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REGULAR ARTICLE

Real-time polymerase chain reaction, in situ hybridization and immunohistochemical localization of insulin-like growth factor-I and myostatin during development of *Dicentrarchus labrax* (Pisces: Osteichthyes)

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Abstract The distribution of insulin-like growth factor-I (IGF-I) and myostatin (MSTN) was investigated in sea bass (*Dicentrarchus labrax*) by real-time polymerase chain reaction (PCR), in situ hybridization (ISH) and immunohistochemistry. Real-time PCR indicated that IGF-I mRNA increased from the second day post-hatching and that this trend became significant from day 4. ISH confirmed a strong IGF-I mRNA expression from the first week post-hatching, with the most abundant expression being detected in the liver of larvae and adults. Real-time PCR also showed that the level of MSTN mRNA increased significantly from day 25. The expression of MSTN mRNA was higher in muscle and almost absent in other anatomical regions in both larvae and adults. Interestingly, the lateral muscle showed a quantitative differential expression of IGF-I and MSTN mRNAs in red and white muscle, depending on the developmental stage examined. IGF-I immunoreactivity was detected in developing intestine at hatching and in skeletal muscle, skin and yolk sac. MSTN immunostaining was evident in several tissues and organs in both larvae and adults. Both IGF-I and MSTN proteins were detected in the liver from day 4 post-hatching and, subsequently, in the kidney and heart muscle from day 10. Our results suggest, on the basis of a combined methodological approach, that IGF-I and MSTN are involved in the regulation of somatic growth in the sea bass.

Keywords Insulin-like growth factor-I · Myostatin · Development · Red and white muscle fibres · Sea bass, *Dicentrarchus labrax* (Teleostei)

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Introduction

Development and growth in teleost fish are highly dynamic and puzzling processes. Indeed, fish live in an environment with a large range of physical habitats and with many different types of hydrodynamic regimes that change rapidly during early stages of larval life (Webb 1988). Furthermore, some fish show a determinate final size (Biga and Goetz 2006), whereas other fish, such as sea bass, are characterized by rapid growth and a large adult size; this is reached by means of continuous hyperplastic mechanisms that occur by the apposition of new fibres along proliferative zones (Patruno et al. 1998; Rowlerson and Veggetti 2001). Recently, increasing attention has been drawn to molecules, i.e. insulin-like growth factors (IGFs) and transforming growth factor-β (TGF-β), that show an endocrine and a paracrine/autocrine action in controlling

fish growth. The regulation of these growth factors is dependent upon many aspects, such as the developmental stage considered, the environment, the swimming behaviour, the mechanisms of growth and the final adult size reached.

IGF-I, a highly conserved 70-amino-acid polypeptide showing a structural sequence similarity to both IGF-II and insulin, plays a central role in the regulation of development and growth by its potent metabolic and mitogenic action (for a review, see Reinecke and Collet 1998). In fish, as in mammals, the main site of IGF-I mRNA expression is the liver, although expression in several other tissues has also been observed (for reviews, see Duan 1998; Reinecke and Collet 1998; Reinecke et al. 2005; Wood et al. 2005). The mitogenic effect of IGF-I in fish has also been described in cultured muscle cells from rainbow trout (Castillo et al. 2004). IGF-I genes have been characterized in several fish species (Reinecke et al. 1997; Moriyama et al. 2000; Duval et al. 2002; Patruno et al. 2006) and the expression of its mRNA has been observed in tissues from larvae, fry and adults (Duguay et al. 1996; Greene and Chen 1997, 1999; Perrot et al. 1999; Ayson et al. 2002; Berishvili et al. 2006; Patruno et al. 2006). To date, scarce information exists regarding the cellular localization of teleost IGF-I protein, with only a few studies in sea bream (Perrot et al. 1999), shi drum (Radaelli et al. 2003a) and tilapia (Shved et al. 2005; Berishvili et al. 2006).

Myostatin (MSTN), also known as "growth and differentiation factor-8" or GDF-8, is a member of the growth factor TGF-β superfamily and is expressed mainly in the skeletal muscle. This protein has therefore been suggested to be involved in the regulation of skeletal muscle growth (McPherron et al. 1997; Joulia-Ekaza and Cabello 2006; Funkenstein and Rebhan 2007; Patruno et al. 2007). Hypertrophy of skeletal muscle because of mutations in the coding sequence of bovine MSTN has been observed in two breeds of double-muscled cattle, Belgian Blue and Piedmontese (Grobet et al. 1997, 1998; Kambadur et al. 1997; McPherron and Lee 1997). McPherron et al. (1997) have demonstrated the inhibitory effect of MSTN during skeletal muscle development and growth by generating MSTN null mice, which show a marked increase in skeletal muscle mass. In mammals, MSTN expression has also been reported in cardiomyocytes and Purkinje fibres of the heart (Sharma et al. 1999; Matsakas et al. 2006) and in mammary gland (Ji et al. 1998) and adipose tissue (McPherron et al. 1997). Although the predicted TGF-β amino acid sequences of many vertebrate and invertebrate species are identical in the active carboxy-terminal region (McPherron et al. 1997; Patruno et al. 2003; Radaelli et al. 2003b), the presence of two distinct MSTN genes (MSTN-1 and MSTN-2) in teleosts confirms the evolutionary complexity of this superfamily of growth factors. Indeed, an early MSTN gene duplication in the fish lineage resulted in the presence of two distinct MSTN genes in sea bream (Maccatrozzo et al. 2001a) and zebrafish (Biga et al. 2005; Kerr et al. 2005), whereas four MSTN paralogues have been described in salmonids (Garikipati et al. 2007).

In fish, the majority of studies have been conducted on the MSTN-1 orthologue (as suggested by Kerr et al. 2005), which, differently from MSTN-2, has been cloned in numerous species, such as the sea bream, the trout, the Atlantic salmon, the channel catfish, the sea bass, the spotted grouper and the zebrafish (Maccatrozzo et al. 2001b; Østbye et al. 2001; Rescan et al. 2001; Roberts and Goetz 2001; Rodgers et al. 2001; Kocabas et al. 2002; Xu et al. 2003; Amali et al. 2004; Biga et al. 2005; Terova et al. 2006; Ko et al. 2007). In contrast to mammals, the MSTN-1 gene and protein are expressed in several fish organs, such as brain, eyes, exocrine and endocrine pancreas, gills, gonads, heart, intestine, kidney, liver, oesophagus, pharynx, skin, spleen, and stomach (Maccatrozzo et al. 2001a, b; Østbye et al. 2001; Rescan et al. 2001; Roberts and Goetz 2001; Kocabas et al. 2002; Radaelli et al. 2003b; Gregory et al. 2004; Garikipati et al. 2006, 2007; Terova et al. 2006; Ko et al. 2007) and in muscle explants of fish (Funkenstein et al. 2006).

Generally, the great bulk of MSTN studies have been carried out on mRNA expression. Scarce information exists about the cellular localization of MSTN protein, with only one study having been carried out in sea bream, sole and zebrafish (Radaelli et al. 2003b).

We have investigated the expression of IGF-I and MSTN during the development and growth of the teleost fish *Dicentrarchus labrax* (sea bass), a species that shows an indeterminate final size and is of great interest in aquaculture. We have chosen these particular growth factors in order to investigate their role in fish since, in mammals, they have an opposite function, especially on skeletal muscle differentiation. We have used real-time polymerase chain reaction (PCR) and in situ hybridization (ISH) to detect the expression of IGF-I and MSTN mRNAs in muscle and in other organs from larvae, fry, juveniles and adult animals, and immunohistochemistry to characterize the cellular localization of both IGF-I and MSTN proteins. Furthermore, we have assessed the putative differential expression of MSTN and IGF-I in the two distinct fibre types present in the lateral muscle of fish (deep white and superficial red fibres) in juveniles and adult animals. In many fish species, the lateral muscles continue to grow, even into juvenile and adult life, through hyperplasia and hypertrophy mechanisms (for a review, see Rowlerson and Veggetti 2001). This designates the fish lateral muscle as a particularly good experimental model for studying the expression of growth factors in the two muscle fibre types after metamorphosis.

Materials and methods

Fish samples and tissue processing

Larvae (0, 2, 4, 6, 10, 25, 40 days post-hatching [dph]), fry (80 days), juvenile (6 months) and adult (3 years) sea bass were obtained from the Pellestrina (VE, Italy) fish hatchery and the Bonello (RO, Italy) fish farm. The animals were killed by an overdose of anaesthetic MS222 (Sandoz, Milan, Italy). For both immunohistochemistry and ISH, animals and tissues were fixed in 4% paraformaldehyde prepared in 0.1 M phosphate-buffered saline pH 7.4 (PBS) at 4°C overnight. Small fish (hatching to juveniles) were fixed in toto followed by longitudinal (both frontal and sagittal) and transversal section preparation. Organ and tissue samples were dissected out from large adult fish and processed separately. Samples for RNA expression analysis were stored in RNA Later Reagent (Ambion, USA) at −20°C until required.

Qualitative reverse transcription/PCR and quantitative real-time PCR

RNAs were extracted by using TRIZOL Reagent (Gibco-BRL, Gaithersburg, Md., USA) from a pool of 10–15 fish sampled at the stages described above (n=6) and from the following adult (3 years) tissues and organs (100 mg samples): red muscle, white muscle, skin, gut, liver, heart and gills (n=9). Red and white muscles were also dissected out from juveniles (6 months, n=9). To assess the integrity and the amount of the RNA extracted, agarose gel electrophoresis and spectrophotometric $A_{260/280}$ readings were performed. Total RNA (2 μg) was retrotranscribed into cDNA. First-strand cDNAs were synthesized by using Superscript II RNase H^- reverse transcriptase protocols (Invitrogen, Life technologies, UK) and a mixture of random hexamers as primer (synthetized by MWG-Biotech, Ebersberg, Germany). The obtained cDNAs were used as templates for PCR expression analysis. PCRs (32 cycles at: 95°C for 45 s, 55°C for 45 s and 72°C for 45 s) were carried out in order to check the specificity of the following primers designed by using Primer Express 3.0 software (Applied Biosystems): IGF-I (forward) 5′ GTG TGT GGA GAG AGA GGC TTT TA, (reverse) 5′ CCA ATG CAC GGC GGT C designed from GenBank, accession no. AY800248; MSTN (forward) 5′ GAG ATT AAC GCC TTC GAT TCG AG, (reverse) 5′ GAC TTT GGC TGG GAC TGG ATT A designed from GenBank, accession no. AY839106. To verify the efficiency of the reverse transcription (RT) and to exclude genomic DNA contamination, a fragment of β-actin cDNA (accession no. AJ493428) was amplified with primers designed to span an intron: forward 5′ ACC CAG TCC TGC TCA CAG AG and reverse 5′ GGG AGT CCA TGA CAA TAC CAG TG. PCR products were electrophoresed on a 1.5% agarose gel and visualized under UV light. Two additional IGF-I primers were used to generate RNA probes for the ISH (see below): forward, 5′ AGC GCT CTC TCC TTT CAG TG; reverse, 5′ TTT GTC TTG TCT GGC TGC TG.

Quantification assays were performed to detect the relative expression of IGF-I and MSTN mRNA among different post-hatching developmental stages of sea bass larvae and juveniles (white and red muscles) and among the various organs and tissues from adults sea bass (as described earlier). This expression analysis was carried out by using the ABI 7500 Real-Time PCR System (Applied Biosystems) as described by Caelers et al. (2004) and Patruno et al. (2006, 2007). A sample without cDNA template was used to verify that the master mix was free from contaminants. Real-time conditions were: 2 min at 50°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Wells contained 30 μl PCR mixture including 3 μl cDNA at a dilution of 1:5 for larvae (60 ng/well) and at a dilution of 1:8 for adult tissues and organs (40 ng/well). The β-actin gene was amplified in separate tubes as an active endogenous reference to normalize quantification of a mRNA target. Three replicates of each sample and endogenous control were amplified.

Real-time PCRs for target and reference genes were run in the same RT reaction. Data from SYBR Green I PCR amplicons were collected with ABI 7500 System SDS Software. The fluorescence signal baseline and threshold were set manually for each detector (IGF-I, MSTN and β-actin), generating a threshold cycle (Ct) for each sample. An amplification plot graphically displayed the fluorescence detected over the number of cycles that were performed.

Standard curves for both targets and the endogenous reference gene, created on the basis of the linear relationship between the Ct value and the logarithm of the starting amount of cDNA, showed acceptable slope values (included between −3.8 and −3.3). Standard curves were obtained by using serial dilutions of sample cDNA (1:2, 1:4, 1:8, 1:16, 1:32).

Before using the ΔΔCt method for relative quantification (comparative method), a validation experiment was required in order to demonstrate that the efficiency of the target amplification and that of the reference amplification were almost equal. All PCR efficiencies were measured and found to be adequate (slope <0.1).

The difference between Ct values was calculated for each mRNA by taking the mean Ct of triplicate reactions and subtracting the mean Ct of triplicate reactions for the reference RNA measured on an aliquot from the same RT reaction ($\Delta Ct = Ct_{target\ gene} - Ct_{reference\ gene}$). All samples

were then normalized to the ΔCt value of a calibrator sample to obtain a ΔΔCt value ($\Delta Ct_{target}-\Delta Ct_{calibrator}$). The calibrator, defined as the sample used as the basis for comparative results, could represent an untreated control or a particular stage of development (see Chemistry Guide, Applied Biosystem, 2003). During larval development, the hatching stage was chosen as the calibrator sample in order to evaluate the putative differential mRNA expression of target genes during post-hatching development relative to day 0 (hatch, see below). In adults, the intestine or the white muscle were chosen as calibrators, respectively (see below). For the comparative method, relative quantifications were calculated in relation to the concentrations of the calibrator sample ($2^{-\Delta\Delta Ct}$), expressed in arbitrary units and normalized to the endogenous reference gene (β-actin). Therefore, by using the $2^{-\Delta\Delta Ct}$ method, data were recorded as the fold-change in gene expression normalized with the endogenous reference gene and relative to the calibrator sample (Livak and Schmittgen 2001). Dissociation melting curves confirmed the specific amplification of the cDNA target and the absence of nonspecific products.

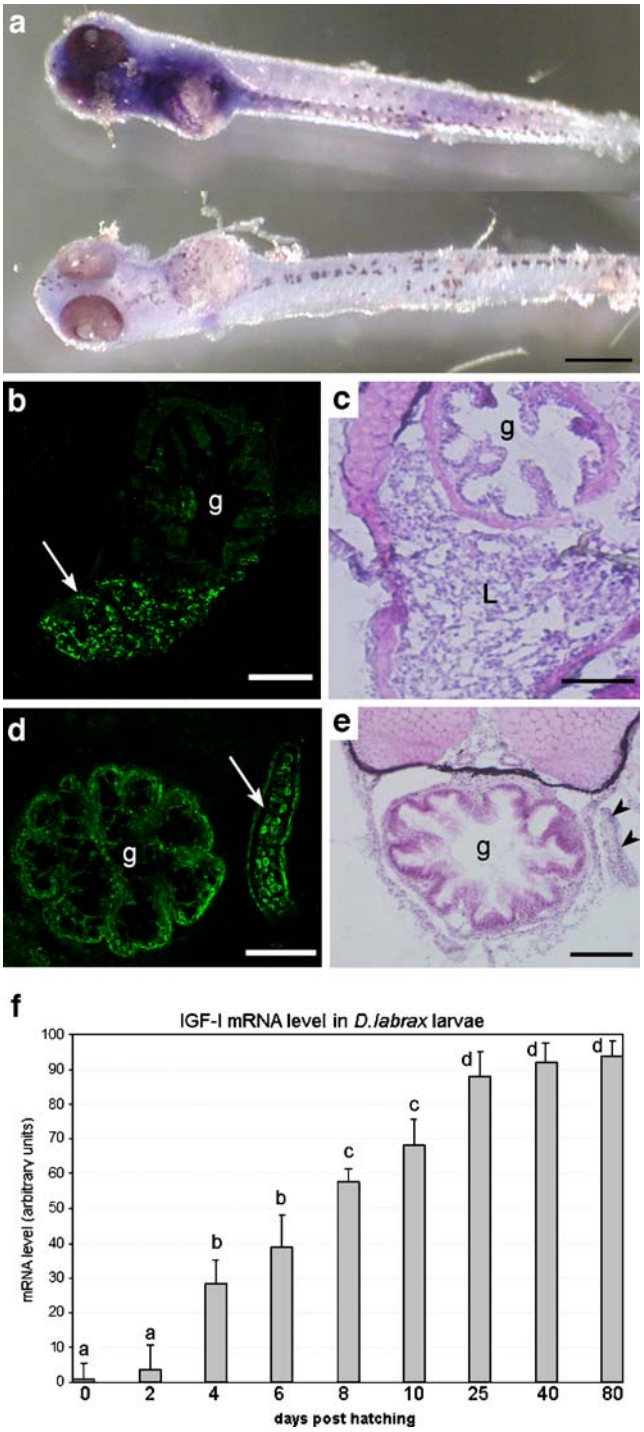
For statistical analysis, the data were expressed as the mean±standard error. Normality of the data was checked with a Kolmogorov-Smirnov test (α=5%). Statistical differences in IGF-I and MSTN mRNA, between calibrator samples (hatching stage for larvae and intestine for adults) and larva samples (at various post-hatching developmental stages) or adult organ samples were detected by one-way analysis of variance (ANOVA) with SPSS software version 11.0 (SPSS, Chicago, Ill., USA). The Tukey honestly post-hoc test was used to characterize statistical differences further among the developmental stages examined. Significant differences in IGF-I and MSTN mRNA levels (see below) between the calibrator sample (white muscle sampled from juveniles and white muscle sampled from adults) *vs* red muscle were determined by using Student's *t*-test for independent samples. The level of statistical significance was set at $P<0.05$ for all analysis.

ISH protocol

Generation of the sea bass probes The PCR products obtained with the IGF-I and MSTN primers (as above) were cloned into the pCR-II plasmid vector using a TOPO-TA cloning kit (Invitrogen) and sequenced by the CRIBI sequencing service (http://bmr.cribi.unipd.it/). Linearized plasmids containing sea bass IGF-I and MSTN fragments were used as templates to generate sense and anti-sense RNA probes (Nieto et al. 1996). The reagents were added, at room temperature, in the following order: 10 μl sterile distilled water, 4 μl 5× transcription buffer (Promega, Italy), 2 μl 0.1 M dithiothreitole (Promega), 2 μl digoxigenin (DIG) nucleotide mix pH 8.0 (Roche, Italy), 1 μl linearized IGF-I plasmid (1 μg/μl), 0.5 μl ribonuclease inhibitor (100 U/μl) (Roche), 1 μl T7 or SP6 RNA polymerase (10 U/μl, Promega). After incubation at 37°C for 2 h, 2 μl ribonuclease-free DNase I (Roche) was added and incubation was continued for an additional 15 min. Precipitation of the synthesized RNA was achieved by the addition of 100 μl TE buffer (10 mM TRIS, 1 mM EDTA, pH 8.0), 10 μl 4 M lithium chloride, 300 μl ethanol and incubated at −20°C for 30 min. The RNA was then purified and air-dried. The RNA was reconstituted in 50 μl TE and 5 μl were run on a 1% denaturing formaldehyde agarose/TBE gel (TBE=0.09M TRIS-borate, 0.002 M EDTA, pH 8.3) to assess its integrity and concentration. The RNA probe was diluted to 0.1 μg/μl and stored at −80°C.

Whole-mounts Sea bass larvae (0, 2, 4, 6, 8, 10, 25, 40 dph) were fixed in 4% paraformaldehyde for 1 h, washed in PBS twice, dehydrated and stored in methanol at −20°C. Following hydration in graded methanol, larvae were processed as described earlier (Radaelli et al. 2005) with slight modifications. Briefly, after incubation in 6% H_2O_2 for 15 min, larvae were permeabilized by treatment with 10 μg/ml proteinase K (Roche) in PBS containing 0.1% Tween 20 (PBT) for about 5 min (duration of treatment depended on the size of the sample), washed in PBT and fixed again in 4% paraformaldehyde plus 0.2% glutaraldehyde for 20 min before the hybridization step. After being washed in PBT, larvae were rinsed with 1:1 PBT/hybridization solution and then incubated with hybridization solution, pre-warmed to 60°C, for 30 min. This was replaced with fresh hybridization solution containing 0.1 μg/μl RNA antisense probe (or sense probe for controls) and incubation continued overnight at 60°C. After several washes in 2× SSC (sodium chloride/sodium citrate) followed by PBS washes, transcripts were identified by using the DIG nucleic acid detection kit (anti-DIG AP-coupled Ab, Roche). The hybridized larvae were viewed by using a stereomicroscope connected to a digital camera (Olympus). Following ISH, larvae were kept in PBT containing 100 mM EDTA at 4°C.

Frozen sections Sections of larvae (as above) and fry (80 dph) were processed for ISH experiments as described elsewhere (Radaelli et al. 2005) with the following modifications. The riboprobes (500 ng/ml final concentration) were resuspended in the following hybridization buffer: 50% (deionized) formamide, 1× SSC, 10% dextran sulfate, 1× Denhardt's solution, 0.67 M NaCl, 0.1 μg/μl yeast tRNA, and 0.1 μg/μl herring sperm DNA. Riboprobes were then heated at 80°C for 5 min before the hybridization step. Approximately 50 μl hybridization buffer containing the probe were used per slide, overlaid with a coverslip. Hybridization was performed overnight



◀ **Fig. 1** IGF-I: in situ hybridization (ISH) and real-time PCR in various post-hatching stages during sea bass development. **a** In a whole-mount larva at 6 days post-hatching (dph), intense IGF-I hybridization signal (*top*) is found mainly in the cephalic region and in the trunk. *Bottom* Sense control experiment. **b** Transverse section of an 8-dph larva (*g* gut) showing a positive fluorescent signal in the liver (*arrow*). **c** Serial frozen section to that in **b** showing the haematoxylin-eosin staining of liver (*L*) and gut (*g*). **d** Transverse section of a 25-dph larva showing a positive fluorescent signal in the gut (*g*) and in the growing cartilage (*arrow*). **e** Serial frozen section to that in **d** showing the haematoxylin-eosin staining of gut (*g*) and cartilage (*arrowheads*). *Bars* 500 μm (**a**), 90 μm (**b**), 75 μm (**c**, **d**, **e**). **f** IGF-I mRNA levels, detected by real-time PCR, show a significant increase from the fourth day post-hatching. Data are calculated as the relative expression with respect to hatching (0 dph). *Bars* designated with different *letters* are significantly different ($P<0.05$)

at 45–50°C in a humidified incubator. Coverslips were removed by rinsing in 6× SSC, followed by two high-stringency steps at 50°C for 20 min in 0.5× SSC and 20% formamide and two rinses in 2× SSC at room temperature. Unhybridized probe was digested with 2 μg/ml RNase A in 0.5 M NaCl, 10 mM TRIS-HCl, pH 8.0, at 37°C for 30 min, followed by five washes in 2× SSC at room temperature and another high-stringency wash for 10 min. The sections were rinsed twice with 2× SSC and maleic buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and twice in blocking solution (15 min for each wash). The samples were then blocked with 5% inactivated BSA (bovine serum albumin; Sigma) in maleic buffer for 60 min at room temperature. Methods for the detection of hybridized probes were adapted from the manufacture's protocols with the anti-DIG antibody conjugated with fluorescein (FITC; Roche). After several washes in PBS, sections were mounted with Vectashield (Vector Laboratories, Burlingame, Calif., USA). Images were obtained with a Leica TCS-SP2 confocal laser scanning microscope. Sense probes were used as negative controls in all experiments.

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 by using a microtome.

Antisera For IGF-I, polyclonal antibodies against recombinant sea bream IGF-I (Fine et al. 1997) were raised in mouse and used at a dilution of 1:100. The anti-MSTN antibody was produced in mouse against sea bream MSTN-a, which is expressed in several tissues of this species (Radaelli et al. 2003b).

Immunohistochemical procedures Immunohistochemical staining was performed by using the Envision system (goat-anti-mouse immunoglobulins conjugated to peroxidase-labelled complex; DAKO, Italy). Endogenous peroxidase activity was blocked by 3% H_2O_2 in PBS prior to the application of the primary antibody. Non-specific binding sites were blocked by incubating the sections in 1:5 dilution of mouse serum (Dako, Italy). The immunoreactive sites were visualized by using diaminobenzidine (Sigma, Italy)

Table 1 Immunohistochemical localization of IGF-I in sea bass (– not detectable, +/– slight staining above background levels, + moderate, ++ marked staining, +++ strong staining, *x* tissue not found on the sections examined at this stage, ^{R} red muscle fibres, ^{W} white muscle fibres, NA not applicable)

Tissue	0–4 days	5–10 days	25–40 days	80 days	Adults
Skeletal muscle	++	++	+	$++^{R}$, $+/-^{W}$	$+++^{R}$, $++^{W}$
Heart	x	+/–	+	+/–	++
Gut epithelium	+++	++	++	+	+
Kidney epithelium	x	+	+/–	+	+
Gill epithelium	–	–	++	++	++
Skin	++	++	++	++	++
Pancreas	–	–	–	–	–
Liver	++	++	++	++	++
Yolk sac	+++	NA	NA	NA	NA

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) antisera pre-absorbed with an excess of synthetic peptides (3 μg/μl) before incubation of the sections. The results of these controls were negative, i.e. staining was abolished.

Results

Expression of IGF-I mRNA in sea bass larvae as detected by ISH and real-time PCR

Whole-mount ISH showed strong IGF-I mRNA expression after the first week post-hatching (Fig. 1a). In particular, IGF-I mRNA was distributed mainly in the cephalic region of the larvae (Fig. 1a, top). The same technique used on frozen sections revealed that the liver (Fig. 1b) was the only organ in which IGF-I mRNA was localized at early stages of growth (6–8 days; Fig. 1b,c). The intestine showed a positive signal from day 25, as did the growing cartilage (Fig. 1d,e).

Real-time PCR experiments confirmed that IGF-I mRNA levels increased significantly (Fig. 1f) from day 4 post-hatching. After this stage, a constant increase of IGF-I mRNA expression was monitored up to day 80 (Fig. 1f). The real-time PCR data were recorded as the expression relative to the hatching stage (0 dph) as the calibrator sample (see Materials and methods).

Localization of IGF-I protein in sea bass larvae by immunohistochemistry

The immunohistochemical localization of IGF-I is summarized in Table 1.

Epithelial cells of the yolk sac (when still present) exhibited strong immunoreactivity to IGF-I antiserum (Table 1). IGF-I immunoreactivity could be detected in the epithelium of developing intestine and in the developing skeletal muscle in larvae staged at day 0 (Fig. 2a). At all stages examined, epithelial cells of the epidermis exhibited a marked immunoreaction, whereas mucous cells were negative (Fig. 2b). At day 4, IGF-I expression was detected in liver parenchyma (Table 1). From day 10, immunostaining was evident in the epithelium of renal tubules and faint reactivity was detected in the heart musculature and in the epithelium of the pharynx and oesophagus (Table 1, Fig. 2c). From day 40, immunostaining was detected in the epithelial cells of the stomach and in the gastric pits (Fig. 2d). In the 80-dph fry, faint immunoreactivity was present in the white fibres of skeletal muscle, whereas red fibres exhibited a marked positivity (Fig. 2e). The epithelium of the gills exhibited immunostaining for IGF-I in both larvae and fry (Fig. 2f).

Expression of IGF-I in various adult tissues and organs of sea bass as detected by real-time PCR and immunohistochemistry

Various tissues and organs sampled from adult animals were processed in order to perform a relative analysis of the IGF-I mRNA content (Fig. 3a). IGF-I mRNA was abundantly expressed in the liver, although a large amount was also found in other organs such as the gills, lateral muscle and skin (Fig. 3a). In order to ascertain the IGF-I mRNA content between the deep white and the superficial red musculature, a real-time PCR approach was carried out in juvenile (6 months) and adult (3 years) sea bass. In juveniles, the level of IGF-I mRNA was higher in white muscle, whereas in adults, the opposite was observed (Fig. 3b). In adults, immunohistochemistry experiments showed marked IGF-I protein expression in the liver (Fig. 4a), gill epithelium (Fig. 4b) and heart muscle (Fig. 4c). In skeletal muscle, strong immunostaining was observed in red muscle fibres (Fig. 4d). The white deep muscle fibres exhibited a mosaic of negative and immunostained fibres (inset of Fig. 4d).

Expression of MSTN mRNA in sea bass larvae as detected by ISH and real-time PCR

Whole-mount ISH performed with the MSTN mRNA probe revealed a signal in the trunk muscle (Fig. 5a, top); in particular, the staining was evident at the border (epiaxial and hypoaxial regions) of the myomeres of the 25-dph larva

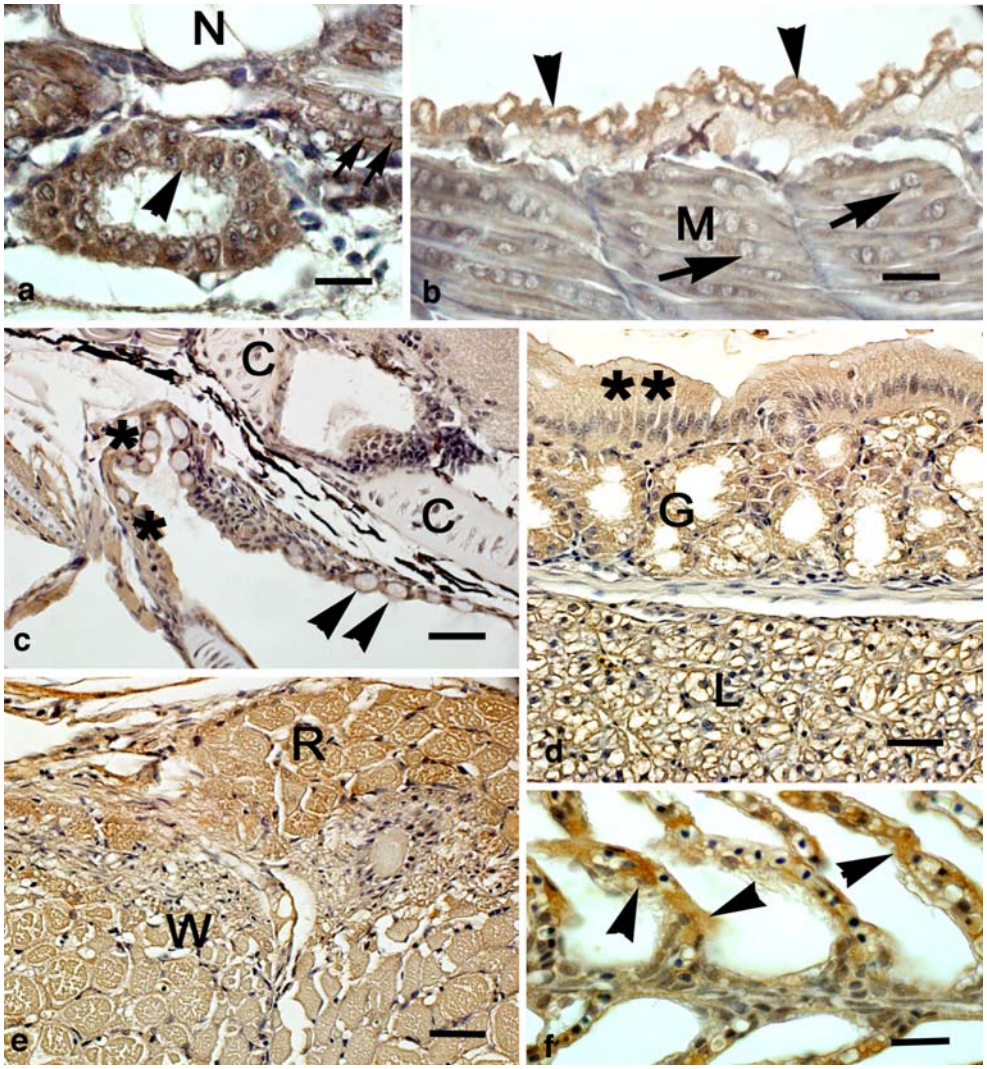


Fig. 2 Immunohistochemical localization of IGF-I in sea bass larvae and fry. All micrographs show counterstaining with haematoxylin. **a** At hatching, strong IGF-I immunoreactivity is present in the cytoplasm of the intestinal epithelium (*arrowhead*). Marked immunostaining is also present in the new muscle fibres that are produced during the myofibrillogenesis process, as indicated by the presence of many centrally aligned swollen nuclei (*arrows*). *Top* Notochord (*N*). **b** Parasagittal section at the trunk level in a 4-dph larva (*M* myomere in which muscle fibres are immunopositive, *arrows* centrally aligned swollen nuclei characterizing the new muscle fibres produced during the myofibrillogenesis process, *arrowheads* immunopositivity in the skin). **c** Sagittal section of a 10-day larva. Immunostaining is found in the epithelium of the pharynx (*asterisks*). Note the negative mucous cells (*arrowheads*). Cartilage (*C*) is also negative. **d** Sagittal section of the stomach of a 40-dph larva showing immunopositivity in the epithelium (*asterisks*) and in the gastric pits (*G*). Liver (*L*) parenchyma is also positive. **e** Transverse section of a 80-dph fry. Marked IGF-I immunostaining is present in the trunk musculature at the level of red muscle fibres (*R*), whereas white muscle fibres (*W*) show faint positivity. **f** In a 80-dph fry, immunostaining is present in the epithelium of the gill filaments (*arrowheads* positive cells located at the base of the secondary lamellae). *Bars* 10 μm (**a–c**, **e**), 20 μm (**d**, **f**)

(Fig. 5a). ISH experiments performed on frozen tissue sections on fish at 80 days revealed that MSTN was only detectable in the muscle tissue, especially in the superficial monolayer and in the proliferative area underneath (composed of developing red and white fibres), adjacent to the horizontal septum (Fig. 5b,c). Real-time PCR experiments showed that the level of MSTN mRNA expression increased significantly at 25 dph (Fig. 5d). At 40 and 80 dph, the level of MSTN mRNA was significantly greater with respect to earlier stages of growth (Fig. 5d). Larvae at stage 2 dph were chosen as calibrators, since the 0 dph stage showed an almost complete absence of MSTN transcript.

Localization of MSTN protein in sea bass larvae by immunohistochemistry

The immunohistochemical localization of MSTN is summarized in Table 2.

The epithelium of the yolk sac (when still present) showed moderate immunoreactivity to MSTN antiserum (Table 2).

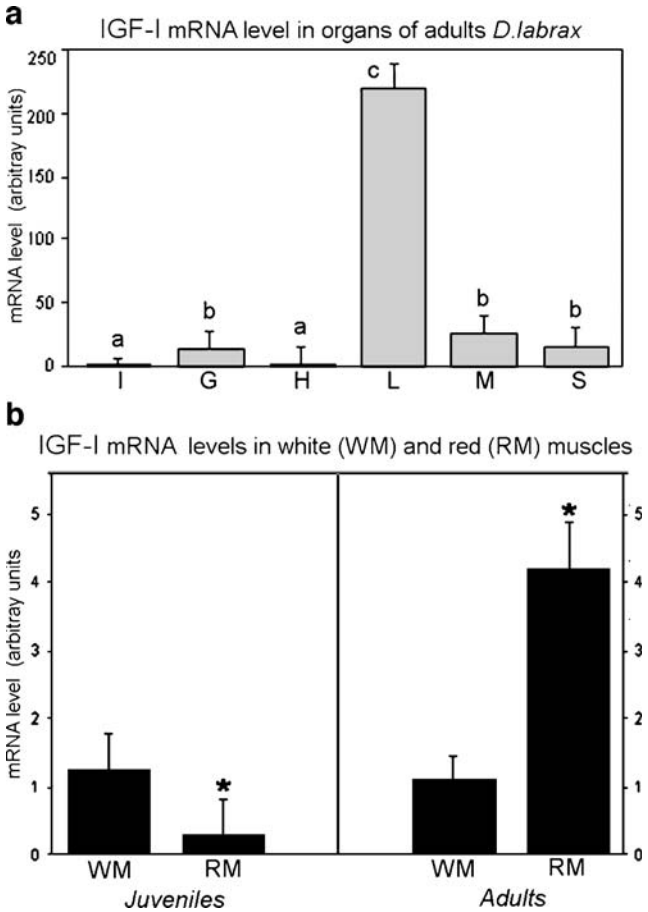


Fig. 3 Expression of IGF-I mRNA in various adult tissues and organs of sea bass, detected by real-time PCR. **a** In adult animals, the liver (*L*) showed the highest level of expression for IGF-I. The intestine (*I*) was used as the calibrator sample (*G* gills, *H* heart, *M* muscle, *S* skin). *Bars* designated with different *letters* are significantly different ($P<0.05$). **b** IGF-I mRNA levels were measured in white and red muscles collected from juvenile and adult fish. The relative amount of IGF-I mRNA was higher in the deep white muscle (*WM*) in juvenile fish compared with that in red superficial muscle (*RW*). The latter showed a higher IGF-I mRNA content in adult animals. A Student's *t*-test for independent samples was used to evaluate significant differences (*$P<0.05$) in IGF-I mRNA expression between the two types of muscle. White muscle was chosen as the calibrator in both juvenile and adult samples

At all stages examined, epithelial cells of the epidermis were immunoreactive to MSTN antiserum (Fig. 6a), whereas mucous cells were negative (Fig. 6e). From day 4, marked immunostaining was present in the epithelium of the developing intestine and in skeletal muscle (Table 2). In larvae aged 5–10 days, faint immunoreactivity was observed in the parenchyma of the liver and pancreas (Fig. 6b) and in the heart muscle (Fig. 6c). In larvae aged 25–40 days, strong MSTN immunostaining was observed in the epithelium of the gill filaments (Fig. 6d). In gut epithelium, strong immunostaining was evident in larvae aged 40 days (Fig. 6e). From day 40, MSTN immunoreactivity was observed in the epithelium of the renal tubules (Fig. 6f). In skeletal muscle of fry, marked immunostaining was observed in white muscle fibres, whereas red fibres were negative (Table 2).

Expression of MSTN in various adult tissues and organs of sea bass as detected by real-time PCR and immunohistochemistry

The same samples and the same real-time methods as used for the IGF-I assay were employed for the relative quantification of MSTN. Among the organs and tissues examined, a high level of MSTN mRNA was exclusively observed in the lateral muscle, although the skin also showed a modest amount of MSTN mRNA (Fig. 7a). The MSTN mRNA content between the deep white and the superficial red musculature was also measured in juvenile and adult fish (Fig. 7b). Real-time PCR results showed that juveniles (6 months) exhibited a similar level of MSTN mRNA between deep white fibres and superficial red fibres, whereas adults showed a higher value for MSTN mRNA in the red muscle fibres (Fig. 7b).

In adults, immunohistochemistry experiments showed moderate MSTN labelling in the liver (Fig. 8a), whereas the gill epithelium exhibited significant immunostaining (Fig. 8b). Weak immunostaining was detected in the muscle fibres of the heart (Fig. 8c). In skeletal muscle, marked immunostaining was observed in red muscle fibres (Fig. 8d), whereas the white deep muscle fibres were negative (*inset* in Fig. 8d).

Discussion

This study reports the expression of mRNA for IGF-I and MSTN throughout the life cycle of sea bass. Moreover, the cellular localization of IGF-I and MSTN proteins has also been studied from hatching to adult stages by immunohistochemistry with polyclonal antisera raised against sea bream IGF-I (Fine et al. 1997) and MSTN (Radaelli et al. 2003b).

Expression and cellular localization of IGF-I in sea bass

The ubiquitous expression of fish IGF-I has been confirmed in this report, although its intensity and appearance (especially at the mRNA level) depended on the organ and the developmental stage considered.

Generally, the liver is the main source of endocrine IGF-I. In sea bass, our ISH experiments have revealed that only this organ shows detectable mRNA expression from 6 dph. Real-time PCR has confirmed the ISH data indicating that IGF-I mRNA increases from the second day post-hatching and that this trend becomes statistically significant from day 4. The observation that IGF-I mRNA is not expressed in large amounts before day 4 is in agreement with earlier

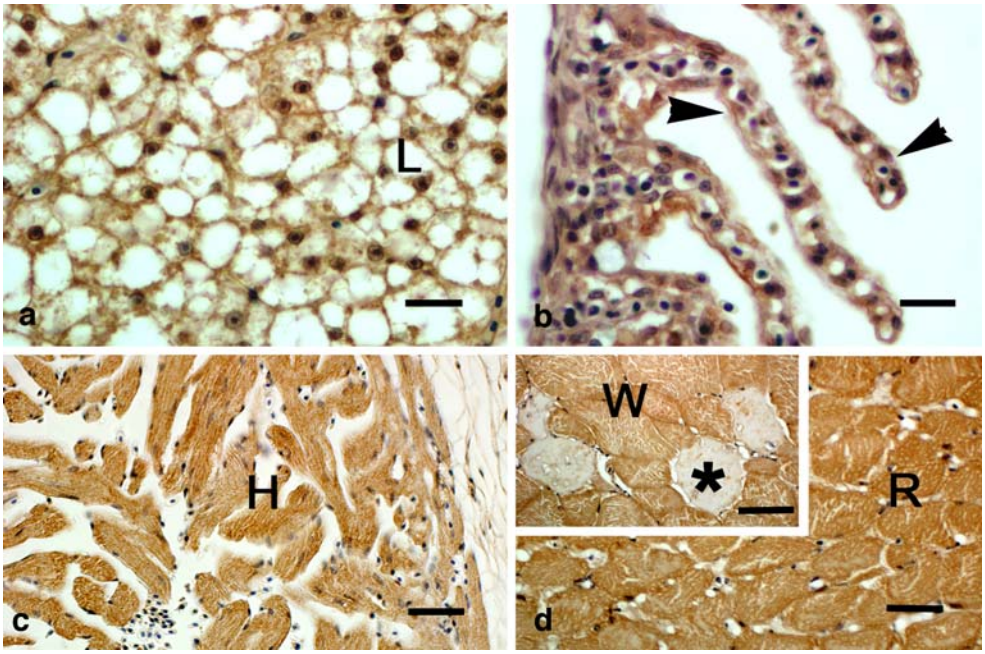
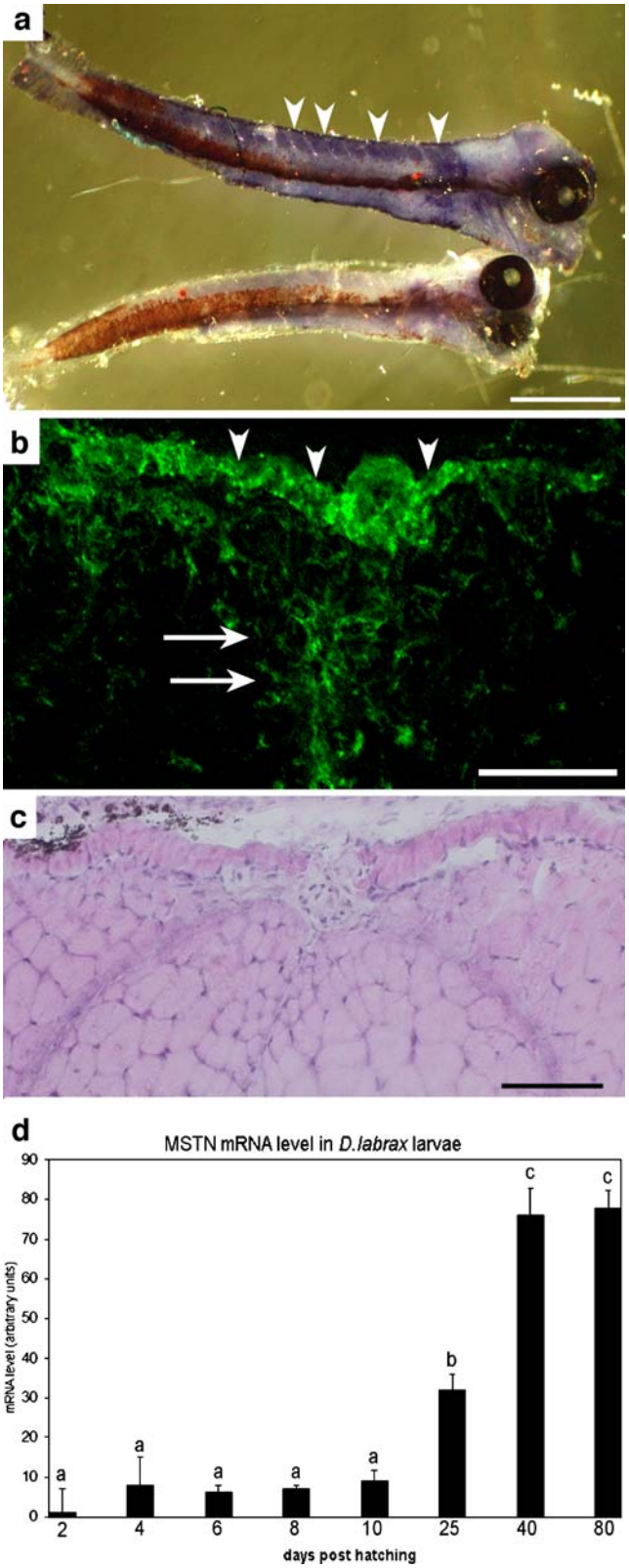


Fig. 4 Immunohistochemical localization of IGF-I in sea bass adults. All micrographs show counterstaining with haematoxylin. **a** Liver (*L*) exhibits marked IGF-I immunostaining in the hepatocytes. **b** In gills, IGF-I immunostaining is present in both primary and secondary (*arrowheads*) lamellae. **c** Striated myocardial fibres of the heart (*H*) showing marked IGF-I immunostaining. **d** Transverse section of the lateral muscle showing strong immunostaining in red fibres (*R*). *Inset*: Mosaic of white muscle fibres (*W*). Most of the fibres exhibit marked immunoreactivity (*asterisk* negative fibre). *Bars* 20 μm

studies that have shown that IGF-II, and not IGF-I, is the most precocious insulin-like factor to be expressed in mammals and fish (Wood et al. 2005). The intensity of IGF-I immunoreactvity observed in the sea bass liver is strong from early developmental stages throughout life, as its mRNA expression. However, in some fish, as in tilapia and in barramundi, the evident expression of IGF-I mRNA in the adult liver does not correspond to IGF-I immunoreactivity in hepatocytes (Richardson et al. 1995; Schmid et al. 2000). If the IGF-I is immediately released from fish liver into the circulation, as is observed in mammals (Hansson et al. 1988) and has been suggested for tilapia and barramundi (Berishvili et al. 2006), then the high level of IGF-I mRNA and protein found in adult sea bass may be attributable to the high IGF-I turnover present in hepatocytes of this species. However, another factor involved in the translational regulation of IGF-I mRNA might be the concentration of circulating growth hormone (GH). Indeed, Pierce et al. (2004) have found, in a primary hepatocyte culture system established from salmon, a biphasic response curve, since the high GH level down-regulates GH signaling and, consequently, IGF-I production.

Paracrine IGF-I is widely distributed and, indeed, our investigations have shown an abundance of IGF-I mRNA and protein in various organs of larvae and of adults. ISH experiments have also revealed that IGF-I mRNA expression is abundant in the intestine and in the cartilage of larvae. However, this expression is not observed until the third week post-hatching. This may be because the mRNA level in the intestine and in the cartilage is too low to be detected by our ISH technique. Most likely, in these organ and tissue, the mitogenic effect of IGF-I is required once the organ is formed and the gut epithelium and chondrocytes need to increase their proliferative ratio by growth mechanisms. We have found IGF-I immunoreactivity in the epithelial layer of the pharynx, oesophagus, stomach and intestine, corroborating earlier observations (Perrot et al. 1999; Radaelli et al. 2003a; Berishvili et al. 2006) and suggesting a paracrine/autocrine action of IGF-I in promoting the epithelial cells proliferation of these tracts. The presence of IGF-I protein in the epithelia of gills and renal tubules also suggests that IGF-I is involved in osmoregulation. Although IGF-I immunoreactivity has been described in the gills of several fish species (Perrot et al. 1999; Radaelli et al. 2003a; Berishvili et al. 2006), its expression in the epithelium of renal tubules has been reported only in tilapia (Berishvili et al. 2006). Our study has demonstrated the presence of IGF-I protein in the heart musculature from larvae to adults; this data is in agreement with that from the shi drum (Radaelli et al. 2003a). The prolonged expression pattern found in the heart, together with the observations of Biga et al. (2004) indicating that the treatment of tilapia with recombinant bovine somatotropin decreases the mRNA level of the IGF-I receptor A, indicates that a complex, poorly understood autocrine/paracrine mode of action of IGF-I occurs in the fish heart. However, of importance, Berishvili et al. (2006) have detected IGF-I immunoreactivity in cardiomyocytes only up to 70 days post-fertilization, suggesting a particular physiological impact of IGF-I, especially during heart development.

In this study, immunoreaction for IGF-I has been found in sea bass skeletal muscle throughout the life cycle. Similar results have been observed in sea bream, shi drum



◀ **Fig. 5** MSTN: ISH and real-time PCR in various post-hatching stages during sea bass development. **a** In a 25 dph whole-mount larva, the MSTN hybridization signal is found in the apical part of the myomeres along the trunk musculature (*top*, *arrowheads*). *Bottom* Sense control experiment with no signals in the trunk and tail. **b** In a transverse section of a fish at 80 dph, a positive hybridization signal is present at the level of the superficial monolayer (*arrowheads*) and in the proliferative area underneath (formed by red and white fibres), adjacent to the horizontal septum (*arrows*). **c** Serial frozen section to that in **g** showing the haematoxylin-eosin staining of the area in which MSTN is expressed. *Bars* 900 μm (**a**), 100 μm (**b**, **c**). **d** MSTN mRNA levels detected by real-time PCR increased from the stage at 25 dph. Data are calculated as the relative expression with respect to the 25 dph stage. *Bars* designated with *different* letters are significantly different ($P<0.05$)

and tilapia (Perrot et al. 1999; Radaelli et al. 2003a; Berishvili et al. 2006) suggesting that IGF-I acts as a growth regulator for both red and white fibres. However, in the adult white muscle, large-diameter differentiated fibres show an absence of IGF-I staining, thus indicating that this factor plays an active role, especially during muscle development and growth. The real-time PCR data corroborate these results, since the amount of IGF-I mRNA is higher in developing white muscle fibres of juveniles (at this stage, white fibres show a higher growth ratio with respect to red fibres) and in developing red muscle fibres of adults (at this stage, red fibres show a higher growth ratio with respect to white fibres).

Expression and cellular localization of MSTN in sea bass

The fish MSTN gene was duplicated during evolution (as shown by the existence of two clades; Kerr et al. 2005) giving the MSTN-1 and MSTN-2 paralogue genes. In salmonids, a second gene duplication occurred resulting in four MSTN paralogues (Garikipati et al. 2007). In sea bass, gene duplication for MSTN has not yet been reported. Therefore, we performed a BLAST and ClustalW search by using the unique sea bass (*D. labrax*) MSTN sequence (AY839106; Terova et al. 2006) against the fish database in order to assess which MSTN we had amplified in this study. The alignment revealed a 93% identity of our fragment with the MSTN-1 gene of zebrafish (NM_131019.3) and 100% identity with the sea bream MSTN-a (or MSTN-1, AF258447.1) but only a 75% identity with the MSTN-2 gene of zebrafish (DQ451548.1) and 59% with the sea bream MSTN-b (or MSTN-2, AY046314.1). Therefore, we assume that the sea bass MSTN gene examined in this report belongs to the MSTN-1 clade. This is also supported by the finding that MSTN-1 and MSTN-2 are differentially expressed during zebrafish development (Kerr et al. 2005), the MSTN mRNA expression found in our study resembling

Table 2 Immunohistochemical localization of MSTN in sea bass (– not detectable, +/– slight staining above background levels, + moderate, ++ marked staining, +++ strong staining, *x* tissue not found on the sections examined at this stage, ^{R} red muscle fibres, ^{W} white muscle fibres, *NA* not applicable)

Tissue	0–4 days	5–10 days	25–40 days	80 days	Adults
Skeletal muscle	+	+	++	$-^{R}$, $++^{W}$	$++^{R}$, $-^{W}$
Heart	x	+/–	+	x	+/–
Gut epithelium	++	+	+++	+	+
Kidney epithelium	x	+	+	+/–	+
Gill epithelium	–	–	+++	++	++
Skin	++	++	+++	++	+++
Pancreas	–	+/–	–	–	–
Liver	+/–	+/–	+	+/–	+
Yolk sac	+	NA	NA	NA	NA

the expression pattern of zebrafish MSTN-1 (zfMSTN-1; Helterline et al. 2007). Levels of zfMSTN-1 rise during late stages of somitogenesis, whereas the zfMSTN-2 peaks rapidly at early stages of somitogenesis and then drops almost immediately (Helterline et al. 2007). Although we have investigated post-hatching phases of development in sea bass, we have detected a low level of MSTN mRNA as early as 2 dph (and none before), with a significant mRNA increase being observed at later stages of development (from day 25).

Because MSTN is expressed at low levels during early post-hatching development and the earlier expression of MSTN is generally of maternal origin (Vianello et al. 2003; Biga et al. 2005), visualization of its mRNA expression by ISH at these stages is extremely difficult (Kerr et al. 2005). Our real-time PCR result confirms the lack of a proper

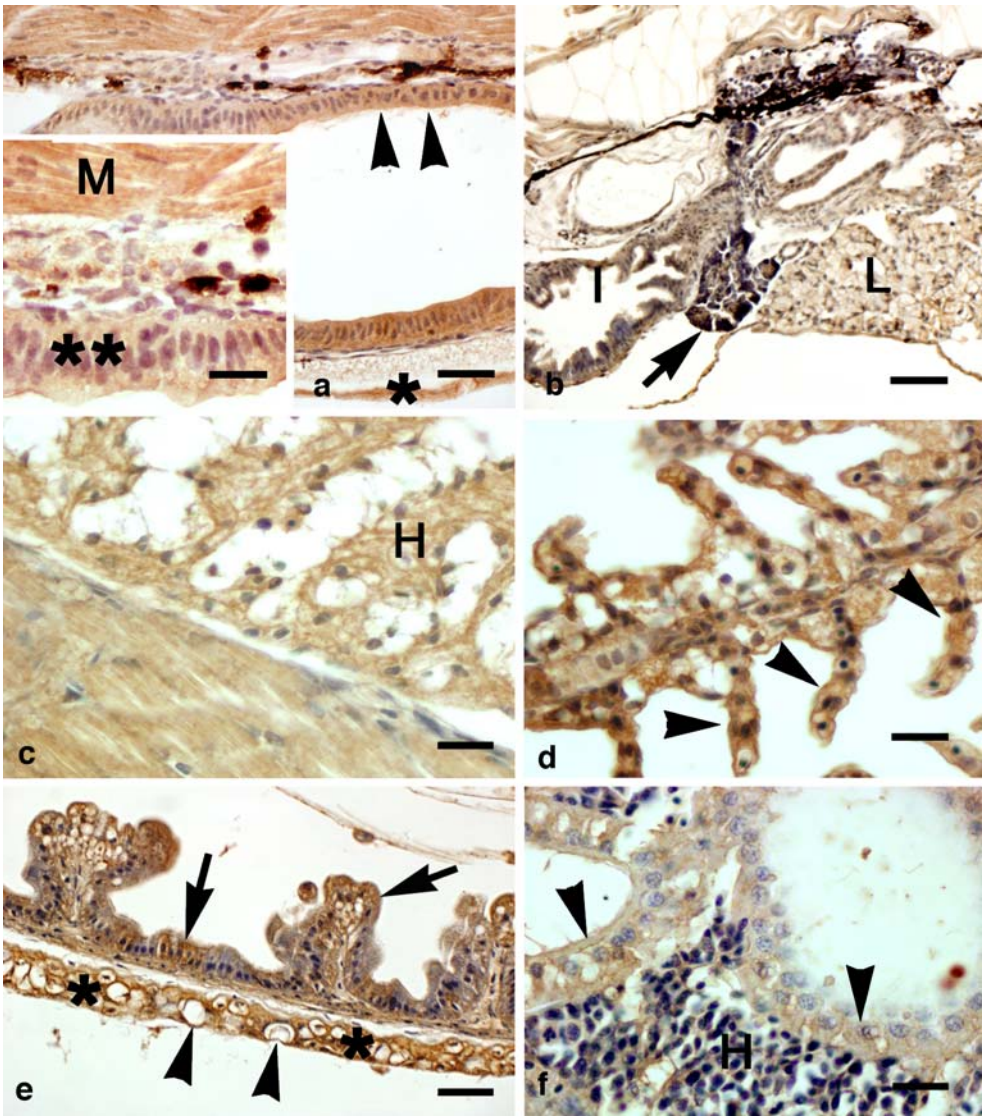


Fig. 6 Immunohistochemical localization of MSTN in sea bass larvae and fry. All micrographs show counterstaining with haematoxylin. **a** Sagittal section a 4-dph larva. Immunostaining is found in the epithelium of developing intestine (*arrowheads*). Skin (*asterisk*) is also positive. *Inset*: Detail of **a** showing immunostaining in the epithelium (*asterisks*) and in the lateral muscle (*M*). **b** Sagittal section of a 10-dph larva showing faint immunoreactivity in pancreas (*arrow*) and liver (*L*). Intestine (*I*) is also positive. **c** Heart (*H*) of a 25-dph larva showing moderate MSTN immunoreactivity in the striated myocardial fibres. **d** In a 25-dph larva, strong immunoreactivity is detectable in the epithelium of the gill filaments (*arrowheads* immunopositive cells located at the level of secondary lamellae). **e** Strong MSTN immunostaining is present in the epithelium of intestine (*arrows*) and in the skin (*asterisks*) of a 40-dph larva. In skin, mucous cells are negative (*arrowheads*). **f** Kidney of a 80-day fry (*H* haemopietic tissue). Faint MSTN immunostaining is present in the epithelium of the renal tubules (*arrowheads*). *Bars* 10 μm (**c**, **d** **f**, *inset* in **a**), 20 μm (**a**, **e**), 40 μm (**b**)

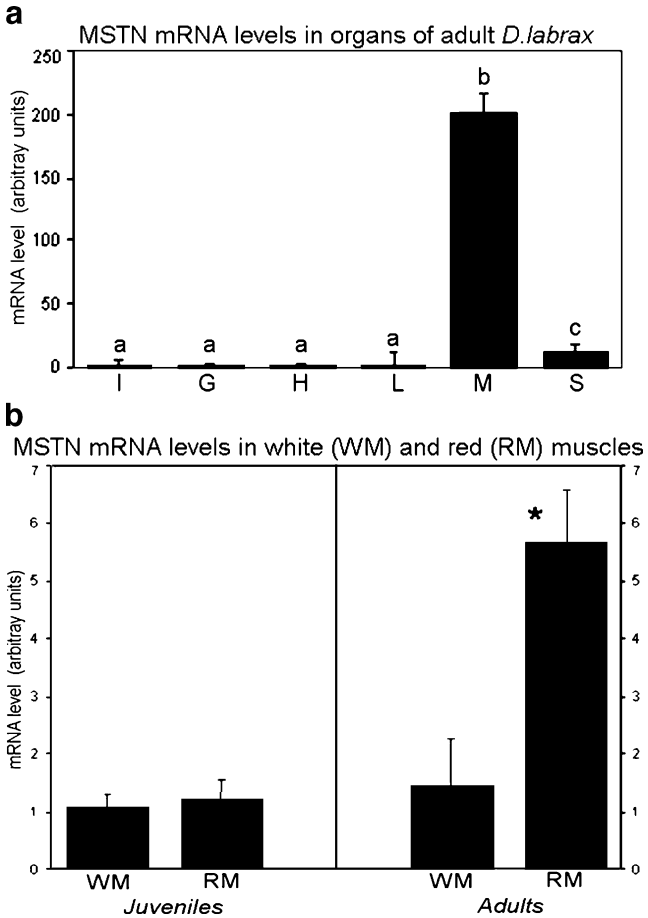


Fig. 7 Expression of MSTN mRNA in various adult tissues and organs of sea bass as detected by real-time PCR. **a** Lateral muscle showed the most elevated expression of MSTN. The intestine (*I*) was used as the calibrator sample (*G* gills, *H* heart, *M* muscle, *S* skin). *Bars* designated with different *letters* are significantly different ($P<0.05$). **b** MSTN mRNA levels were measured in white and red muscles collected from juvenile and adult fish. In juveniles, the two types of muscles exhibited a similar MSTN mRNA level, whereas in adults, the red muscle (*RM*) showed a higher level of mRNA compared with the white muscle (*WM*). A Student's *t*-test for independent samples was used to evaluate significant differences (*$P<0.05$) in MSTN mRNA expression between the two types of muscle. White muscle was chosen as the calibrator in both juvenile and adult samples

MSTN mRNA signal up to the third week from hatching; following this period, mRNA expression can be detected in the trunk lateral muscle of young larvae only in the epiaxial and hypoaxial proliferative muscle zones. ISH performed on frozen sections of a 80-dph sea bass has revealed the presence of MSTN mRNA in the superficial monolayer (future red muscle under the skin) and in the proliferative zone below it, adjacent to the horizontal septum. The latter area is a region of hyperplastic growth characterized by small-diameter fibres among large fibres (future red and white fibres), known as the mosaic area (Patruno et al. 1998; Rowlerson and Veggetti 2001). Interestingly, MSTN immunostaining carried out on sections at the same stage (80 dph) has revealed that the white muscle only exhibits intense reaction (see Table 2 and supplemental material). These observations prompted us to establish a real-time PCR assay for measuring MSTN mRNA levels in red and white muscles from juvenile and adult fish, stages in which the two muscle types can readily be dissected (see below).

In adults, MSTN mRNA is expressed in all organs examined, as detected by standard PCR. The real-time PCR approach shows that MSTN mRNA is expressed at high levels in the lateral muscle, at low levels in the skin and at very low levels in all other organs examined. However, when the muscle is not considered in the relative calculation, higher amount of MSTN mRNA are also detected in the gills, heart, intestine and liver (data not shown). The wide distribution of MSTN mRNA in sea bass organs is supported by the presence of the relative MSTN protein.

In our previous immunohistochemical studies, the use of an antiserum raised against sea bream MSTN allowed us to detect the cellular distribution of MSTN protein in three different teleosts. This demonstrates the high degree of similarity between MSTNs from different fish species, leading to cross-species immunoreactivity (Radaelli et al. 2003b). Similar results have been obtained in the present study in which MSTN protein has been observed not only in the muscle, but also in several other tissues. The presence of MSTN in heart musculature is in agreement with the data observed in sole and zebrafish (Radaelli et al. 2003b), indicating that MSTN plays an important role during cardiac muscle development. Of interest, in fish, there may be a direct link between IGF-I and MSTN in cardiomyocytes, as hypothesized by Shyu et al. (2005) for mammals; MSTN could, indeed, represent a chalone molecule of the IGF-I pathway in the cardiac muscle.

In skeletal muscle, MSTN immunostaining is present, even during larval life, as observed in other fish species (Radaelli et al. 2003b). During post-larval development, fry exhibit negative red fibres and positive white fibres, whereas adults show the opposite profile. In order to study MSTN expression in these two highly different types of muscles further, we have carried out a quantitative relative assay by means of real-time PCR. Such data in the literature are scarce and not quantitative, with Northern analysis showing that (1) MSTN mRNA is higher in the red muscle of adult brook trout, king mackerel and yellow perch, (2) MSTN is expressed predominantly in white muscle in little tunny and (3) MSTN is expressed equally in both muscle types in mahi-mahi (Roberts and Goetz 2001). Furthermore, Østbye et al. (2001) have demonstrated that, in salmon, the mRNA of MSTN is detectable in red muscle only (by standard PCR assay). The real-time PCR assay has

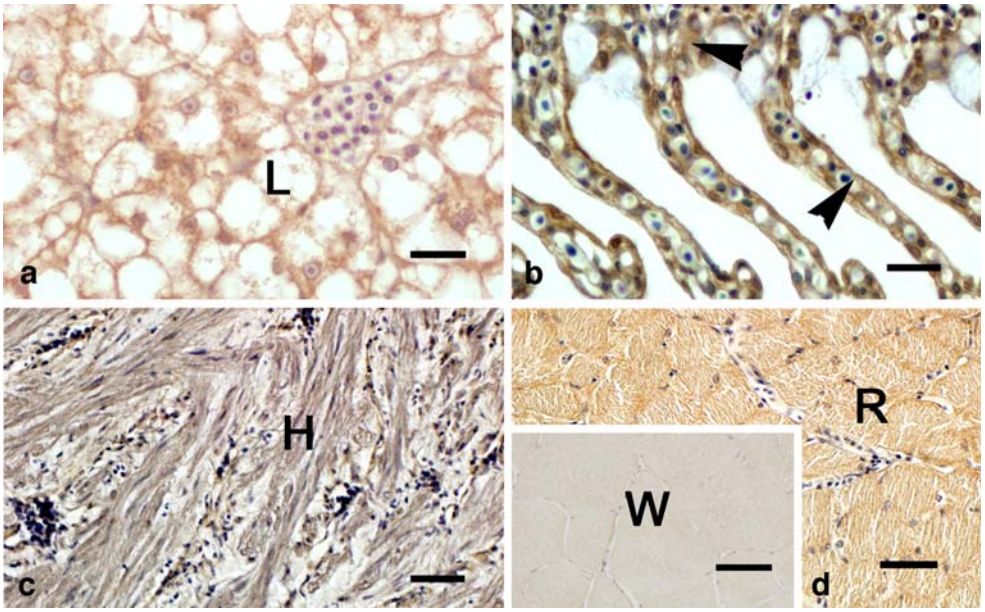


Fig. 8 Immunohistochemical localization of MSTN in sea bass adults. All micrographs show counterstaining with haematoxylin. **a** Liver (*L*) exhibits MSTN immunostaining in the hepatocytes. **b** In gills, MSTN immunostaining is present in both primary and secondary (*arrowheads*) lamellae. **c** Striated myocardial fibres of the heart (*H*) showing faint MSTN immunostaining. **d** Transverse section of the lateral muscle showing marked immunostaining in red fibres (*R*). *Inset*: Immunonegative white muscle fibres (*W*). *Bars* 20 μm

confirmed our immunohistochemical data, especially in adult sea bass in which the level of mRNA is much higher in the red/slow portion of lateral muscle than in the white/fast muscle (this is also the case in shi drum and sea bream; M. Patruno et al., unpublished observations), whereas the mRNA levels in juveniles are about the same. This pattern of MSTN expression, which is influenced by the developmental stage considered, is analogous to that observed in mammals. Indeed, in the latter animals, MSTN mRNA levels are higher in adult fast-glycolytic fibres compared with those in slow fibres (Carlson et al. 1999; Matsakas et al. 2006), whereas during myogenesis (towards the end of the gestation period), MSTN is expressed in slow fibres only (Patruno et al. 2007). In sea bass, red muscle grows slowly compared with the fast-growing white muscle. The lack of MSTN protein in the red muscle of fry (as opposed to the high level of expression found in the same muscle in adults) suggests that MSTN plays a role as a negative growth regulator in this muscle type. However, only a single study has demonstrated that MSTN suppression (by RNAi) increases the size of zebrafish because of an increase of muscular mass (Acosta et al. 2005). Therefore, we cannot exclude other possible functions for MSTN in the fish muscle or in other tissues in which it is expressed.

Concluding remarks

Our study demonstrates that the IGF-I mRNA and protein expression patterns are consistent with previous findings (Vong et al. 2003; Biga et al. 2004; Pierce et al. 2004) indicating that IGF-I functions in an autocrine/paracrine manner that stimulates cell proliferation in various tissues and organs, especially from the first week post-hatching. Another indication of the importance of the local production of IGF-I on general growth is found in the lateral muscle in which IGF-I mRNA fluctuations during development are strictly linked to the proliferative condition of either the white or red muscle fibres. Recently, Eppler et al. (2007) have shown that the enhanced growth of GH-overexpressing tilapia probably depends on the action of IGF-I on extrahepatic sites, in particular on the skeletal muscle. Therefore, during intense phases of growth, IGF-I mRNA may be produced continuously in the muscle (red or white or both) and immediately released into the circulation or translated locally; this could also explain the discrepancy between IGF-I mRNA and protein expression profiles observed in the lateral muscle.

We have also confirmed the wide expression of sea bass MSTN-1 mRNA and protein, suggesting that, in fish, MSTN has additional functions other than regulating muscle growth. However, of interest, the muscle tissue of sea bass exhibit a quantitative differential expression of MSTN in red and white muscle in adults but not during earlier phases of development. The MSTN mRNA levels observed in the two muscle types of sea bass, together with previous ambiguous results obtained in other fish species (Østbye et al. 2001; Roberts and Goetz 2001), suggest that MSTN is developmentally regulated (McFarlane et al. 2005) and that a rapid up- or down-regulation of this factor is necessary during both slow (adults) and rapid (juveniles) growing phases of sea bass.

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References

Acosta J, Carpio Y, Borroto I, González O, Estrada MP (2005) Myostatin gene silenced by RNAi show a zebrafish giant phenotype. J Biotechnol 119:324–331

Amali AA, Lin CJ, Chen YH, Wang WL, Gong HY, Lee CY, Ko YL, Lu JK, Her GM, Chen TT, Wu JL (2004) Up-regulation of muscle-specific transcription factors during embryonic somitogenesis of zebrafish (*Danio rerio*) by knock-down of myostatin-1. Dev Dyn 229:847–856

Ayson FG, de Jesus EG, Moriyama S, Hyodo S, Funkenstein B, Gertler A, Kawauchi H (2002) Differential expression of insulin-like growth factor I and II mRNAs during embryogenesis and early development in rabbitfish, *Siganus guttatus*. Gen Comp Endocrinol 126:165–174

Berishvili G, Shved N, Eppler E, Clota F, Baroiller JF, Reinecke F (2006) Organ-specific expression of IGF-I during early development of bony fish as revealed in the tilapia, *Oreochromis niloticus*, by in situ hybridization and immunohistochemistry: indication for the particular importance of local IGF-I. Cell Tissue Res 325:287–301

Biga PR, Goetz FW (2006) Zebrafish and giant danio as models for muscle growth: determinate vs. indeterminate growth as determined by morphometric analysis. Am J Physiol Regul Integr Comp Physiol 291:1327–1337

Biga PR, Schelling GT, Hardy, RW, Cain KD, Overturf K, Ott TL (2004) The effects of recombinant bovine somatotropin (rbST) on tissue IGF-I, IGF-I receptor, and GH mRNA levels in rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol 135:324–333

Biga PR, Roberts SB, Iliey DB, McCauley LA, Moon JS, Collodi P, Goetz FW (2005) The isolation, characterization, and expression of a novel GDF11 gene and a second myostatin form in zebrafish, *Danio rerio*. Comp Biochem Physiol [B] Biochem Mol Biol 141:218–230

Caelers A, Berishvili G, Meli ML, Eppler E, Reinecke M (2004) Establishment of a real-time RT-PCR for the determination of absolute amounts of IGF-I and IGF-II gene expression in liver and extrahepatic sites of the tilapia. Gen Comp Endocrinol 137:196–204

Carlson CJ, Booth FW, Gordon SE (1999) Skeletal muscle myostatin mRNA expression is fibre-type specific and increases during hindlimb unloading. Am J Physiol 277:601–606

Castillo J, Codina M, Martínez ML, Navarro I, Gutiérrez J (2004) Metabolic and mitogenic effects of IGF-I and insulin on muscle cells of rainbow trout. Am J Physiol Integr Comp Physiol 286:935–941

Duan C (1998) Nutritional and developmental regulation of insulin-like growth factor in fish. J Nutr 128:306–314

Duguay SJ, Lai-Zhang J, Steiner DF, Funkenstein B, Chan SJ (1996) Developmental and tissue-regulated expression of IGF-I and IGF-II mRNAs in *Sparus aurata*. J Mol Endocrinol 16:123–132

Duval H, Rousseau K, Elies G, Le Bail PY, Dufour S, Boeuf G, Boujard D (2002) Cloning, characterization, and comparative activity of turbot IGF-I and IGF-II. Gen Comp Endocrinol 126:269–278

Eppler E, Caelers A, Shved N, Hwang G, Rahman AM, Maclean N, Zapf J, Reinecke M (2007) Insulin-like growth factor I (IGF-I) in a growth-enhanced transgenic (GH-overexpressing) bony fish, the tilapia (*Oreochromis niloticus*): indication for a higher impact of autocrine/paracrine than of endocrine IGF-I. Transgenic Res 16:479–489

Fine M, Almuly R, Sandowski R, Marchant TA, Chan SJ, Gertler A, Funkenstein B (1997) Recombinant gilthead seabream (*Sparus aurata*) insulin-like growth factor-I: subcloning, expression in *Escherichia coli*, purification and characterization. J Endocrinol 153:139–150

Funkenstein B, Rebhan Y (2007) Expression, purification, renaturation and activation of fish myostatin expressed in *Escherichia coli*: facilitation of refolding and activity inhibition by myostatin prodomain. Prot Expr Purif 54:54–65

Funkenstein B, Balas V, Skopal T, Radaelli G, Rowlerson A (2006) Long-term culture of muscle explants from *Sparus aurata*. Tissue Cell 38:399–415

Garikipati DK, Gahr SA, Rodgers BD (2006) Identification, characterization, and quantitative expression analysis of rainbow trout myostatin-1a and myostatin-1b genes. J Endocrinol 190:879–888

Garikipati DK, Gahr SA, Roalson EH, Rodgers BD (2007) Characterization of rainbow trout myostatin-2 genes (rtMSTN-2a and -2b): genomic organization, differential expression, and pseudogenization. Endocrinology 148:2106–2115

Greene MW, Chen TT (1997) Temporal expression pattern of insulin-like growth factor mRNA during embryonic development in a teleost, rainbow trout (*Onchorynchus mykiss*). Mol Mar Biol Biotechnol 6:144–151

Greene MW, Chen TT (1999) Quantitation of IGF-I, IGF-II, and multiple insulin receptor family member messenger RNAs during embryonic development in rainbow trout. Mol Reprod Dev 54:348–361

Gregory DJ, Waldbieser GC, Bosworth BG (2004) Cloning and characterization of myogenic regulatory genes in three ictalurid species. Anim Genet 35:425–430

Grobet L, Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Menissier F, Massabanda J, Fries R, Hanset R, Georges M (1997) A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. Nat Genet 17:71–74

Grobet L, Poncelet D, Royo LJ, Brouwers B, Pirottin D, Michaux C, Menissier F, Zanotti M, Dunner S, Georges M (1998) Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. Mamm Genome 9:210–213

Hansson HA, Nilsson A, Isgaard J, Billig H, Isaksson O, Skottner A, Andersson IK, Rozell B (1988) Insulin-like growth factor I in the pancreas of normal and diabetic adult rats. Acta Physiol Scand 132:569–576

Helterline DL, Garikipati D, Stenkamp DL, Rodgers BD (2007) Embryonic and tissue-specific regulation of myostatin-1 and -2 gene expression in zebrafish. Gen Comp Endocrinol 151:90–97

Ji SQ, Losinski RL, Cornelius SG, Frank GR, Willis GM, Gerrard DE, Depreux FFS, Spurlock ME (1998) Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. Am J Physiol Regul Integr Comp Physiol 44: R1265–R1273

Joulia-Ekaza D, Cabello G (2006) Myostatin regulation of muscle development: molecular basis, natural mutations, physiopathological aspects. Exp Cell Res 312:2401–2414

Kambadur R, Sharma M, Smith TPL, Bass JJ (1997) Mutations in myostatin (GDF8) in double muscled Belgian Blue and Piedmontese cattle. Genome Res 7:910–915

Kerr T, Roalson EH, Rodgers BD (2005) Phylogenetic analysis of the myostatin gene sub-family and the differential expression of a novel member in zebrafish. Evol Dev 7:390–400

Ko CF, Chiou TT, Chen TT, Wu JL, Chen JC, Lu JK (2007) Molecular cloning of myostatin gene and characterization of

tissue-specific and developmental stage-specific expression of the gene in orange spotted grouper, *Epinephelus coioides*. Mar Biotechnol 9:20–32

Kocabas AM, Kucuktas H, Dunham RA, Liu Z (2002) Molecular characterization and differential expression of the myostatin gene in channel catfish (*Ictalurus punctatus*). Biochim Biophys Acta 1575:99–107

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta Ct)}$ method. Methods 25:402–408

Maccatrozzo L, Bargelloni L, Cardazzo B, Rizzo G, Patarnello T (2001a) A novel second myostatin gene is present in teleost fish. FEBS Lett 509:36–40

Maccatrozzo L, Bargelloni L, Radaelli G, Mascarello F, Patarnello T (2001b) Characterization of the myostatin gene in the gilthead sea bream (*Sparus aurata*): sequence, genomic structure, and expression pattern. Mar Biotechnol 3:224–230

Matsakas A, Bozzo C, Cacciani N, Caliaro F, Reggiani C, Mascarello F, Patruno M (2006) Effects of swimming exercise and training on myostatin expression in red and white gastrocnemius muscle and in the heart of the rat. Exp Physiol 91:983–994

McFarlane C, Langley B, Thomas M, Hennebry A, Plummer E, Nicholas G, McMahon C, Sharma M, Kambadur R (2005) Proteolytic processing of myostatin is auto-regulated during myogenesis. Dev Biol 283:58–69

McPherron AC, Lee S-J (1997) Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci USA 94:12457–12461

McPherron AC, Lawer AM, Lee S-J (1997) Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature 387:83–90

Moriyama S, Ayson FG, Kawauchi H (2000) Growth regulation by insulin-like growth factor-I in fish. Biosci Biotech Biochem 64:1553–1562

Nieto MA, Patel K, Wilkinson DG (1996) In situ hybridization analysis of chick embryos in whole mount and tissue sections. Methods Cell Biol 51:219–235

Østbye TK, Falck Galloway T, Nielsen C, Gabestad I, Bardal T, Andersen Ø (2001) The two myostatin genes of Atlantic salmon (*Salmo salar*) are expressed in a variety of tissues. Eur J Biochem 268:5249–5257

Patruno M, Radaelli G, Mascarello F, Candia Carnevali MD (1998) Muscle growth in response to changing demands in the teleost *Sparus aurata* (L) during development from hatching to juvenile. Anat Embryol 198:487–504

Patruno M, McGonnell I, Graham A, Beesley P, Candia Carnevali MD, Thorndyke M (2003) Anbmp2/4 is a new member of the transforming growth factor-beta superfamily isolated from a crinoid and involved in regeneration. Proc Biol Sci 270:1341–1347

Patruno M, Maccatrozzo L, Funkenstein B, Radaelli G (2006) Cloning and expression of insulin-like growth factors I and II in the shi drum (*Umbrina cirrosa*). Comp Biochem Physiol [B] Biochem Mol Biol 137:137–151

Patruno M, Caliaro F, Maccatrozzo L, Sacchetto R, Martinello T, Toniolo L, Reggiani C, Mascarello F (2007) Myostatin shows a specific expression pattern in pig skeletal and extraocular muscles during pre-natal and post-natal growth. Differentiation (OnlineEarlyArticles, DOI 10.1111/j.1432-0436.2007.00189.x)

Perrot V, Moiseeva EB, Gozes Y, Chan SJ, Ingleton P, Funkenstein B (1999) Ontogeny of the insulin-like growth factor system (IGF-I, IGF-II, and IGF-IR) in gilthead seabream (*Sparus aurata*): expression and cellular localization. Gen Comp Endocrinol 116:445–460

Pierce AL, Dickey JT, Larsen DA, Fukada H, Swanson P, Dickhoff WW (2004) A quantitative real-time RT-PCR assay for salmon IGF-I mRNA, and its application in the study of GH regulation of IGF-I gene expression in primary culture of salmon hepatocytes. Gen Comp Endocrinol 135:401–411

Radaelli G, Domeneghini C, Arrighi S, Bosi G, Patruno M, Funkenstein B (2003a) Localization of IGF-I, IGF-I receptor and IGFBP-2 in developing *Umbrina cirrosa* (Pisces: Osteichthyes). Gen Comp Endocrinol 130:232–244

Radaelli G, Rowlerson A, Mascarello F, Patruno M, Funkenstein B (2003b) Myostatin precursor is present in several tissues in teleost fish: a comparative immunolocalization study. Cell Tissue Res 311:239–250

Radaelli G, Patruno M, Rowlerson A, Maccatrozzo L, Funkenstein B (2005) Cellular localisation of insulin-like growth factor binding protein-2 (IGFBP-2) during development of the marine fish, *Sparus aurata*. Cell Tissue Res 319:121–131

Reinecke M, Collet C (1998) The phylogeny of the insulin-like growth factors. Int Rev Cytol 183:1–94

Reinecke M, Schmid A, Ermatinger R, Loffin-Cueni D (1997) Insulin-like growth factor I in the teleost *Oreochromis mossambicus*, the tilapia: gene sequence, tissue expression, and cellular localization. Endocrinology 138:3613–3619

Reinecke M, Biörnsson BT, Dickhoff WW, McCormick SD, Navarro I, Power DM, Gutiérrez J (2005) Growth hormone and insulin-like growth factors in fish: where we are and where to go. Gen Comp Endocrinol 142:20–24

Rescan P-Y, Jutel I, Ralliere C (2001) Two myostatin genes are differentially expressed in myotomal muscles of the trout (*Oncorhynchus mykiss*). J Exp Biol 204:3523–3529

Richardson NA, Chamberlain CG, McAvoy JW (1995) Localization of insulin-like growth factor-I immunoreactivity in larval and juvenile barramundi (*Lates calcarifer*). Gen Comp Endocrinol 100:282–292

Roberts SB, Goetz FW (2001) Differential skeletal muscle expression of myostatin across teleost species, and the isolation of multiple myostatin isoforms. FEBS Lett 491:212–216

Rodgers BD, Weber GM, Sullivan CV, Levine MA (2001) Isolation and characterization of myostatin complementary deoxyribonucleic acid clones from two commercially important fish: *Oreochromis mossambicus* and *Morone chrysops*. Endocrinology 142:1412–1418

Rowlerson A, Veggetti A (2001) Cellular mechanisms of post-embryonic muscle growth in aquaculture species. In: Johnston IA (ed) Muscle development and growth. Fish physiology, vol 18. Academic Press, San Diego, pp 103–140

Schmid AC, Naf E, Kloas W, Reinecke M (2000) Primary cultured hepatocytes of the bony fish, *Oreochromis mossambicus*, the tilapia: a valid tool for physiological studies on IGF-I expression in liver. J Endocrinol 166:265–273

Sharma M, Kambadur R, Matthews KG, Somers WG, Devlin GP, Conaglen JV, Fowke PJ, Bass JJ (1999) Myostatin, a transforming growth factor-beta superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. J Cell Physiol 180:1–9

Shved N, Berishvili G, D'Cotta H, Baroiller JF, Eppler E, Segner H, Reinecke M (2005) A survey on the expression of IGF-I in the early developing bony fish with special emphasis on the tilapia, *Oreochromis niloticus*. Ann N Y Acad Sci 1040:469–471

Shyu KG, Ko WH, Yang WS, Wang BW, Kuan P (2005) Insulin-like growth factor-I mediates stretch-induced upregulation of myostatin expression in neonatal rat cardiomyocytes. Cardiovasc Res 68:405–414

Terova G, Bernardini G, Binelli G, Gornati R, Saroglia M (2006) cDNA encoding sequences for myostatin and FGF6 in sea bass (*Dicentrarchus labrax*, L.) and the effect of fasting and refeeding on their abundance levels. Domest Anim Endocrinol 30:304–319

Vianello S, Brazzoduro L, Dalla Valle L, Belvedere P, Colombo L (2003) Myostatin expression during development and chronic stress in zebrafish (*Danio rerio*). J Endocrinol 176:47–59

Vong QP, Chan KM, Cheng CH (2003) Quantification of common carp (*Cyprinus carpio*) IGF-I and IGF-II mRNA by real-time PCR: differential regulation of expression by GH. J Endocrinol 178:513–521

Webb PW (1988) Simple physical principles in vertebrate aquatic locomotion. Am Zool 28:709–725

Wood AW, Duan C, Bern HA (2005) Insulin-like growth factor signaling in fish. Int Rev Cytol 243:215–285

Xu C, Wu G, Zohar Y, Du S-J (2003) Analysis of myostatin gene structure, expression and function in zebrafish. J Exp Biol 206:4067–4079