibodies (Fig. 2B). Some epithelial cells (about 5-10%) showed IR-iNOS-like (Fig. 2C). These patterns were detected at all levels of the gut without regional differences. In the middle gut of 5 month-old specimens the IR-nNOS-like and the IR-iNOS-like disappeared from the epithelial cells. The IR-nNOS-like (Fig. 2D) was localized only in intramural neurons and fibers of the wall at all levels of the middle gut.

The controls were negative in all stages both for histochemistry and immunohistochemistry.

Western blot analysis and determination of NOS activity

To characterize the NOS isoforms we analyzed cross reactivity of crude homogenate of decapitated larvae and adult guts by Western blotting with polyclonal antibodies against nNOS and iNOS. In the soluble fraction of larvae both nNOS and iNOS immunoreactive protein bands were present. No immunoreactive bands were present in the larvae particulate fraction. Western blot analysis revealed an immunoreactive nNOS protein band (Fig. 3) with an apparent molecular mass of 150 kDa with the same mobility of the nNOS immunoreactive band of rat cerebellum used as positive control and an immunoreactve iNOS protein band (Fig. 4) with a molecular mass of 130 kDa with the same mobility of hepatic iNOS from LPS pretreated rat liver used as positive control.

In the adult gut a very weak nNOS immunoreactive protein band, visible to the naked eye, was present both in soluble (Fig. 3) and in particulate fractions of crude homogenate. The immunoreactive band was not quantifiable by densitometry. No immunoreactive iNOS protein bands were present either in the adult gut soluble or particulate fraction.

In the soluble fraction of larval homogenate the total NOS activity measured by the conversion of radiolabeled arginine in citrullline was 0.81 ± 0.07
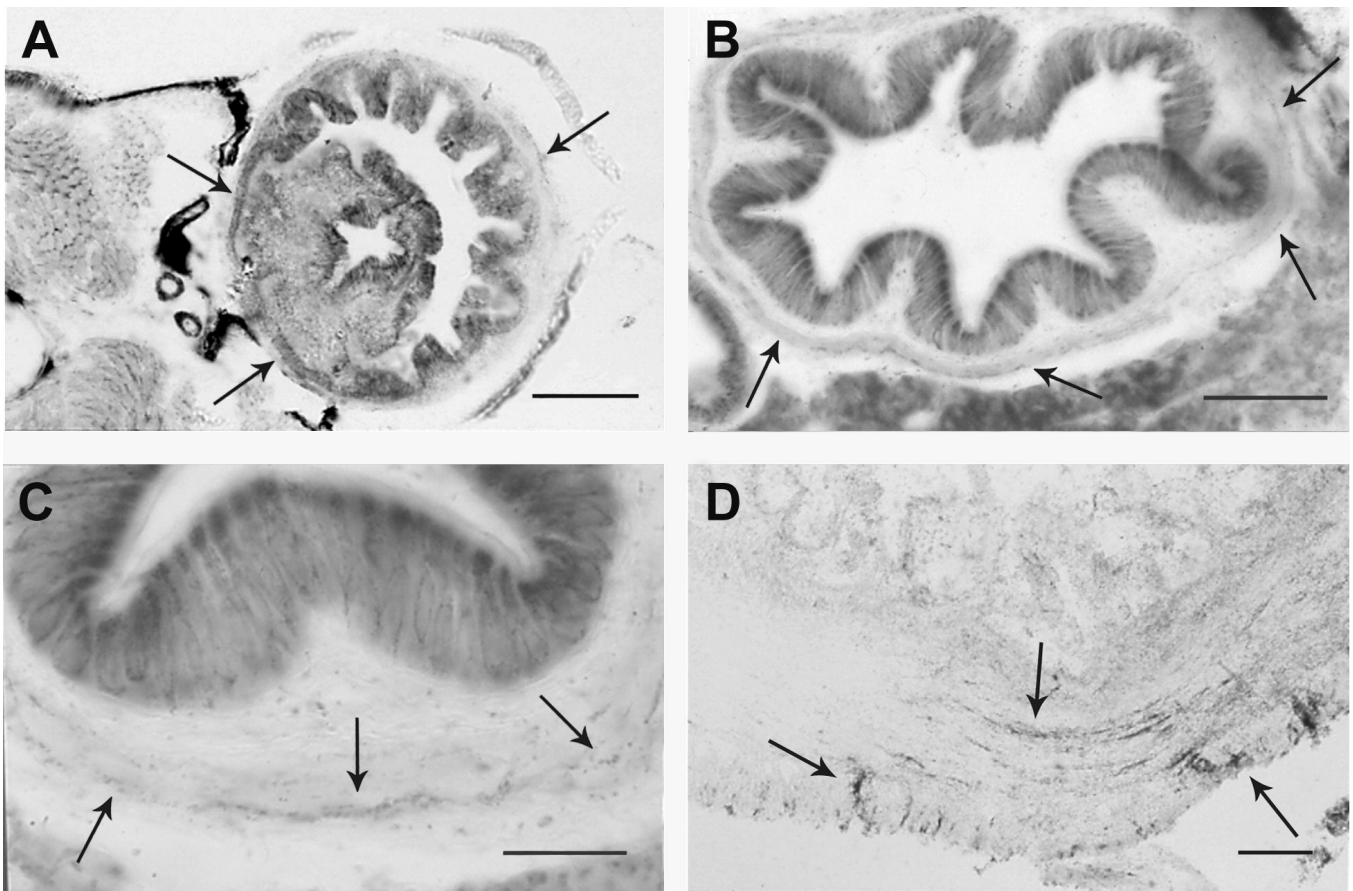

Fig. 1. Histochemical reaction for NADPH-diaphorase activity. **A.** Transversal section of gut at valve structure opening into the rectum level of 8-day-old larva of *Dicentrarchus labrax* with positivity to reaction in all epithelial cells and nerve fibers (arrows). Bar: 50 μm. **B.** Transversal section of middle gut of 24-day-old larva of *Dicentrarchus labrax* with positivity to reaction in all epithelial cells and nerve fibers (arrows). Bar: 50 μm. **C.** Enlargement of B. Bar: 150 μm. **D.** Transversal section of 5 month-old *Dicentrarchus labrax* gut. Positive nerve fibers to NADPH-diaphorase in the middle gut wall are indicated by arrows. Bar: 50 μm.

nmol/min/g of lysate. [3H]–citrulline formation decreased with increasing concentrations of the NOS inhibitor L-NMMA. With 10 mM L-NMMA concentration complete inhibition was obtained (enzyme activity lower than 1%) at 30 µM substrate concentration. In the particulate fraction of larval homogenate and in soluble and particulate fractions of adult gut, NOS activity was not detectable with this assay. The NOS activity in the larval solution fraction was significantly reduced by the addition of 6mM EGTA
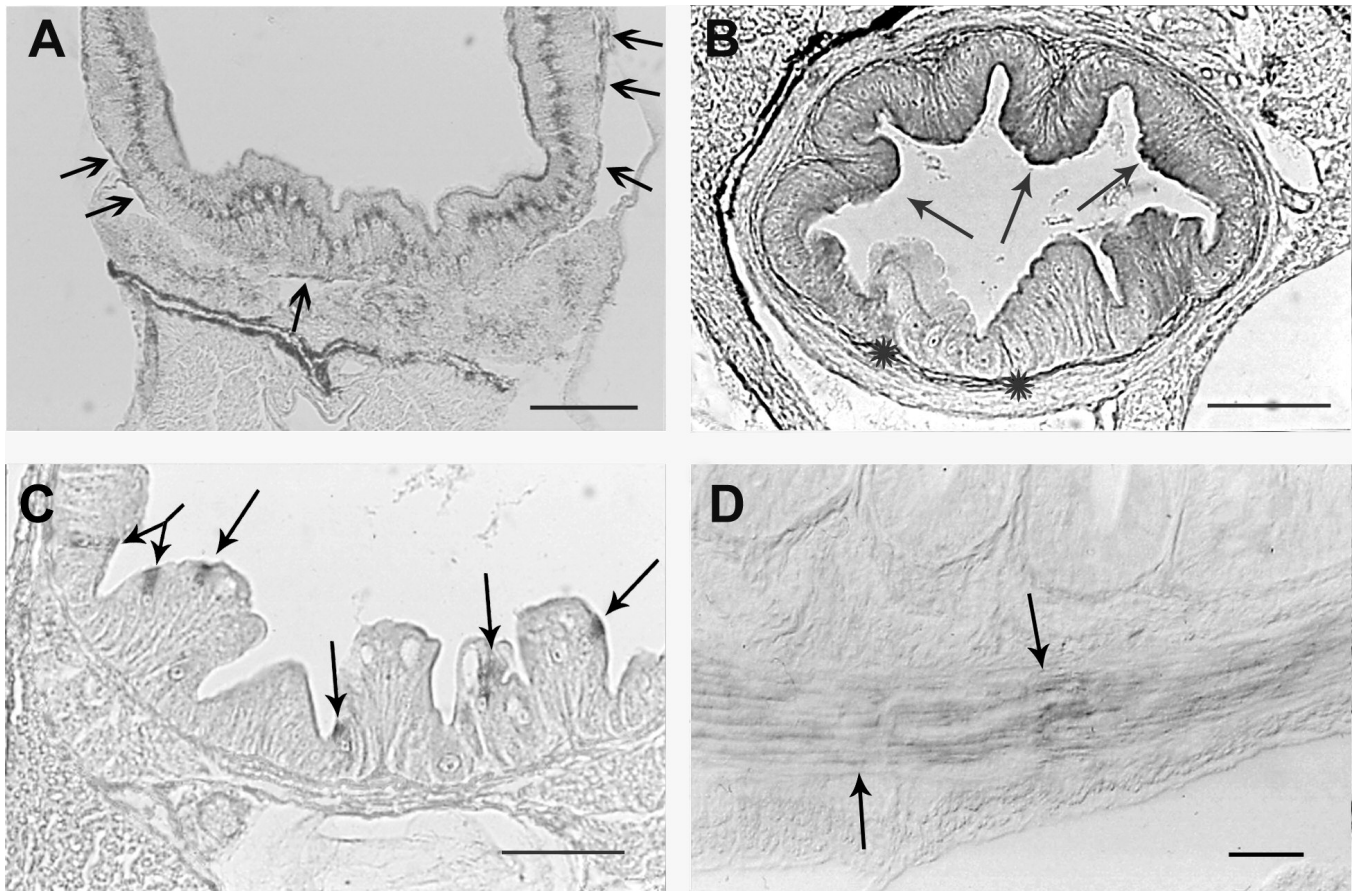
Fig. 2. Immunoreactivity to anti-NOS. **A.** Transversal section of middle gut in 8-day-old larvae of *Dicentrarchus labrax*. Immunopositivity to anti-nNOS in epithelial cells and nerve fibers (arrows). Bar: 50 µm. **B**. Arrows and asterisks indicate respectively many epithelial cells and several nerve fibers immunoreactive to anti-nNOS in the stomach of 24-day-old larva of *Dicentrarchus labrax*. Nomarski interference. Bar: 50 µm. **C.** A few epithelial cells immunoreactive to anti-iNOS in the middle gut of 24-day-old larva of *Dicentrarchus labrax* are indicated by arrows. Bar: 100 µm. **D.** Immunohistochemical reaction with anti-nNOS in nerve fibers of the middle gut wall (arrows) of 5 month-old *Dicentrarchus labrax* gut. Bars: 50 µm.
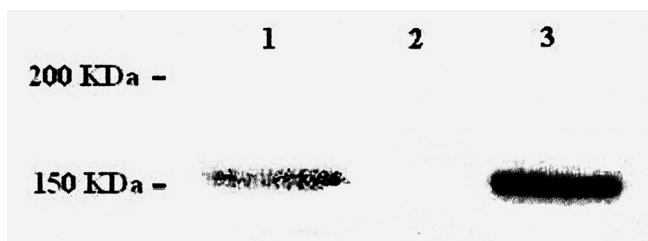
Fig. 3. Immunodetection of nNOS in the soluble fraction of *Dicentrarchus labrax*. Lane 1: *D. labrax* decapitated larvae (24-day-old); lane 2: *D. labrax* 5-month-old gut; lane 3: rat cerebellum.
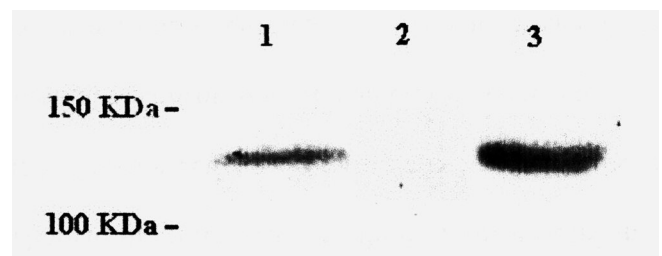
Fig. 4. Immunodetection of iNOS in the soluble fraction of *Dicentrarchus labrax*. Lane 1: *D. labrax* decapitated larvae (24-day-old); lane 2: *D. labrax* 5-month-old gut; lane 3: LPS pretreated rat liver.

which decreases the free Ca^{2+} concentration to about 20 nM (0.43±0.03 nmol/min/g of lysate). The fall in the free Ca2+ concentration to about 3 nM does not further decrease the enzyme activity. These results showed that about 55% of the total larval NOS activity is Ca^{2+} independent.

Discussion

It is well know from many years that all known NOS are NADPH-d, but that NOS represents only a fraction of the total cellular NADPH-d activity. Therefore the NADPH-d reaction may be regarded only as an indicative method for NOS in the nervous system (Schmidt et al., 1992; Matsumoto et al., 1993; Furness et al., 1994; Timmermans et al., 1994; Spessert and Claassen, 1998; Cuoghi et al., 2002). In the sea bass a very high discrepancy between histochemical and immunohistochemical results has been observed in the epithelial cells of the larval gut, whereas a quite good overlap of two methods has been detected in the enteric nervous system.

The data showed that both the calcium-calmodulin dependent nNOS and calcium-independent iNOS were present in the larval gut of sea bass.

In particular, the immunohistochemical results showed that nNOS-IR was present in the epithelial cells and intramural nerve cells of gut both in the 8-day-old specimens and in the 24-day-old-larvae. In the adult nNOS-IR disappeared form epithelial cells, remaining in the intramural neurons and fibers. The iNOS-IR was present in the epithelial cells of 24-day-old-larvae and was not detectable in the adult gut.

Western blot analysis also demonstrated the presence of the two NOS isoforms, nNOS and iNOS, in the gut of 24-day-old specimens. The results were confirmed by the NOS activity determination with radiolabeled arginine. When the NOS activity was assayed at very low calcium concentration it significantly decreased but about 55% of the enzyme activity remained suggesting the presence of a calcium independent NOS activity. In other fish the presence of Ca^{2+}-dependent (Conte and Ottaviani, 1998; Conte, 2003) and Ca^{2+}-independent (Conte, 2003) NOS isoforms has been demonstrated in brain and liver of *Cyprinus carpio* and *Carassius auratus*.

The NO produced by the two isoforms of enzyme presumably play two different roles during the development of sea bass gut.

Hormones, autonomic nerves and the presence of food control peristalsis in vertebrates, including fish. The bolus may cause release of 5-HT from endocrine cells which stimulate sensory nerve endings of intrinsic neurons located in the myenteric or submucous plexus (Furness and Costa, 1987; Kunze and Furness, 1999; see also Olsson and Holmgren, 2001). The sensory neurons activate ascending and descending interneurons. Ascending projecting interneurons release acetylcholine and tachykinins, acting on excitatory motoneurons

innervating the circular muscle (Karila and Holmgren, 1995; Timmermans et al., 1997; Karila et al., 1998; Kunze and Furness, 1999). Descending projecting interneurons release NO and VIP/PACAP in addition to acetylcholine (Olsson and Karila, 1995; Karila and Holmgren, 1997; Timmermans et al., 1997; Kunze and Furness, 1999). NO often acts as a mediator or potentiator of the inhibitory effect of neurotransmitters, or stimulates the release of inhibitory transmitters (Olsson and Holmgren, 2001). In mammals NO may mediate the inhibitory responses to neurotransmitters, such as GABA, ATP, calcitonin gene-related peptide (CGRP), serotonin and CCK, and also stimulates acid gastric secretion and intestinal ion transport (see Olsson and Holmgren, 2001).

In a previous work (Pederzoli et al., 2004) we suggested that SP and VIP play an important role as neuromodulators, influencing the motility of the digestive tract, from the early stages of the gut development of *D. labrax*, also prior to exotrophic feeding. In particular, we demonstrated that the appearance of SP-IR in the peptidergic nervous system begins from the rectum and follows a distal to proximal gradient, whereas VIP-IR begins proximally and progresses along a proximal to distal gradient. The calcium-calmodulin dependent nNOS isoform is present in the gut wall neurons of the same larval stages of *D. labrax*, demonstrating that all three components of the motility control system (SP, VIP, NO) are already present in early stages of gut development.

In zebrafish both excitatory and inhibitory pathways, mediated by acetylcholine, PACAP and neurokin A, develop at an early stage in the gut, independent of exogenous feeding (Holmberg et al., 2004). Moreover, between 40 and 55 hours post fertilization, nNOS mRNA expression started in peripheral organs, forming distinct cell populations after hatching within or in the vicinity of several organs, including enteric ganglia, gut and nephritic ducts (Holmquist et al., 2004).

Recently it has been suggested that in zebrafish a nitrergic inhibitory tonus develops shortly before or at the time for onset of exogenous feeding. The guts of zebrafish larvae begin responding to the inhibitory effects of NO at about the same time that they become responsive to acetylcholine, with a probable co-functionality between these two pathways (Holmberg et al., 2006).

In the sea bass the maturation of cell-mediated immune responses and humoral immune system takes place respectively around the first and second month post hatching (Scapigliati et al., 2002). Considering that ACTH is one of the main actors in the stress response (Ottaviani et al., 1997), the immunohistochemical detection of ACTH-like material in the same regions of the sea bass gut in which the gut-associated lymphoid tissue (GALT) will differentiate, suggests a role for ACTH in early defence mechanisms in sea bass, before the establishment of cell-mediated immune responses in GALT (Mola et al., 2004). Other research shows a

precocious ACTH-like immunopositivity during the development of *D. labrax* (from 2 to 24 days post hatching) into various hemopoietic and/or lymphopoietic organs, such as yolk sac, pronephros tubules, thymus, liver, spleen and blood veins, as well as areas of the external environment (skin), and a modified pattern of immunoreactivity in the bacterial products (LPS)-treated samples, confirming that ACTH could be involved in the immune defence mechanisms. The distribution of the ACTH- and ACTH receptor-like molecules indicates a paracrine and/or autocrine way of action (Mola et al., 2005).

Vertebrate NO is an effector component of cell-mediated immune response and many factors, such as cytokines, glucorticoids, LPS, regulate the expression of iNOS (Ottaviani et al., 1997). Moreover, the immune, stress and inflammation responses appear to be mediated by a common pool of molecules, among which are ACTH and NO, and complex and intriguing relationships among these molecules are known (Ottaviani et al., 1997).

The present results on the occurrence of iNOS isoform, which appeared in the epithelial cells of 24-day-old-larvae and was not detectable in the adult gut, could be set in this background. Thus, the presence of inducible NOS in the same regions of the sea bass gut in which the GALT will differentiate, may also suggests a role for NO in early defence mechanisms, before the establishment of immune responses in GALT, possibly toghether with ACTH.

Compared to mammalian studies there are relatively few studies on roles of NO in the fish immune responses. The presence of an inducible NOS isoform has been demonstrated in liver and kidney of rainbow trout indicative of NO involvement in the immune response in fish gut (Barroso et al., 2000). An increased number of nerve components immunoreactive to NOS and typical gastrointestinal peptides antibodies has been found in the gut of *Leuciscus cephalus* infected by Acanthocephala (Bosi et al., 2005). Concerning the early immune response, there is evidence of an active role of NO, together with different cytokines, in early antiviral immune defence of rainbow trout (Tafalla et al., 2005). Moreover, 2 days post fertilization embryos of *Cyprinus carpio* responded to LPS with upregulation of iNOS and some cytokines, implying a functional embryonic innate defence system (Huttenhuis et al., 2006). Finally, a possible role of NO in stress-induced drinking of larval and juvenile fish has recently been suggested, hypothesising that NO could have an important environmental influence for aquatic organisms (Eddy, 2005).

We cannot exclude other roles for NO in the gut of *D. labrax* larvae. The disappearance of the NOS, both of isoform inducible and isoform constitutive, from the gut epithelium in the adult (at the end of larval phase) could suggest a regulatory role in development and differentiation of the fish gut.

It has been demonstrated that NO is involved in the

differentiation of neuronal elements during embryonic development of the gut in various species (Van Ginneken et al., 1998, 2001; Bagyanszki et al., 2000; Belai and Burnstock 2000; Bayram et al., 2002). Moreover NO is also involved in the gastrointestinal development of non neuronal tissues: nNOS expression in the rat stomach occurs early during embryonic life and the development of the gastric wall i.e. smooth muscle layer, epithelium, and blood vessels, may be closely associated with nNOS-immunoreactive neuronal elements (Bayram et al., 2002). Tissue- and development- expressional control of nNOS has been described (Lee et al., 1997; Förstermann et al., 1998) demonstrating that biological activity of nNOS is highly and specifically regulated by spatial and temporal modulation of its expression. The indications that the expression of this synthase is dynamic and adaptive (Van Ginneken et al., 2001; Bayram et al., 2002) could explain the high decrease of nNOS in enzymatic and Western blot assays for sea bass adults.

iNOS isoform also may be involved in gut development of *D. labrax*. In differentiating PC12 neuronal cells the development of the differentiated phenotype is preceded by an elevation of NOS activity and a substantial part of NO is produced by an inducible form (Peunova and Enikolopov, 1995).

For fish a possible involvement of NO in processes such as neurogenesis, organogenesis and early physiology has already been advanced in zebrafish by Holmquist et al. (2004). Developmental and regional differences in nNOS and iNOS expession in the gut of *D. labrax* could suggest an involvement of NO as an important mediator also in the gastrointestinal development of this fish.

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