Immunohistochemical localization of IGF-I, IGF-II and MSTN proteins during development of triploid sea bass (*Dicentrarchus labrax*)

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Abstract

The cellular localization of IGF-I, IGF-II and MSTN proteins was investigated during ontogenesis of triploid sea bass (*Dicentrarchus labrax*) by an immunohistochemical approach. The results were compared with those observed in diploids. IGF-I immunostaining was mainly observed in skin, skeletal muscle, intestine and gills of both diploids and triploids. From day 30 of larval life, IGF-I immunoreactivity observed in skeletal muscle, intestine, gills and kidney was stronger in triploids than in diploids. At day 30, triploids exhibited a standard length significantly higher than the one of diploids. Although IGF-II and MSTN immunoreactivity was detectable in different tissues and organs, no differences between diploids and triploids were observed. The spatial localization of IGF-I, IGF-II and MSTN proteins detected in this study is in agreement with previous findings on the distribution of these proteins in diploid larvae and fry. The highest IGF-I immunoreactivity observed in triploids suggests a possible involvement of ploidy in their growth performance.

Introduction

In the last years, the use of chromosome set manipulation techniques to induce triploidy has been employed to increase growth performance in both freshwater and marine fish species.[1-5] Although triploids are morphologically similar to diploids, they are functionally sterile and may give economical advantage of a potential faster post-puberal growth.[6] However, comparing the growth performance between triploid and diploid animals, different results have been observed in relation to the species examined, the age and the rearing conditions.[7] The European sea bass (*D. labrax*) is a marine teleost fish of great interest in aquaculture, due to its meat quality and the large size that it can reach. Sea bass represents one of the numerous marine species that has been subjected to triploidization[3,8-9] in order to study the growth performance.[7] By evaluation of body weight, Felip *et al.*[3,10] observed that in juveniles, diploids reached similar growth rates as those of triploids, whereas adult triploids grew slower than adult diploids although they exhibited similar fork length. No papers report on the cellular localization of growth factors such as IGFs and MSTN during ontogenesis of triploid fish.

It is well known that a complex of endo-, para- and autocrine ways of action regulates growth. In fish, the Insulin-like Growth Factor (IGF) complex and myostatin (MSTN) play a key role in the growth regulation.[11-14] The IGF complex 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 IGF-binding proteins (IGFBP-1 to -6).[15-17] IGF-I is produced mainly in the bony fish liver, although numerous other organs express this molecule as well.[16,18-28] IGF-II shows structural sequence similarity to IGF-I and in fish exhibits an ubiquitous expression, where it acts mainly as a growth factor.[21,25,27,29-40] Myostatin (MSTN) is a member of the TGF-β superfamily and in fish its expression has been observed in several organs such as brain, eyes, exocrine and endocrine pancreas, gills, gonads, heart, intestine, kidney, liver, oesophagus, pharynx, skin, spleen, stomach[12,41-53] and muscle fish explants.[54]

Since the data concerning the growth performance of triploids are mainly focused on body weight and fork length, the aim of this study was to evaluate whether diploid and triploid sea bass (*D. labrax*) differed in terms of immunohistochemical localization of IGF-I, IGF-II and MSTN proteins. For this purpose, the cellular sites of protein distribution were examined during development and growth in larvae and fry of both diploid and triploid animals.

Materials and Methods

Animals

Sea bass (*D. labrax*) eggs and milt were obtained from broodstock held in a fish farm at Pellestrina (Venice, Italy). Diploids and triploids were obtained as described by Colombo *et al.*[8] Briefly, females were stimulated with a single dose of 10 µg kg⁻¹ of GnRH analogue des-Gly[10], [D-Ala[6]]-LH-RH ethylamide (Sigma, USA). Males were not hormonally treated. Nearly 50-60 h after hormone treatment, fish were netted, anaesthetized and gametes were collected by a gentle abdominal compression in both males and females. Within 5 min from fertilization, eggs were rapidly rinsed from sperm and submitted to a cold shock (0-2°C) for 20 min to prevent the extrusion of the second polar body. An equivalent part of fertilized egg mass was untreated and used as diploid control. Ploidy of animals was determined by flow-cytometry assessment of the nuclear DNA content in *in toto* larvae.[55]

Diploid and triploid eggs were reared, till hatch, into two different 80 L incubators, under the same hydrological conditions (temperature range:15.5-22.8°C). Larvae hatched in 36 h and, after yolk sack absorption, were transferred into two separate 1 m³ tank and fed accordingly to rearing protocol. At 2, 6, 10, 30, 45, 60, 74 days post hatch (dph) a pool of larvae from each ploidy was sampled and euthanasized with a lethal dose of MS-222 (Sandoz, Italy).

Growth performance

At each age class (except at 2 dph), the standard length (LS, the distance from snout to the tip of the notochord or hypural plate) of 10-12 larvae or juveniles *per ploidy* was measured under a binocular microscope.

Fixation and embedding

Animals were fixed in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS,

0.1 M, pH 7.4) at 4°C overnight, 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.

Immunohistochemical procedure

All the antibodies used for this study are detailed in Table 1. Immunohistochemical staining was done using the Elite ABC KIT system (Vector Laboratories, Inc., Ca, USA). Before applying the primary antibody, endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in PBS. The non-specific binding sites were blocked by incubating the sections in normal goat serum (Dako, Italy). Then, sections were incubated with specific primary antisera (see Table 1) overnight at 4°C. After washing with PBS, sections were incubated with biotin-conjugated anti-mouse Ig antibodies (Dako), washed with PBS and reacted with peroxidase-labeled avidin-biotin complex (Vector Laboratories, Inc., Ca, USA). 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: i) PBS instead of the specific primary antibodies (see Table 1); ii) preimmune sera instead of the primary antisera; iii) PBS instead of the secondary antibodies and iv) by absorption of the antisera with excess of synthetic peptides (3 μg/L) before incubation with sections. The results of these controls were negative (i.e. staining was abolished).

Statistical analysis

A Student's t-test for independent samples was used to determine any significant differences between mean LS of diploids and triploids at each age class. Statistical significance was taken as $P<0.05$.

Results

Ploidy in the examined animals

All the putative triploid sea bass were characterized by 1.5 fold the nuclear DNA amount of the diploid fish (data not shown), thus confirming their triploid status and the success of triplodization.

Table 1. Antibodies used in the current study.

Antibody name and origin	Immunogen and dilution	Source and references for use with fish tissues	Significance
Anti-IGF-I; mouse polyclonal anti-insulin-like growth factor-I	IGF-I from fish (*S. aurata*) 1:100	Perrot *et al.*[21]	Proliferation and differentiation of satellite cells, induced during muscle regeneration
Anti-IGF-II; mouse polyclonal anti-insulin-like growth factor-II	IGF-II from fish (*S. aurata*) 1:500	EuroGentec, Belgium; Radaelli *et al.*[35]	Proliferation of satellite cells, induced during muscle regeneration
Anti-MSTN; mouse polyclonal anti-myostatin	MSTN from fish (*S. aurata*) 1:800	EuroGentec, Belgium; Radaelli *et al.*[47]	Expression of MSTN precursor, occurring soon after muscle differentiation

Table 2. Immunohistochemical localization of IGF-I in diploids (D) and triploids (T) of sea bass.

Tissue	2 dph D	2 dph T	6 dph D	6 dph T	10 dph D	10 dph T	30-45 dph D	30-45 dph T	60 dph D	60 dph T	74 dph D	74 dph T
Gill epithelium	*	*	-	-	-	-	+	++	+	++	+	++
Heart	*	*	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Gut epithelium	++	+/-	++	++	+	+	+	++	+	++	+	++
Liver	*	*	+	+	+	+	+	+	+	+	+	+
Kidney	*	*	+	+	+	+/-	+/-	+	+/-	+	+/-	+
Pancreas	*	*	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-
Skin	+	+	++	++	+	+	++	++	+	+	+	+
Skeletal muscle	++	+	++	++	+	+	+	++	+/-	+	+	++
Yolk sac	++	+/-										

Staining: -, not detectable; +/-, slight but above background levels; + moderate; ++, marked staining. *Tissue not found on the sections examined at this stage.

Table 3. Immunohistochemical localization of IGF-II in diploids (D) and triploids (T) of sea bass.

Tissue	2 dph D	2 dph T	6 dph D	6 dph T	10 dph D	10 dph T	30-45 dph D	30-45 dph T	60 dph D	60 dph T	74 dph D	74 dph T
Gill epithelium	*	*	-	-	-	-	+	+/-	+	+	+	+/-
Heart	*	*	-	-	-	-	+	+/-	+/-	+/-	+/-	+/-
Gut epithelium	+	+/-	+	+/-	++	++	+	+	+/-	+/-	+/-	+/-
Liver	*	*	+	+/-	+/-	+/-	+	+/-	+	+/-	+	+/-
Kidney	*	*	+/-	-	+/-	-	-	-	-	-	-	-
Pancreas	*	*	-	-	-	-	-	-	-	-	-	-
Skin	+	+	+	+	+	+	+	+	+	+	+	+
Skeletal muscle	+	+	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Yolk sac	+/-	+/-										

Staining: -, not detectable; +/-, slight but above background levels; + moderate; ++, marked staining. *Tissue not found on the sections examined at this stage.

Growth performance

Mean values of LS in diploid and triploid fish for each age are shown in Figure 1.

The statistical analysis highlighted a difference only in 30 dph fish with triploids significantly greater than diploids (P<0.01).

Immunohistochemical localization of IGF-I, IGF-II and MSTN proteins

General

Immunohistochemical localization of IGF-I, IGF-II and MSTN in different tissues of diploid and triploid sea bass is summarized in Tables 2-4.

IGF-I

In larvae aged 2 days, IGF-I immunoreactivity was detected in the epithelia of developing intestine and skin, as well as in lateral muscle and yolk sac of both diploids and triploids (Table 2). In larvae aged 6 days, skeletal muscle of both diploids and triploids showed a marked immunostaining (Figure 2A, B). At this developmental stage, a marked immunoreactivity was also observed in the epithelia of gut and skin (Table 2). In larvae aged 6-10 days, a moderate immunostaining was also detected in liver and developing kidney, whereas heart musculature exhibited a faint reactivity (Table 2). From day 30, skeletal muscle as well as the epithelia of gills, gut and kidney of triploids exhibited an immunoreactivity higher than the one of diploids (Table 2, Figure 2C-F).

IGF-II

In both diploids and triploids aged 2 days, a moderate IGF-II immunostaining was observed in the epithelia of skin and developing intestine (Table 3), as well as in skeletal muscle (Figure 3A, B). At this developmental stage, a faint IGF-II immunoreactivity was also observed in the yolk sac (Table 3). From day 6, IGF-II reactivity was also detected in liver of both diploids and triploids and in developing kidney of diploids (Table 3). From day 30, heart musculature and gill epithelium exhibited an IGF-II immunostaining, whereas no reactivity was detected in kidney (Table 3). At all stages, tissues exhibited an IGF-II immunostaining ranging from faint to moderate, although the gut epithelium of larvae aged 10 days showed a marked reactivity (Table 3, Figure 3C, D). No significant differences between diploids and triploids were observed in immunoreactivity.
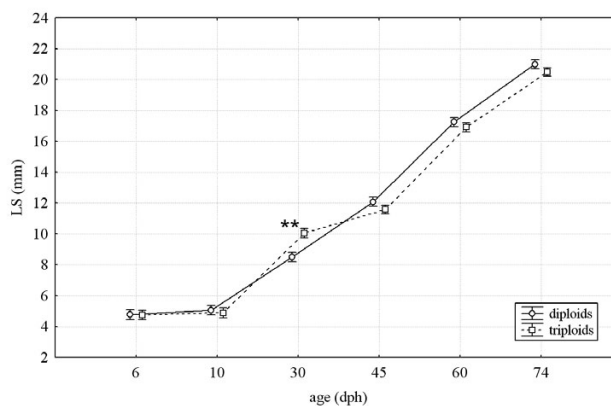
MSTN

In both diploids and triploids aged 2-10 days, a MSTN immunostaining was observed in skeletal muscle and in the epithelia of skin and developing intestine (Table 4, Figure 4A-F). From day 6, MSTN immunoreactivity was also detected in liver, heart musculature and in developing kidney, although the staining was different between diploids and triploids (Table 4, Figure 4C-F). Pancreas exhibited a MSTN immunostaining only in larvae aged 10 days (Figure 4F). From day 30 the epithelium covering gill filament was stained, too (Table 4). No significant differences between diploids and triploids were observed in immunoreactivity.

Discussion

The present study reports novel information on the cellular localization of IGF-I, IGF-II and MSTN proteins during development and growth of diploid and triploid sea bass (*D. labrax*). Although triploids are morphologically similar to diploids, they have larger but fewer cells in most tissues and organs and they are sterile, thus increasing their appealing for aquaculture as a mean to protect somatic growth, survival and flesh quality from the negative effects of sexual maturation.[5,6,56]

A general opinion is that triploids should grow faster than diploids, since their genes are more numerous and their cells are larger.[56] However, the literature concerning the growth performance of triploids and their diploid counterparts is often contradictory. Several studies have reported that growth of triploid fish was similar to the diploids one, during the juvenile period,[3,10,57-59] whereas it was higher at their sexual maturation time.[60-63] Moreover, Razak *et al.*[64] found that transgenic diploids of tiliapia were superior in growth performance, followed by transgenic triploids, non transgenic diploids and non transgenic triploids. In terms of growth performance, the literature of triploids is mainly limited to the evaluation of growth rate and fork length, whereas no papers report on the cellular localization of growth factors, such as IGFs and MSTN, during ontogenesis. In the present study the cellular localization of IGF-I, IGF-II and MSTN proteins was studied from hatching to juvenile stages by immunohistochemistry using polyclonal antisera raised against sea bream IGF-I,[21] IGF-II[35] and MSTN.[47] In general, the cellular sites of immunoreactivity observed in triploids were identical to those found in diploids for all tested antibodies.

Cellular localization of IGF-I

The pattern of immunostaining for IGF-I observed in both diploids and triploids was

Table 4. Immunohistochemical localization of MSTN in diploids (D) and triploids (T) of sea bass.

Tissue	2 dph		6 dph		10 dph		30-45 dph		60 dph		74 dph	
	D	T	D	T	D	T	D	T	D	T	D	T
Gill epithelium	*	*	-	-	-	-	+	+/-	+	+/-	+	+/-
Heart	*	*	+/-	-	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-
Gut epithelium	+	+	+/-	+	++	+/-	+	+	+	+	+	+
Liver	*	*	+	+/-	+/-	-	+/-	-	+/-	-	+/-	-
Kidney	*	*	+/-	-	+/-	-	+/-	-	+/-	+/-	+/-	+/-
Pancreas	*	*	-	-	+/-	+	-	-	-	-	-	-
Skin	+	+	+	+	+	+	+	+/-	+	+/-	+	+/-
Skeletal muscle	+	+	+	+	+/-	+/-	+	+/-	+/-	+/-	+	+/-
Yolk sac	+	+/-										

Staining: -, not detectable; +/-, slight but above background levels; + moderate; ++, marked staining. *Tissue not found on the sections examined at this stage.

Figure 1. Mean standard length of diploid and triploid fish at each age class. Vertical bars represent standard errors of the mean and asterisks indicate significant difference between groups.

similar to that observed in diploids of *S. aurata*,[21] *U. cirrosa*,[23,27] *D. labrax*,[28] demonstrating that the sequence similarity between IGF-I from different fish species is sufficient to allow cross-species immunoreactivity. A similar pattern of immunostaining was also observed in *O. niloticus* by Berishvili *et al.*[26]

During early larval life, IGF-I immunoreactivity was mainly detected in the epithelia of gut, skin and kidney, as well as in skeletal musculature and liver. No significant differences between diploids and triploids were observed in immunoreactivity. Interestingly, from day 30, skeletal muscle and the epithelia of gills, gut and renal tubules of triploids exhibited an immunostaining higher than that of diploids. In our experimental conditions, the growth of triploids measured in terms of standard length was similar to that of diploids, although at day 30 triploids exhibited a significantly higher length. As mentioned above, triploid fish have larger but fewer cells in their tissues and the reduction in the cell number could be compensated by a higher protein synthesis.[56] In fish, body growth is mainly correlated to that of skeletal lateral muscle, which continues to grow significantly even into juvenile life in many species, through continuous hyperplasia and hypertrophy.[65] Johnston *et al.*[66] found that triploid *Salmonidae* exhibited a reduced hyperplasia as a consequence of a decrease in the satellite cell number. They suggested that the reduced number of myocytes detected in triploids was compensated by their rates of hypertrophic growth, greater than the diploids ones.[66]

Moreover, Alonso *et al.*[67] observed that during fin regeneration of *O. mykiss*, the protein synthesis (per unit muscle mass) was higher in triploids than in diploids.
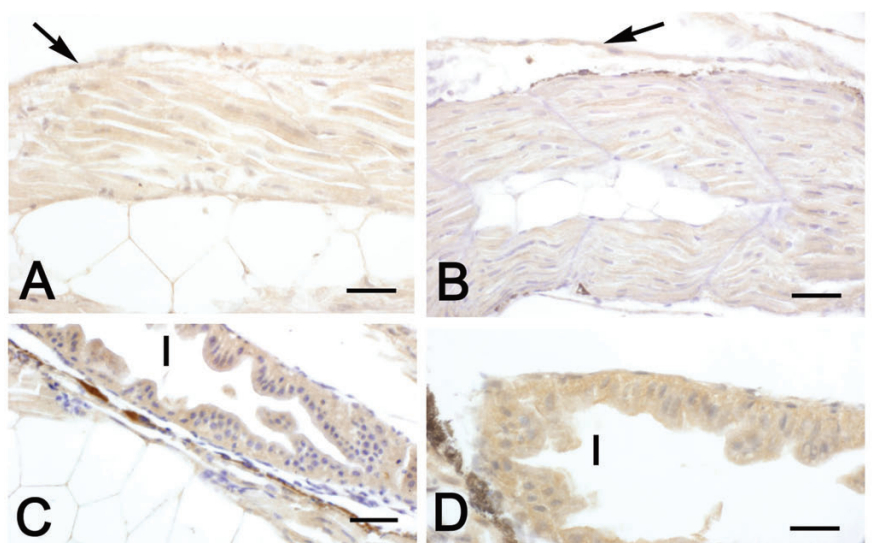
Cellular localization of IGF-II

In our previous immunohistochemical study, the use of an antiserum raised against sea bream IGF-II gave us the possibility to detect the cellular distribution of IGF-II protein in *D. labrax*,[28] as well as in *U. cirrosa*,[27] demonstrating that there is a high degree of similarity between IGF-II proteins from different fish species and therefore allows cross-species immunoreactivity.

In young larvae, IGF-II immunostaining was found in skeletal muscle, liver, the epithelia of skin and developing intestine of both diploids and triploids, as well as in the epithelium of renal tubules of diploids. From day 30, heart musculature and the epithelium of gill filaments exhibited an IGF-II immunostaining. The pattern of IGF-II immunopositivity was similar to the one observed in diploids of several fish species.[21,27,35,40] However, in the present work we did not observe significant differences between diploids and triploids in terms
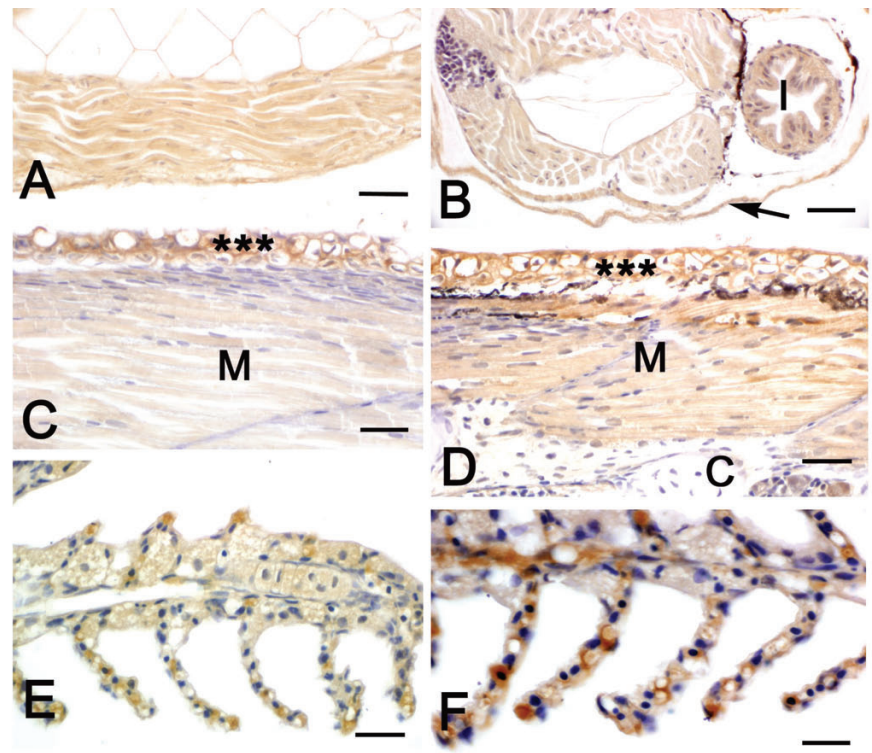
Figure 2. Immuno-histochemical localization of IGF-I in sea bass larvae and fry. All panels are counter-stained with haematoxylin. A, C, E: diploid animals; B, D, F: triploid animals. A Sagittal section of a 6-day larva. A marked IGF-I immunostaining is present in the trunk musculature. B Transverse section of a 6-day larva. A marked IGF-I immunostaining is present in the trunk musculature, intestine (I) and skin (arrow). C-D Sagittal sections of 45-day larvae. Skin epithelium (asterisks) exhibits a marked positivity in both diploids and triploids. An immunostaining is also present in skeletal muscle (M), although in triploids (D) the reactivity is stronger than in diploids (C). Cartilage (c) is negative. E-F Gills of 60-day fry. Immunostaining is present in the epithelium of the gill filaments and the reactivity is stronger in triploids (F) than in diploids (E). Bars (A) 20 μm, (B) 20 μm, (C) 15 μm, (D) 15μm, (E) 12.5 μm, (F) 12.5 μm.

Figure 3. Immunohistochemical localization of IGF-II in sea bass larvae. All panels are counterstained with haematoxylin. A, C: diploid animals; B, D: triploid animals. A-B Sagittal sections of 2-day larvae. In both diploids and triploids a moderate immunostaining is present in skeletal muscle and skin (arrows). C-D Sections of 10-day larvae. In both diploids and triploids a marked immunostaining is detectable in the intestinal epithelium (I). Bars (A) 15 μm; (B) 20 μm, (C) 20 μm, (D) 12.5 μm.
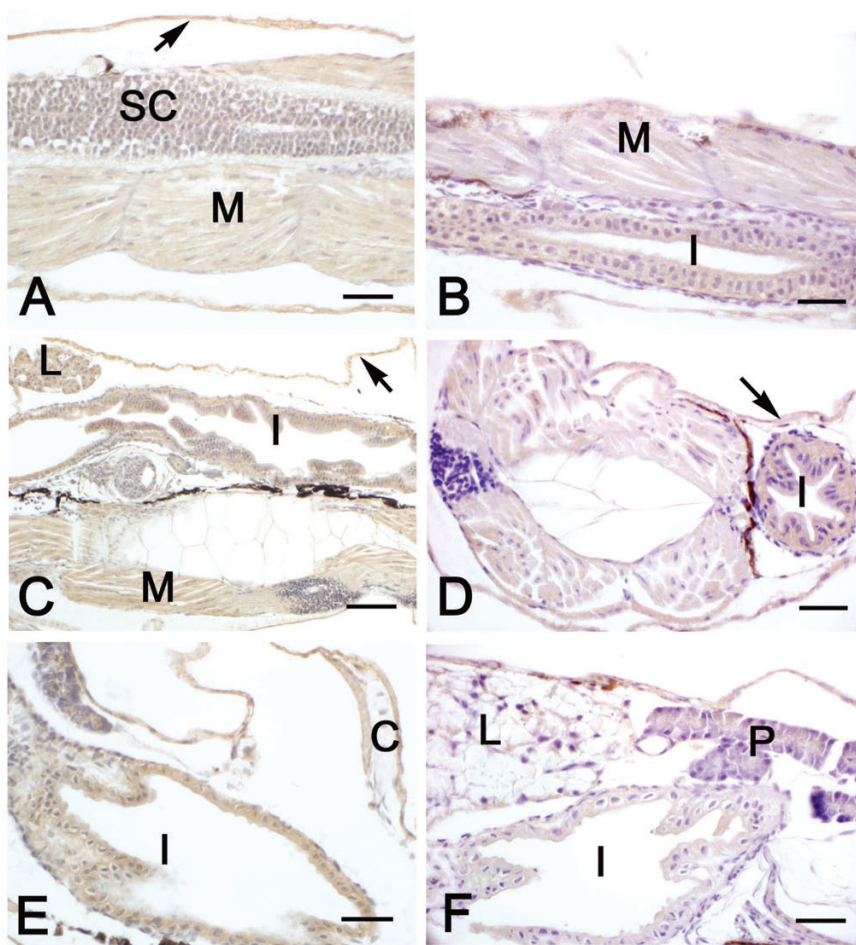
Figure 4. Immunohistochemical localization of MSTN in sea bass larvae. All panels are counter-stained with haematoxylin. A, C, E: diploid animals; B, D, F: triploid animals. A-B Sagittal sections of 2-day larvae. In both diploids (A) and triploids (B), a moderate immunostaining is found in the epithelium of developing intestine (I), in skeletal muscle (M) and in the epithelium of skin (arrow). Spinal cord (SC) is negative. C-D) Sections of 6-day larvae of both diploids (C) and triploids (D), showing a moderate immunoreactivity in skeletal muscle (M), intestinal epithelium (I), liver (L) and skin (arrows). E-F Sagittal sections of 10-day larvae of both diploids (E) and triploids (F), showing immunostaining in intestinal epithelium (I) and pancreas (P). Cartilage (c) is negative. L: liver. Bars (A) 20 μm, (B) 20 μm, (C) 40 μm, (D) 20 μm, (E) 20 μm, (F) 20 μm.

of IGF-II immunoreactivity, suggesting that a similar amount of IGF-II protein is detectable in the tissues of both diploids and triploids.

Cellular localization of MSTN

The high degree of similarity between MSTNs from different fish species allowed us to use an antiserum raised against sea bream MSTN to detect the cellular distribution of the protein in different fish species, including *D. labrax*.[28,40] In the present work, the pattern of MSTN immunostaining was similar to the one observed for IGF-I and IGF-II. A co-localization of IGF-I, IGF-II and MSTN proteins has been observed in cultured muscle explants from *S. aurata*,[54] suggesting an autocrine-paracrine action of these factors in regulating development and growth of fish. As for IGF-II, we did

not observe significant differences between diploids and triploids in terms of MSTN immunoreactivity, suggesting that a similar protein amount is present in the tissues of both diploids and triploids.

The results we report here show for the first time the immunohistochemical localization of IGF-I, IGF-II and MSTN proteins during ontogenesis of diploids and triploids of *D. labrax*. The spatial localization of all examined growth factors is identical in both diploids and triploids although from day 30, skeletal muscle, as well as the epithelia of gills, gut and kidney of triploids exhibited an IGF-I immunoreactivity higher than the one of diploids. Interestingly, at day 30, the standard length of triploids was significantly higher than the diploids one. We intend to further investigate, by Real-Time

PCR, the IGF-I espression in these tissues, in mind to better understand whether triploidy can result in any growth advantage.

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