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Cellular localization of insulin-like growth factor-II protein in the sea bass (*Dicentrarchus labrax*) from hatching to adult

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Summary. The cellular localization of IGF-II protein was investigated during larval and postlarval developmental stages of sea bass (*Dicentrarchus labrax*) by immunohistochemistry using antisera raised against *Sparus aurata* IGF-II. At hatching, IGF-II immunoreactivity was already present in the skin, developing intestine and skeletal muscle. During larval life IGF-II protein was also observed in heart musculature, in kidney and gill epithelia as well as in liver. In fry skeletal muscle a moderate IGF-II immunostaining was detected in red fibres, whereas white muscle fibres exhibited a faint immunoreactivity. In adults, a marked IGF-II immunostaining was observed in red muscle fibres. A moderate immunoreactivity was also present in white fibres as well as in heart striated myocardial fibres. These results are in agreement with previous findings on the spatial localization of IGF-II and IGF type 1 receptor in *S. aurata* and *Umbrina cirrosa*, confirming the role of IGF system during development and growth of fish.

Key words: IGF-II, Immunohistochemistry, *D. labrax*

Introduction

Growth as a biological phenomenon is controlled by a complex of endo-, para-, and autocrine control mechanisms. The insulin growth factor (IGF) complex plays a key role in growth regulation together with insulin, thyroid hormones, sex steroids, and the growth hormone (Lowe, 1991; Jones and Clemmons, 1995; Reinecke and Collet, 1998).

The IGF system consists of peptides closely related family which includes the two highly conserved primary ligands, IGF-I and IGF-II, high-affinity transmembrane receptors that belong to the insulin/IGF receptor family, and six specific high-affinity IGF-binding proteins (IGFBP-1 to -6) (reviewed in Peter and Marchant, 1995; Reinecke and Collet, 1998; Moriyama et al., 2000). IGFs are growth-promoting peptides, which are structurally homologous with insulin, and also their biological effects are similar to those of insulin. Insulin is synthesized exclusively in the pancreatic islets of Langerhans, while IGFs are synthesized in several tissues throughout the body. In mammals, IGF-I is a mediator of growth hormone action and it is important during the postnatal growth, having a significant role in many conditions, including normal organ development and mechanisms associated with tissue function and maintenance (Reinecke and Collet, 1998). As in mammals, IGF-I is produced mainly in the liver of bony fish, although numerous other organs also express IGF-I (Funkenstein et al., 1997; Reinecke et al., 1997, 2005; Duan, 1998; Reinecke and Collet, 1998; Perrot et al., 1999; Schmid et al., 1999; Radaelli et al., 2003b; Wood et al., 2005; Berishvili et al., 2006a,b; Eppler et al., 2007; Hevrøy et al., 2007). IGF-II is a 67-amino acid single chain polypeptide that shows a structural sequence similar to IGF-I. In mammals, IGF-II mRNA transcripts are present early during development, suggesting a main role for this protein during fetal development (Han et al., 1988; Daughaday and Rotwein, 1989, De Chiara et al., 1990; Singh et al., 1991). The presence of IGF-II mRNA in larvae, fry and adults of bony fishes is in contrast with the situation observed in mammals (for review, see Reinecke and Collet, 1998). In bony fishes IGF-II mRNA has been detected in liver as well as in numerous other organs (Duguay et al., 1996; Collet et al., 1997; Perrot et al., 1999; Loffing-Cueni et al., 1999; Degger et al., 2001; Ayson et al., 2002; Tse et al., 2002; Radaelli et al., 2003a; Vong et al., 2003; Caelers et al., 2004; Carnevali et al., 2005; Wood et al., 2005; Funes et al., 2006; Patruno et al., 2006; Hevrøy et

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al., 2007) and its expression is under the control of GH (Reinecke et al., 2005). Recently, it has been demonstrated that IGF-II is involved in starvation in rabbitfish (Ayson et al., 2007) and rainbow trout (Gabillard et al., 2006). Moreover, the nutritional regulation of IGF-II has been observed in Atlantic salmon (Wilkinson et al., 2006). Recently, Wilkinson et al. (2006) demonstrated that handling and confinement resulted in significant decreases in circulating levels of IGF-I and IGF-II in Atlantic salmon and rainbow trout.

In contrast to IGF-I, the information regarding the cellular localization of IGF-II protein during development and growth of fish is very scarce. Only our previous studies reported the localization of IGF-II protein during ontogeny in *S. aurata* (Radaelli et al., 2003a) and *Umbrina cirrosa* (Patruno et al., 2006).

The aim of this study was to localize the cellular sites of IGF-II peptide during development and growth of sea bass (*D. labrax*) by immunohistochemistry using a polyclonal antibody against S. aurata IGF-II.

Materials and methods

Fish samples and tissue processing

Larvae (0, 2, 4, 6, 10, 25, 40 days), fry (80 days) and adults of sea bass were obtained from the Pellestrina (VE, Italy) fish hatchery and from the Bonello (RO, Italy) fish farm, respectively, and were killed by an overdose of MS222 (Sandoz, Milan, Italy) anaesthesia. Animals and tissues were fixed in 4% paraformaldehyde prepared in PBS (0.1 M, pH 7.4) at 4°C overnight. Small fish (hatching to juveniles) were fixed *in toto* and longitudinal (both frontal and sagittal) and transversal sections were cut. Organ and tissue samples were dissected out from large adult fish and processed separately.

Immunohistochemistry

Fixation and embedding

Samples fixed as described above were washed in PBS, dehydrated through a graded series of ethanol and embedded in paraffin. Sections were cut at a thickness of 4 µm using a microtome.

Antisera

A polyclonal antibody against *S. aurata* IGF-II, raised in mouse (Eurogentec, Brussels, Belgium), was used at a dilution of 1:500 (Radaelli et al., 2003a).

Immunohistochemical procedure

Immunohistochemical staining was done using the Envision system (goat-anti-mouse immunoglobulins conjugated to peroxidase-labeled complex, Dako, Italy). Before applying the primary antibody, endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in PBS. Non-specific binding sites were blocked by incubating the sections in 1:5 dilution of mouse serum (Dako, Italy). The immunoreactive sites were visualized using diaminobenzidine (DAB) (Sigma, Italy) as the chromogen. To ascertain structural details, sections were counterstained with Mayer's haematoxylin.

Controls

The specificity of the immunostaining was verified by incubating sections with: (1) PBS instead of the specific primary antibodies (see above); (2) preimmune sera instead of the primary antisera; (3) PBS instead of the secondary antibodies; (4) by absorption of the antisera with excess of synthetic peptides (3 mg/ml) before incubation with sections. The results of these controls were negative (i.e. staining was abolished).

Results

Immunohistochemical localization of IGF-II

General

Immunohistochemical localization of IGF-II in different tissues is summarized in Table 1.

Larval stages

At hatching, IGF-II immunoreactivity was present in the epithelia of skin and developing intestine (Fig. 1A). A faint IGF-II positivity was also present in the new muscle fibres produced during the myofibrillogenesis process (Fig. 1A). At all stages examined, epithelial cells of the epidermis exhibited an immunostaining, whereas mucous cells were negative (Fig. 1A). Epithelial cells of the yolk sac (when still present) showed a moderate

Table 1. Immunohistochemical localization of IGF-II in sea bass.

Tissue	0-4 days	5-10 days	25-40 days	80 days	Adults
Skeletal Muscle	±	+	+	$+^{R}$ $\pm^{W}$	$++^{R}$ $+^{W}$
Heart	*	-	++	+	+
Gut epithelium	+	++	++	+	+
Kidney epithelium	*	+	+	*	±
Gill epithelium	-	-	++	++	++
Skin	+	+	++	++	+
Pancreas	-	±	-	-	-
Liver	±	±	++	++	++
Yolk sac	+				

Staining: -, not detectable; ±, slight but above background levels; +, moderate; ++, marked staining; +++, very strong. *: tissue not found on the sections examined at this stage. $^{R, W}$: red and white muscle fibers.

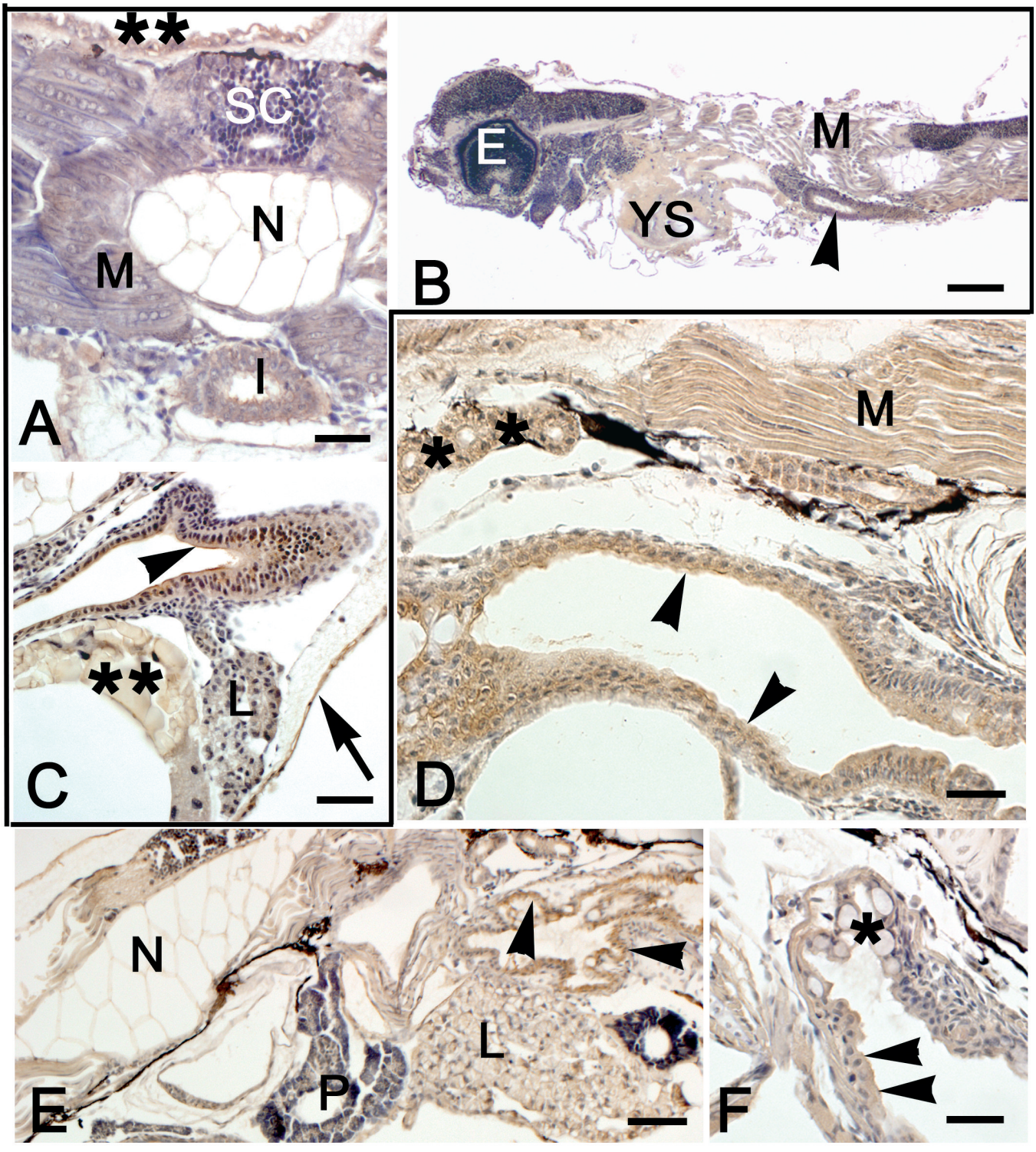

Fig. 1. Immunohistochemical localization of IGF-II in sea bass larvae. Panels **A-C** (boxed area) represent larvae aged 0-4 days; panels **D-F** (unboxed area) represent larvae aged 6-10 days. All panels are counterstained with haematoxylin. **A.** Transverse section of a larva at hatching. Immunostaining is found in the epithelia of developing intestine (I), and skin (asterisks). A faint IGF-II positivity is also present in the new muscle fibres (M) that are produced during the myofibrillogenesis process. SC, spinal cord; N, notochord. **B.** Sagittal section of a 2-day larva. Arrowhead indicates immunopositive intestine. E, eye; M, lateral muscle; YS, yolk sac. **C.** In a 4-day larva, epithelium of intestine and brush border (arrowhead) are positive, whereas liver parenchyma (L) shows a faint IGF-II immunoreactivity. Epithelium of skin (arrow) is positive too. Asterisks, yolk sac. **D.** Sagittal section of a 6-day larva. Immunoreactivity is present in developing intestinal epithelium (arrowheads) and in lateral muscle (M). Asterisks indicate developing renal tubules which show IGF-II immunostaining. **E.** Sagittal section of a 10-day larva showing IGF-II immunostaining in the epithelium of intestine (arrowhead). A very faint immunostaining is also present in the parenchyma of liver (L) and pancreas (P). N, notochord. **F.** Pharynx of a 10-day larva showing IGF-II immunostaining in the epithelium (arrowheads). Asterisk indicates negative mucous cells. Bars represent: A, C, D, F, 20 µm; B, 100 µm; E, 40 µm

immunoreactivity to IGF-II antiserum (Table 1, Fig. 1B, C). From day 4, IGF-II immunostaining was found in the brush border of intestinal epithelium (Fig. 1C) and in liver parenchyma (Fig. 1C). From day 6, a moderate immunoreactivity was detected in developing renal tubules (Fig. 1D). At around 10 days post-hatching, a faint IGF-II immunoreactivity was detected in the parenchyma of pancreas (Fig. 1E) and in the epithelium of pharynx (Fig. 1F). From day 25, the epithelium of pharynx showed a marked IGF-II immunostaining, although mucous cells were negative (Fig. 2A). At the same stage, a marked IGF-II immunostaining was observed in the epithelium of the intestine (Fig. 2B), in liver parenchyma (Fig. 2B) and in heart striated myocardial fibres (Fig. 2C). From day 40, the gut IGF-II positivity was mainly detected in the intestinal brush border (Fig. 2D).

Postlarval stages

After metamorphosis, a marked IGF-II immunostaining was detected in skin, gill epithelium and liver parenchyma (Table 1). A moderate IGF-II immunostaining was observed in gut epithelium and in heart striated myocardial fibres (Table 1). In skeletal muscle, a moderate immunostaining was observed in the red fibres, whereas in white deep muscle fibres the immunoreactivity was faint (Table 1).

Adults

A marked IGF-II immunostaining was detected in

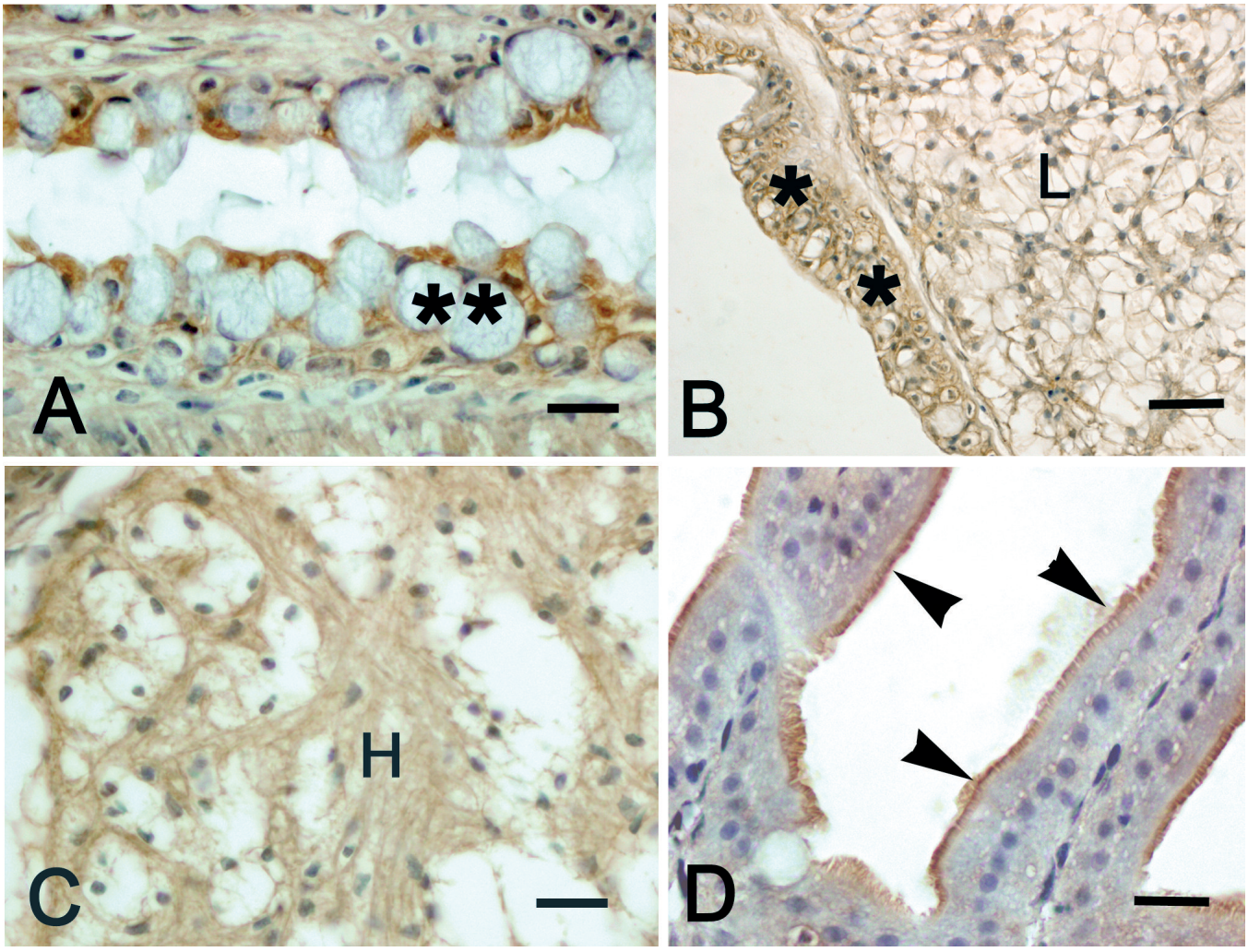

Fig 2. Immunohistochemical localization of IGF-II in sea bass larvae. All panels are counterstained with haematoxylin. **A.** Pharynx of a 25-day larva showing intense immunoreactivity in the epithelium. Mucous cells (asterisks) are negative. **B.** In a 25-day larva, IGF-II immunostaining is present in the epithelium of intestine (asterisks) as well as in liver (L). **C.** Heart (H) of a 25-day larva showing a marked IGF-II immunostaining in the striated myocardial fibres. **D.** In a 40-day larva, IGF-II immunostaining is present in the brush border of intestinal epithelium (arrowheads). Bars represent: A, C, D, 10 µm; B, 20 µm.

the epithelium lining primary and secondary lamellae of gills (Fig. 3A) and in liver parenchyma (Fig. 3B). In the heart, striated myocardial fibres showed a moderate IGF-II immunostaining (Fig. 3C). In the skeletal muscle, a marked immunoreactivity was present in red fibres, whereas white deep fibres exhibited a mosaic of negative and moderately positive fibres (Fig. 3D, insert).

Discussion

The present study reports the cellular localization of IGF-II peptide throughout a complete life cycle, from hatching to adult stages of *D. labrax*, by immunohistochemistry using a polyclonal anti-IGF-II antibody. The efficiency of the antibody, which was raised in mouse against a synthetic peptide, in recognizing *S. aurata* IGF-II has been tested by western blot using recombinant *S. aurata* IGF-II (Radaelli et al., 2003a). *D. labrax* was chosen in order to better understand the cellular immunolocalization of IGF-II in teleost fishes of interest in aquaculture, since the precise distribution of IGF-II peptide has been analyzed only in few works of our group (Radaelli et al, 2003a; Patruno et al., 2006). In general, the pattern of immunostaining for IGF-II observed in *D. labrax* is similar to that obtained in *S. aurata* (Radaelli et al., 2003b) and *U. cirrosa* (Patruno et al., 2006), indicating that the sequence similarity between IGF-II from different fish species is sufficient to allow cross-species immunoreactivity. This study also provides information on changes in the spatial localization of IGF-II during posthatching development in this species, demonstrating the presence of the peptide not only in liver but in extrahepatic tissues too. These results are also in agreement with those observed for type 1 IGF-I receptor (IGF-IR) in *S. aurata* (Perrot et al., 1999) and *U. cirrosa* (Radaelli et al., 2003b) and for IGFBP-2 in *S. aurata* (Radaelli et al., 2005) and *U. cirrosa* (Radaelli et al., 2003b). In the liver, which represents the main site for IGF-II production, the peptide has been detected throughout the complete life cycle of *D. labrax*, although the immunostaining was weaker in early larval than postlarval stages as observed

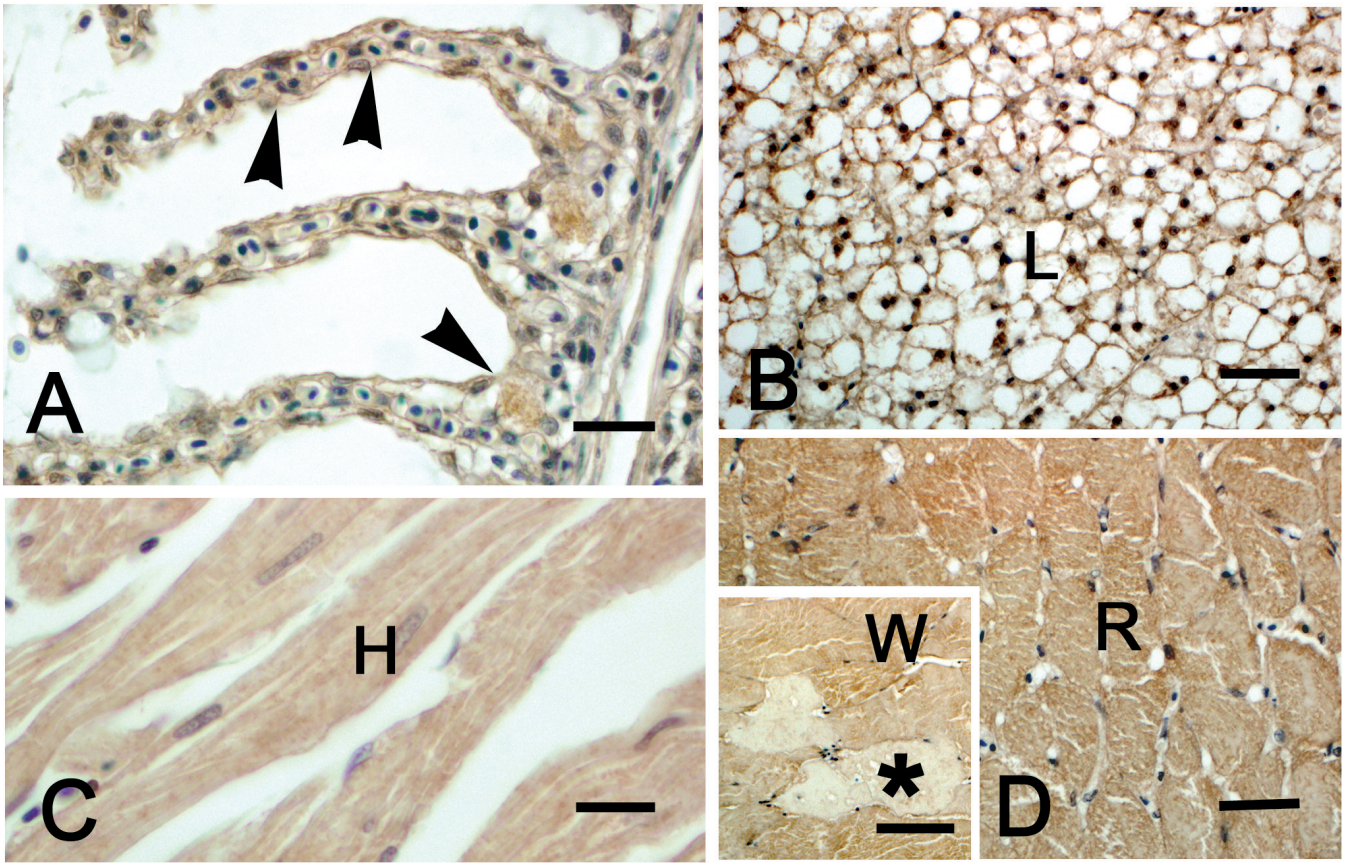

Fig. 3. Immunohistochemical localization of IGF-II in sea bass adults. All panels are counterstained with haematoxylin. **A.** In gills, IGF-II immunostaining is present in the epithelial cells lining the primary and secondary (arrowheads) lamellae. **B.** Hepatocytes of liver parenchyma (L) showing IGF-II immunostaining. **C.** Striated myocardial fibres of the heart (H) showing a moderate IGF-II immunostaining. **D.** Transverse section of the lateral muscle showing a marked immunostaining in red fibres (R). Insert represents a mosaic of white muscle fibres (W) showing a moderate immunostaining. Few fibres are negative (asterisk). Bars represent: A, C, 10 µm; B, D, 20 µm; insert in D, 40 µm.

in *U. cirrosa* (Patruno et al., 2006). Similarly, in humans, the liver seems to be the major site for IGF-II expression, and serum concentrations are low during development, increase after birth, and persist at high levels throughout life (Reinecke and Collet, 1998). A faint IGF-II immunoreactivity was also observed in the exocrine pancreas of larvae aged 10 days, and this result is in agreement with those reported in mammals, where IGF-II mRNA was found in murine and human fetal pancreas, and IGF-II peptide was produced by human fetal pancreas in culture (reviewed in Reinecke and Collet, 1998). A similar pattern of IGF-II cellular localization was also observed in the pancreas of U. cirrosa, although immunoreactivity was detected in both larvae and postlarvae (Patruno et al., 2006). In *D. labrax*, as in mammals, IGF-II seems to regulate pancreas growth only in early developmental stages. Immunoreactive IGF-II in the epithelial layer of pharynx and intestine found in *D. labrax* larvae, fry and adults supports a role for IGF-II in stimulating proliferation of these cells as described in mammals. A similar pattern of IGF-II localization has been described in *S. aurata* (Radaelli et al., 2003a) and *U. cirrosa* (Patruno et al., 2006). Moreover, the present results are in agreement with those observed earlier with respect to IGF-IR (Perrot et al., 1999; Radaelli et al., 2003b) supporting a paracrine/autocrine action of IGF-II in promoting cell proliferation during development of pharynx and intestine. In this study, the presence of IGF-II peptide in the epithelial cells of skin, gill filaments and kidney tubules supports a role for IGF-II in osmoregulation. Immunostaining of IGF-II in the same tissues was found in *S. aurata* (Radaelli et al., 2003a) and *U. cirrosa* (Patruno et al., 2006) where these tissues were also positive for IGF-IR (Perrot et al., 1999; Radaelli et al., 2003b). From day 25, IGF-II immunoreactivity was also found in the heart musculature, confirming our previous results where IGF-II peptide was detected in the heart of *S. aurata* (Radaelli et al., 2003a) and *U. cirrosa* (Patruno et al., 2006). A possible role of IGF-II in the physiology of heart musculature has been described in mammals and chickens (Armstrong et al., 2000; Kotlyar et al., 2001). IGF-II immunostaining detected in the skeletal muscle of young larvae suggests a possible role of this growth factor during the myofibrillogenesis process, confirming our previous data for *S. aurata* (Radaelli et al., 2003a) and *U. cirrosa* (Patruno et al., 2006). In fry and adults, IGF-II peptide was mainly detected in red muscle fibres, whereas white muscle exhibited a mosaic of negative and moderately positive fibres. This result is in contrast with our previous observations in *S. aurata* (Radaelli et al., 2003a) and *U. cirrosa* (Patruno et al., 2006) where white muscle fibres were IGF-II immunonegative, suggesting that in *D. labrax* IGF-II acts as a growth regulator of both red and white fibres. In fish, a possible role of IGF-II during growth has been suggested by Peterson et al. (2004), who demonstrated that levels of IGF-II mRNA was higher in the white muscle of fast growing fish compared to slow growing fish. The importance of IGF-II not only in early stages, but also later in life, has been reported by several authors (Caelers et al., 2003, 2004; Chauvigne et al., 2003; Radaelli et al., 2003a; Vong et al., 2003; Caelers et al., 2004) and this is in agreement with the extensive postlarval muscle hyperplasia which characterizes many fish species (Rowlerson et al., 1995; Rowlerson and Veggetti, 2001). In contrast, few studies describe a possible role of IGF-II only during early stages of development (Méndez et al., 2001; Gabillard et al., 2003; Montserrat et al., 2007). In fish embryos, Méndez et al. (2001) described that IGF-II/M6-P receptors bind IGF-II with high affinity. These authors suggest that the role of the IGF-II/M6-P receptor could be to bind and degrade IGF-II, therefore regulating the effects of the mitogenic peptide. In our work, co-localization of IGF-IR, IGF-I, IGF-II and myostatin (MSTN) peptides, strongly suggests an autocrine-paracrine action of IGFs and MSTN in the cultured muscle explants from S. aurata (Funkenstein et al., 2006), similar to that occurring *in vivo* (Radaelli et al., 2003a,b,c). Moreover, our data demonstrate that IGF-II peptide is detectable in different tissues and organs; these results are in agreement with those found for IGF-I and IGF-II by several authors (Perrot et al., 1999; Radaelli et al., 2003a,b; Berishvili et al., 2006b; Patruno et al., 2006), confirming an autocrine-paracrine action of IGFs in fish. In conclusion, the spatial localization of IGF-II peptide observed in the present study is consistent with previous findings on the cellular distribution of both peptide and IGF type I receptor in *S. aurata* and *U. cirrosa*, indicating that IGF-II role is important throughout the complete life cycle also in *D. labrax*. Moreover, these results suggest that IGF-II stimulates cell proliferation in different tissues and organs in a autocrine/paracrine manner. Our future purposes are to investigate by Real-Time PCR the effects of aquaculture related stressors such as transport, confinement and handling on the expression of IGF-II mRNA. Moreover, by radio-immunoassay, we want to evaluate the effects of the same stressors on circulating levels of IGF-II.

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