Cell Tissue Res (2016) 363:541–554
DOI 10.1007/s00441-015-2254-0

REGULAR ARTICLE

Transient up- and down-regulation of expression of myosin light chain 2 and myostatin mRNA mark the changes from stratified hyperplasia to muscle fiber hypertrophy in larvae of gilthead sea bream (*Sparus aurata* L.)

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Received: 13 March 2015 / Accepted: 6 July 2015 / Published online: 6 August 2015

Abstract Hyperplasia and hypertrophy are the two mechanisms by which muscle develops and grows. We study these two mechanisms, during the early development of white muscle in *Sparus aurata*, by means of histology and the expression of structural and regulatory genes. A clear stage of stratified hyperplasia was identified early in the development of gilthead sea bream but ceased by 35 dph when hypertrophy took over. Mosaic recruitment of new white fibers began as soon as 60 dph. The genes *mlc2a* and *mlc2b* were expressed at various levels during the main phases of hyperplasia and hypertrophy. The genes *myog* and *mlc2a* were significantly up-regulated during the intensive stratified formation of new fibers and their expression was significantly correlated. Expression of *mstn1* and *igf1* increased at 35 dph, appeared to regulate the hyperplasia-to-hypertrophy transition, and may have stimulated the expression of *mlc2a*, *mlc2b* and *col1a1* at the onset of mosaic hyperplasia. The up-regulation of *mstn1* at transitional phases in muscle development indicates a dual regulatory role of myostatin in fish larval muscle growth.

Keywords Gene markers · mlc2 · Muscle cellularity · Myogenic factors · Gilthead sea bream

This research was co-financed by Greek National Funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funding Program: Heracleitus II, Investing in Knowledge Society through the European Social Fund and COST Action FA0801 "LarvaNet".

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Introduction

Muscle develops and grows through two fundamental processes: the recruitment of new fibers (hyperplasia) and the increase in size of existing fibers (hypertrophy). The two processes are differentially regulated throughout the lifecycle in teleosts: hyperplastic muscle growth dominates during the initial stages of development, whereas hypertrophy takes over later on. In teleosts that reach a large adult body size such as the gilthead sea bream (*Sparus aurata* L.), fiber recruitment occurs in three distinct phases during ontogeny: embryonic myogenesis that gives rise to two morphologically and functionally distinct muscle types forming the primary myotome; stratified hyperplasia, during which dorsal and ventral germinal zones add new white muscle fibers in discrete layers apically; and mosaic hyperplasia in which deep myogenic progenitor cells (MPCs) are recruited to fuse and form new myotubes or to contribute to the expansion in size of existing muscle fibers post-metamorphosis. The result is the generation of muscle fibers of variable diameter creating the mosaic appearance of the mature teleost musculature (Rowlerson et al. 1995). The axial musculature or fillet can account for up to 70 % of the fish body mass (Bone 1978) and is made up of serial myotomes of muscle fibers, mainly fast white fibers.

White muscle cellularity (number and size of muscle fibers) is a highly plastic process that depends both on intrinsic factors such as species and strain (e.g., Weatherley et al. 1988;

Valente et al. 1999) and extrinsic factors that include temperature and exercise (for a review, see Johnston 2006), plane of nutrition (e.g., Weatherley et al. 1979; Kiessling et al. 1991; Salze et al. 2014) and dietary nutrient composition (e.g., Fauconneau et al. 1997; Alami-Durante et al. 1997, 2010a, 2010b, 2011, 2014; Johnston et al. 2002; Silva et al. 2009); it also follows seasonal cycle patterns (Alami-Durante et al. 2007) and is strongly linked to the stage of development (for a review, see Johnston 1999). Most importantly, muscle cellularity is strongly correlated with the final size that a fish may attain. For example, Weatherley and colleagues (1988) compared ten different fish species and found that growth capacity largely depended on the maintenance of white muscle fiber recruitment and the number of small-diameter fibers. Similarly, significant differences in the final size of four isolated morphs of Arctic charr (*Salvelinus alpinus*) were strongly correlated with the number of white muscle fibers (Johnston et al. 2004). Furthermore, genetically modified Arctic charr carrying an extra growth hormone gene had significantly higher numbers of white muscle fibers compared with non-genetically modified fish of either the same age or the same size (Pitkänen et al. 2001).

The generation and enlargement of muscle fibers requires the synthesis and deposition of sarcomeric proteins that make up the bulk of differentiated muscle cells. Myosin is a major sarcomeric protein and consists of two heavy chains (MHCs) and four light chains (MLCs) combined in a long coiled α-helical tail and two heads. MHCs and MLCs exist in multiple isoforms that can exhibit both tissue-specific and stage-specific patterns of expression (Whalen et al. 1981; Gauthier et al. 1982) and are regulated by hormonal status and environmental cues (Gerlach et al. 1990; Hirayama et al. 1998; Moutou et al. 2001). Fish myosin expression is also known to be regulated by ration level (Overturf and Hardy 2001), fasting followed by satiation feeding (Bower et al. 2009) and some macro- and micro-nutrients (Hevroy et al. 2006; Alami-Durante et al. 2010b, 2011, 2014; Campos et al. 2010; Betancor et al. 2013).

In gilthead sea bream, two isoforms of *myosin light chain 2a* (*mlc2a*), which encode a 170-amino-acid (aa) peptide, have been isolated and characterized from white muscle (Moutou et al. 2001; Sarropoulou et al. 2006). Three alternative transcripts of *mlc2a* have been isolated that are products of alternative polyadenylation site selection and encode for the same peptide but differ in the length of their 3' untranslated regions (284 bp, *mlc2a*-S; 788 bp, *mlc2a*-M; 876 bp, *mlc2a*-L; Sarropoulou et al. 2006). Preliminary work has shown that the two *mlc2* isoforms exhibit different expression patterns during development and in primary muscle cell cultures. Transcripts of *mlc2a* are detectable at the onset of somitogenesis, increase gradually up to metamorphosis and are down-regulated post-metamorphosis (Moutou et al. 2009). Expression of *mlc2b* starts before hatch and remains at very low levels up until metamorphosis. Similarly, in primary muscle cell cultures, *mlc2a* is the predominant isoform expressed and peaks at differentiation, whereas *mlc2b* is expressed at significantly lower levels in all differentiation states (Moutou et al. 2009). In juvenile fish, the expression pattern of *mlc2a* and *mlc2b* is reversed, with *mlc2b* expression being about 10-fold higher than that of *mlc2a* and they respond differently to growth hormone administration (Moutou et al. 2009).

The improvement of muscle mass is a major goal in aquaculture research. The identification of the molecular mechanisms underlying muscle growth will provide molecular markers that can be used during genetic or environmental selection; such markers are an important tool for the efficient management of growth. In this context, we investigated the way that the expression of the two different *mlc2* isoforms, namely *mlc2a* and *mlc2b*, correlates with hyperplastic and hypertrophic growth and with other structural, regulatory and myogenic genes during the early stages of muscle development by using gilthead seabream larvae as the model.

Materials and methods

Fish and sampling stages

Gilthead sea bream (*Sparus aurata* L.) larvae were obtained from a commercial hatchery at Maliakos Gulf, Greece. Larvae were held in filtered, continuously aerated sea-water (salinity 29 ppt). Larvae were fed in excess twice a day with rotifers up to 23 days post hatch (dph) and newly hatched *Artemia nauplii* were co-fed from 13-25 dph during the nursing period. Rotifers were enriched by 24-h incubation in DHA Master (Inve, Belgium) and *Artemia* metanauplii were enriched by 24-h incubation in S.presso (Inve). Dry commercial diet was introduced on 20 dph (INVE O.range start, wean). The microalgae *Chlorella minutissima* was added during the period of feeding with rotifers. Larvae were kept under a 24-h light regime up to 25 dph and switched to 15:9 light:dark afterwards. The oxygen concentration in the seawater was 7 ±2 mg l^{-1} and water temperature during the embryonic, yolk-sac (0-4 dph) and larval-juvenile (5-60dph) stages was 19.0± 0.1 °C (18.0 - 20.5 °C).

Gilthead sea bream larvae were sampled on 5, 15, 25, 35, 45 and 60 dph. Larvae were anesthetized in 2-phenoxyethanol (1:5000, Sigma-Aldrich, P1126) and the total length of 10 larvae per sampling age was measured individually with a semi-automatic image analyzer (ImageJ software, Abramoff et al. 2004). Larvae for morphometric analyses were dipped in Serra fixation buffer (ethanol 6 vol, formalin 3 vol, acetic acid 1 vol) and then dehydrated up to butanol (Alami-Durante 1990) in which they were kept until histological analyses. Samples for gene expression analyses were

placed in RNAlater Reagent (Sigma-Aldrich, R0901) and held at -20 °C until use.

Quantitative histology

A histological protocol was adapted from protocols drawn for larvae of other fish species (Alami-Durante 1990; Alami-Durante et al. 2006). Muscle morphometric analysis was performed by using 10 fish from the 5-, 15-, 25-, 35-, 45- and 60-dph age groups. Gilthead sea bream larvae of 5, 15 and 25 dph were pre-embedded in agar and then dehydrated by immersion in an ethanol series (for 5 min in each solution) of increasing concentration (0, 25 %, 50 %, 75 % and 100 %) with a final step in butanol in which samples were held until being embedded in paraffin. Inclusion of larvae in paraffin was carried out by incubation for a total of 1 h (5-, 15- and 25-dph larvae) or 2 h (trunco-caudal region of 35-, 45- and 60-dph larvae) in four changes of molten paraffin (58 °C). Serial transverse sections (10 μm) were prepared by using a microtome (Leica, R2125), placed on glass slides and dried at 37 °C for 48 hr.

Sections were stained with Ehrlich hematoxylin and 1 % eosin (Alami-Durante et al. 2007) after the dewaxing of the sections in toluene (two changes for 5 min), rehydration through a graded ethanol series (100 %, 75 %, 50 % and 25 %) and washes in distilled water. Stained sections of 10 individuals per age group were observed by using a light microscope (Leica, DM2000) coupled to a digital camera and captured images were analyzed by using ImageJ software (Abramoff et al. 2004). Cellular analyses were performed in one dorsal epaxial quadrant of myotome located in the vent region of each specimen analyzed. This quadrant of white muscle consisted of newly formed muscle fibers and older fibers and was selected for cellularity measurements, i.e., muscle hypertrophy and hyperplasia. The outline of all individual white muscle fibers was drawn and the white fiber diameter (WFD; diameter of a circle with an area equal to the muscle fiber; hereafter referred to as "fiber diameter"), area (WFA) and perimeter (WFP) were determined. The total number of white fibers (TNF) present in the dorsal quadrant of white muscle was determined for each fish, as was the total cross-sectional area (TCSA) of this quadrant. Sections of 5-dph larvae were of insufficient quality for cellularity measurements and were excluded from the morphometric analysis.

RNA extraction

Total RNA was extracted from individual whole 5-, 15-, 25-, 35-, 45- and 60-dph larvae by using TRI Reagent (Sigma, T9424) according to the manufacturer's instructions. Total RNA was subsequently subjected to DNAse treatment with DNA-free (Ambion, AM1906) to remove traces of contaminating genomic DNA and was stored at -80 °C until further use. cDNA synthesis was performed simultaneously for all samples to ensure the same reaction efficiency. cDNA was generated from 1 μg total RNA by using 200 U/μl SuperScript II reverse transcriptase (Invitrogen, 18064-014), 3 μg random primers (Invitrogen, 48190-011) and 40 U/μl recombinant RNaseOUT ribonuclease inhibitor (Invitrogen, 10777-019) in a total reaction volume of 27 μl.

Relative quantification of gene expression by real-time quantitative polymerase chain reaction analysis

The gene expression levels of *mlc2a*, *mlc2b*, *myogenin* (*myog*), *myostatin 1* (*mstn1*), *mrf4*, *follistatin* (*fst*), *insulin-like growth factor 1* (*igf1*) and *collagen 1a1* (*col1a1*) were determined (Table 1). Real-time polymerase chain reaction (Q-PCR) was conducted in an MXPro 2000 (Stratagene) in duplicate by using the KAPA SYBR FAST qPCR (2×; KAPA Biosystems, KK4602). All reactions comprised 150-300 nmol/l of each primer and 0.17 μg/μl cDNA (1:5 diluted) in a reaction volume of 20 μl. The following PCR conditions were used: an initial denaturation step at 95 °C for 3 min, 40 cycles of amplification (each cycle was 30 s at 95 °C, 1 min at 61 °C, 1 min at 72 °C), followed by the dissociation curve step (1 min at 95 °C, 30 s at 55 °C, 30 s at 95 °C) to verify the amplification of a single product. Efficiency curves were obtained for each cDNA template by plotting CT values against the $\log_{10}$ of six serial dilutions of a cDNA pool created from all samples analyzed. Q-PCR efficiency (E) was calculated according to $E=10^{[-1/\text{slope}]}$ (Pfaffl 2001) and varied between 95 % and 100 %. A series of housekeeping genes (*ef1α*, *b-actin*, *rpl13a*, *rps18*) were validated for use and were rated by using the geNorm VBA applet (Vandesompele et al. 2002). The normalization factor was calculated as the geometric mean of the three most stably expressed housekeeping genes (*b-actin*, *rpl13* and *ef1a*).

Statistical analyses

Gene expression data were square-root-transformed to meet assumptions of normality and/or homogeneity. Morphometric and Q-PCR results are expressed as means±standard error of the mean (SEM). Morphometric data failed to fit the Kolmogorov–Smirnov test for normality and were analyzed by using a non-parametric Kruskal–Wallis analysis of variance median test (Zar 1996). When significant differences were obtained, a post-hoc non-parametric Dunn's test was used to determine the influence of age on larval development. The frequency distribution of <5 μm diameter of white muscle fibers was evaluated with Pearson's chi-squared test (Zar 1996). Spearman's rank correlation was performed to assess the statistical dependence of the two variables (Zar 1996). For all statistical tests, differences were considered to be significant

Table 1 Gene identity, accession number and forward and reverse primers used for real-time polymerase chain reaction analysis of gene expression (*ef1α*, *b-actin*, *rpl13a*, *rps18* housekeeping genes, *mlc2a* myosin light chain 2a isoform A, *mlc2b* myosin light chain 2a isoform B, *myog* myogenin, *mstn1* myostatin 1, *mrf4* myogenic regulatory factor 4, *fst* follistatin, *igf1* insulin-like growth factor 1, *col1a1* collagen 1a1)

Gene	Accession number	Forward primer	Reverse primer
ef1a	AF184170	TCAAGGGATGGAAGGTTGAG	AGTTCCAATACCGCCGAT
b-actin	AF384096	CGACATCCGTAAGGACCTGT	ACATCTGCTGGAAGGTGGAC
rpl13a	CV133427	TCTGGAGGACTGTCAGGGGCATGC	AGACGCACAATCTTAAGAGCAG
rps18	AM490061	AGGGTGTTGGCAGACGTTAC	GAGGACCTGGCTGTATTTGC
mlc2a	AF150904	GCCCCATCAACTTCACCGTCTTT	GGTTGGTCATCTCCTCAGCGG
mlc2b	FG618629	TCCCTTTGCTATTCTGCCTTC	AAATCAGCCCTATTCCCCATA
myog	EF462191	CAGAGGCTGCCCAAGGTG	CAGGTGCTGCCCGAACTGGGC
mstn1	AF258448	TTTTTGACACAACCGATCCA	GTGTGTGTGTTCCTGCATCC
mrf4	N034421	AGCGGGGAKGAGCACGTCCT	ATGGCGCTGMGTAAAATCTCC
fst	AY544167	GTACCAGGGGAAGTGCAAGA	GCATAGATGATCCCGTCGTT
igf1	AY996779	TCTTCAAGAGTGCGATGTGC	GCCGTAGCCAGGTTTACTGA
col1a1	DQ324363	AGACCTGCGTATCCCCAACTC	GCCACCGTTCATAGCCTCTCC

at $P<0.05$. Data were analyzed by using SigmaStat software (version 3.5; STATCON).

Supervised hierarchical clustering was applied (de Hoon et al. 2004) to Q-PCR analysis and each gene was classified according to its expression profile. TreeView software was used to generate a visual representation of the classification.

Results

Larval growth, muscle growth and changes in muscle cellularity

The axial growth of gilthead sea bream larvae recorded as total length was linear between 5 dph and 60 dph ($P\leq0.001$, Fig. 1). The TCSA of dorsal white muscle increased continuously during the period studied (Fig. 2a).

The TNF increased significantly between 15 dph and 35 dph (from 250±34 to 742±30, mean±SEM) compared with other age groups and slowed down in subsequent stages ($P\leq$ 0.001, Fig. 2b). The distribution of white muscle fiber diameter in all age groups was monophasic rather than biphasic and shifted to larger fibers with age increment (Fig. 3a). Hyperplasia was dominant between 15 and 25 dph as revealed by the increase in TNF (+109 %) and the lack of differentiation in the distribution of muscle fiber diameter (Figs. 2b, 3a). As shown in Fig. 3b, hyperplasia was powered in these ages by germinal zones located at the myotome periphery. White muscle fibers with a diameter smaller than 5 μm (WFD <5 μm) increased from 34 % in 15 dph larvae to its maximum value of 40 % in 25 dph larvae and decreased thereafter ($P\leq0.001$, Fig. 4a). Mean WFD doubled from 25 to 35 dph ($P\leq0.001$, Fig. 4b). Mean WFP and WFA did not change between 15 and 25 dph but a significant progressive increase occurred thereafter (for both WFP and WFA, $P\leq0.001$, not shown). Mean fiber diameter was well correlated with mean fiber area (R=1, P= 0.000), mean fiber perimeter (R=0.990, P=0.000) and fish total length (R=0.977, P=0.000) as indicated by the Spearman rank correlation.

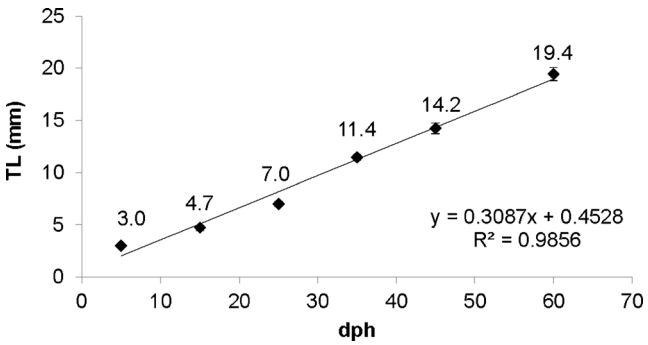

Fig. 1 Increase in total length (*TL*; mm) of gilthead sea bream larvae during the experiment (*dph* days post hatch). Values are given as means ± SEM (n=10/sample)

In 35 dph larvae, white fiber size analysis revealed an overall increase relative to earlier stages (Fig. 3a) and we measured a four-fold increment in mean fiber area ($P\leq0.001$) and a two-fold increase in mean diameter ($P\leq0.001$) and mean perimeter ($P\leq0.001$) compared with 25 dph larvae. The germinal zones of small-diameter white fibers located at the myotome periphery were still evident but the percentage of white muscle fibers smaller than 5 μm was significantly decreased ($P\leq0.001$, Fig. 4a) compared with 25 dph larvae.

In 45 dph larvae, white muscle growth occurred principally by hypertrophy with a two-fold increase in mean area ($P\leq$ 0.001) and an overall significant increase in fiber mean diameter ($P\leq0.001$) and mean perimeter ($P\leq0.001$) compared with

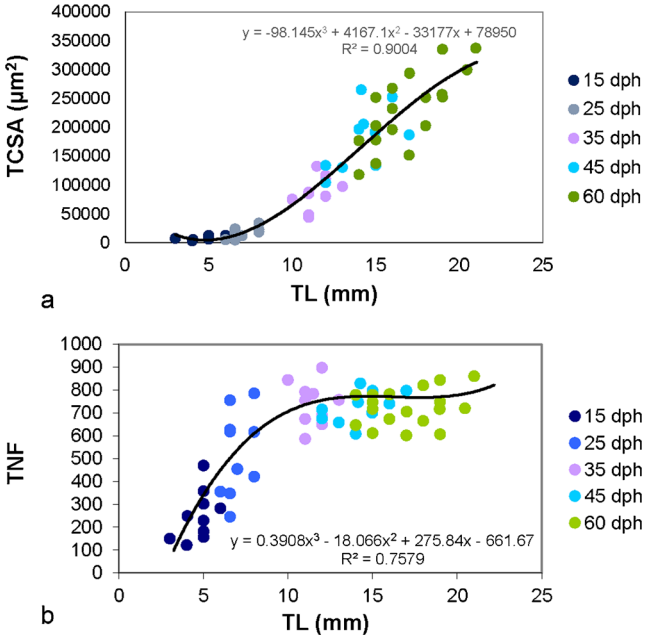

Fig. 2 Increase in (**a**) the total cross-sectional area (*TCSA*; µm^2) of a dorsal quadrant of white muscle and (**b**) the total number of white muscle fibers (*TNF*) in this quadrant as a function of the total length (*TL*) of gilthead sea bream larvae. Values are given as means ± SEM (n=10 larvae/age)

35 dph larvae. The zones of small-diameter white fibers located at the periphery of white muscle were still evident at 45 dph but neither the total white muscle fiber number nor the percentage of white muscle fibers smaller than 5 µm changed compared with 35 dph larvae (Figs. 2b, 4a).

In the deep white muscle of some 60 dph larvae, a new hyperplastic process emerged with recruitment of immature small-diameter fibers that were dispersed in the epaxial quadrant and were not limited to previously identified peripheral proliferative zones. The small-diameter white fibers interspersed between mature white fibers gave rise to mosaic hyperplasia (Fig. 3b). Mean area (P≤0.001), mean perimeter (P≤0.001) and mean diameter (P≤0.001) were all significantly elevated at 60 dph compared with 45 dph in gilthead sea bream larvae.

Gene expression patterns

The expression of structural genes and of myogenic and hormonal factors was monitored by Q-PCR in age classes of sea bream larvae in which substantial cellular changes were observed by histology. The most abundant isoform of *mlc2* during stratified hyperplasia was *mlc2a* and its expression significantly (P<0.001) increased by 2.4-fold between 25 dph and 35 dph (Fig. 5a). Indeed, TNF reached its maximum (742±94; mean±SEM) at 35 dph relative to other age classes. After 35 dph, when dorsal and ventral germinal zones contained few small-diameter fibers, *mlc2a* was significantly down-regulated (P<0.001) relative to earlier stages but significantly increased again on 60 dph at the onset of mosaic hyperplasia (Figs. 3b, 5a).

Isoform *mlc2b* was of low abundance during early hyperplastic growth (5-25 dph) and was significantly up-regulated during hypertrophy (Fig. 5b). A transient down-regulation of *mlc2b* expression occurred at 45 dph before it peaked at 60 dph, at the onset of mosaic hyperplasia. Expression of *col1a1* (Fig. 5c) paralleled and was at similar levels to those of *mlc2b*. Expression of *mlc2b* was significantly lower than *mlc2a* at all sample times (P<0.002), making *mlc2a* the dominant isoform in early development. The expression of all three of the structural genes analyzed was significantly and positively correlated during gilthead sea bream early development (P<0.05, Table 2).

During early rapid hyperplasia (5, 15, 25 dph), *myog* was highly expressed but was significantly down-regulated at 45 dph when the recruitment in apical germinal zones ceased (P<0.001, Fig. 6a). A significant positive and a significant negative correlation was observed between *myog* expression and the expression of *mlc2a* and *mlc2b*, respectively. However, no correlation occurred between *myog* expression and *col1a1 expression*. A significant positive correlation was observed between *myog* expression and other important regulatory genes, namely *mstn1*, *fst* and *igf1* (Table 2).

Expression of *mrf4* remained at low levels until 15 dph when a peak in its expression that coincided with the establishment of apical germinal zones and the accumulation of newly formed fibers occurred (P=0.02, Fig. 6b). The expression of *mrf4* was negatively correlated with all the other genes studied but the correlation was only significant with *mstn1* (Table 2).

Expression of *mstn1* (Fig. 6c) and *igf1* (Fig. 6e) was at low levels up until 25 dph. Subsequently, expression increased with a transient peak occurring at 35 dph and coinciding with the increase in mean fiber diameter and was significantly positively correlated with the transcript abundance of *mlc2a* and *col1a1* (Table 2). In contrast, *fst* was highly abundant at 5 dph and declined progressively during the development of the muscle (P≤0.001, Fig. 6d). Interestingly, *fst* exhibited a significant negative correlation with all three structural genes (Table 2).

Hierarchical gene clustering

Hierarchical clustering was performed on the mean expression levels of the eight genes studied (Fig. 7). Genes were clustered on the basis of the similarity of their expression during sea bream larval development. Two major clusters were found (Fig. 7): cluster I contained all the genes that were significantly up-regulated at the onset of hypertrophy at 35 dph (*mlc2a*, *mlc2b*, *col1a1*, *mstn1* and *igf1*). All structural genes fell within cluster I. Comparison of the expression levels of genes in

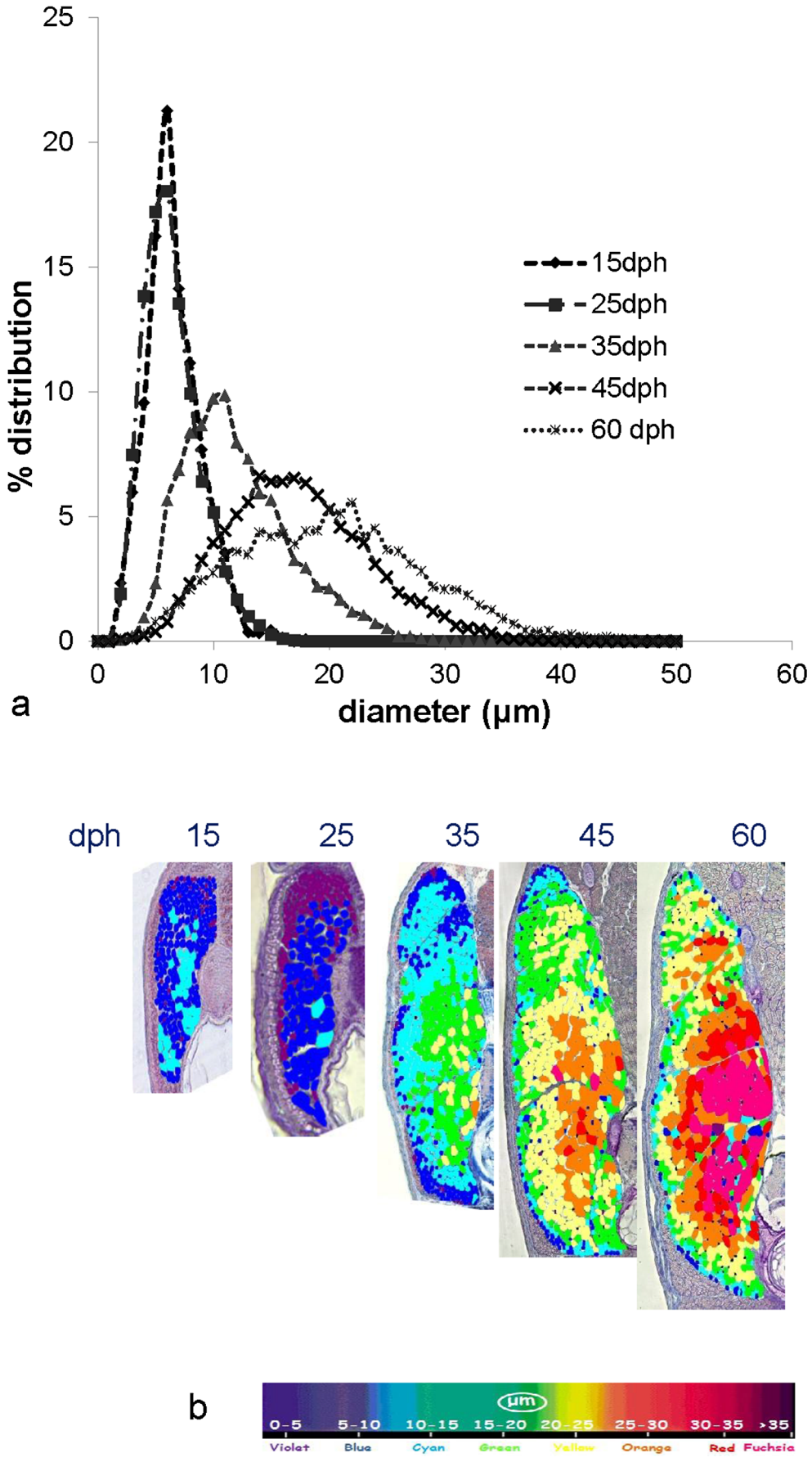

Fig. 3 **a** Changes in the distribution of white muscle fiber diameter in an epaxial white muscle quadrant of gilthead sea bream larvae at 15, 25, 35, 45 and 60 dph. **b** View of the anatomical position of the muscle fibers according to their diameter (smallest fibers in *violet*, largest in *fuchsia*)

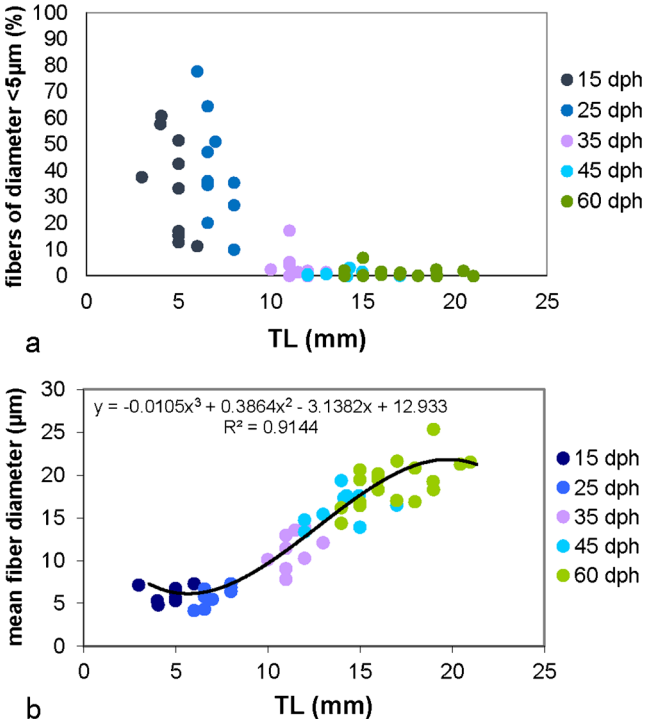

Fig. 4 Changes in (**a**) the percentage of white muscle fibers with a small diameter (<5 μm) as a function of the total length (TL) of gilthead sea bream larvae and (**b**) the mean diameter of white muscle fibers. Values are given as means ± SEM (n=10 larvae/age)

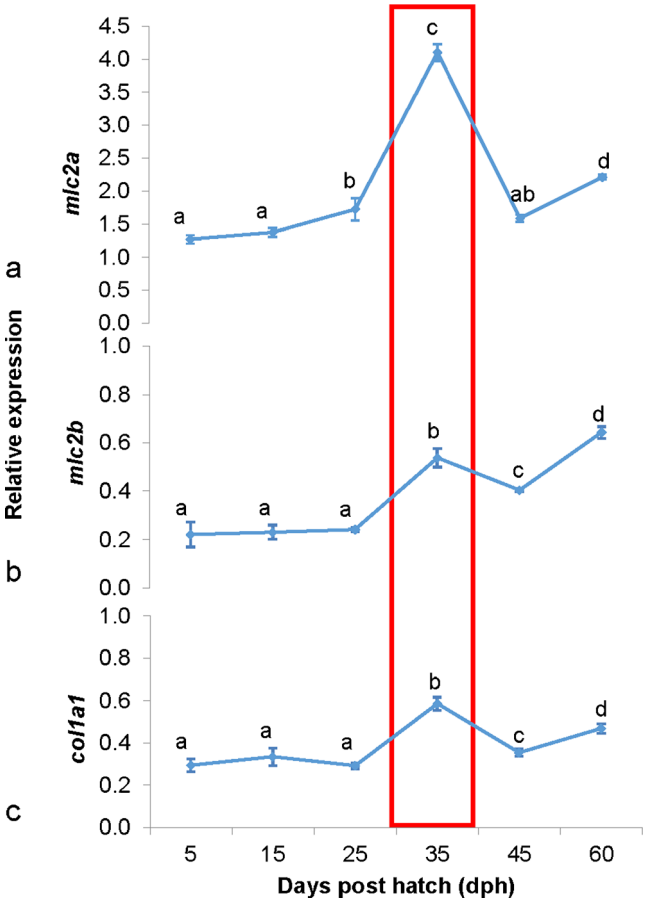

Fig. 5 Expression patterns of structural genes (*mlc2a* myosin light chain 2a isoform A, *mlc2b* myosin light chain 2a isoform B, *col1a1* collagen 1a1) in developing gilthead sea bream larvae; (**a**) *mlc2a*, (**b**) *mlc2b*, (**c**) *col1a1*. Values are given as means of normalized arbitrary expression levels of 10 larvae/age. Values with the same *superscript* are not significantly different at $P<0.05$. *Red box* indicates the onset of hypertrophy

cluster I revealed that *mlc2b* differed from other genes as it exhibited the highest expression on 60 dph and marked the initiation of mosaic hyperplasia during sea bream muscle development. Genes of cluster I went through a transitional phase of decreased expression at 45 dph between the initiation of hypertrophy (35 dph) and the initiation of mosaic hyperplasia (60 dph). Cluster II contained genes highly expressed before muscle hypertrophy (*myog*, *fst*, *mrf4*; Fig. 7). The expression of all genes of cluster II declined significantly as muscle development progressed.

Discussion

In the gilthead sea bream, the early stages in the development of muscle are a crucial period and the fine implementation of hyperplastic and hypertrophic mechanisms is a prerequisite for the successful transition from larvae to juveniles. The present study offers insight into the molecular mechanisms involved in the onset of hypertrophy and mosaic hyperplasia by following the expression patterns of key structural and regulatory genes in these larvae from 5 dph to 60 dph. Our comparison of the gene expression patterns with the changes observed in muscle cellularity revealed significant regulatory contributions to the transitional stages. The expression of *mlc2a* dominates these early stages and marks the formation of new fibers, whereas *mlc2b* is up-regulated at the onset of hypertrophy. Interestingly, *mstn1* appears to be associated with the onset of hypertrophy together with *igf1* and they may act as molecular triggers for the expression of the structural factors *mlc2a*, *mlc2b* and *col1a1* at mosaic hyperplasia post-metamorphosis.

Gilthead sea bream early development and changes in muscle growth dynamics

Many developmental changes that occurred in gilthead sea bream during the period of the study are linked to changing function (Patruno et al. 1998). Larvae first attempt to feed with active darting movements between 1 dph and 9 dph (i.e., between 15 degree-days post hatch [°dph] and 135°dph, with an experimental temperature of 15 °C up to 20°dph and 17-18 °C thereafter); their gut development progresses and they show active feeding and continuous eel-like swimming plus darting movements between 9 dph and 18 dph (135-270°dph). The

Table 2 Spearman rank correlation coefficients between structural (*shaded*) genes and myogenic and regulatory factors in developing gilthead sea bream larvae. *$P<0.05$; **$P<0.01$; ***$P<0.001$

	mlc2b	*col1a1*	*mrf4*	*myog*	*mstn1*	*fst*	*igf1*
mlc2a	0.649***	0.716***	-0.136	0.318*	0.723***	-0.305*	0.557*
mlc2b		0.583***	-0.074	-0.287**	0.591***	-0.455***	-0.023
col1a1			-0.062	-0.007	0.700***	-0.230*	0.367*
mrf4	-			-0.017	-0.208*	-0.195	-0.188
myog	-	-	-		0.366*	0.527***	0.435*
mstn1	-			-		-0.038	0.507***
fst	-		-	-	-		0.114

flexion of the notochord, a major event in larval development, is in progress at 18 dph and is completed by 30-45 dph (475-738°dph) allowing combined eel-like and hydrofoid swimming. Metamorphosis ends before 60 dph (1000°dph), as fish aged 60 dph lose their typical larval features (Patruno et al. 1998).

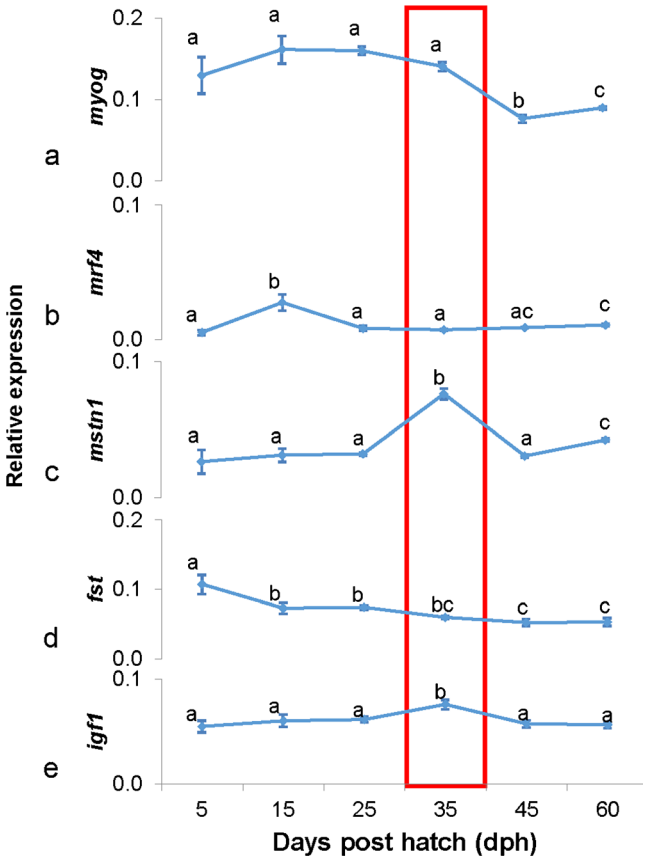

Fig. 6 Expression patterns of myogenic and regulatory genes (*myog* myogenin, *mrf4* myogenic regulatory factor 4, *mstn1* myostatin 1, *fst* follistatin, *igf1* insulin-like growth factor 1) in developing gilthead sea bream larvae; (**a**) *myog*, (**b**) *mrf4*, (**c**) *mstn1*, (**d**) *fst*, (**e**) *igf1*. Values are given as means of normalized arbitrary expression levels of 10 larvae/age. Values with the same *superscript* are not significantly different at $P<0.05$. *Red box* indicates the onset of hypertrophy

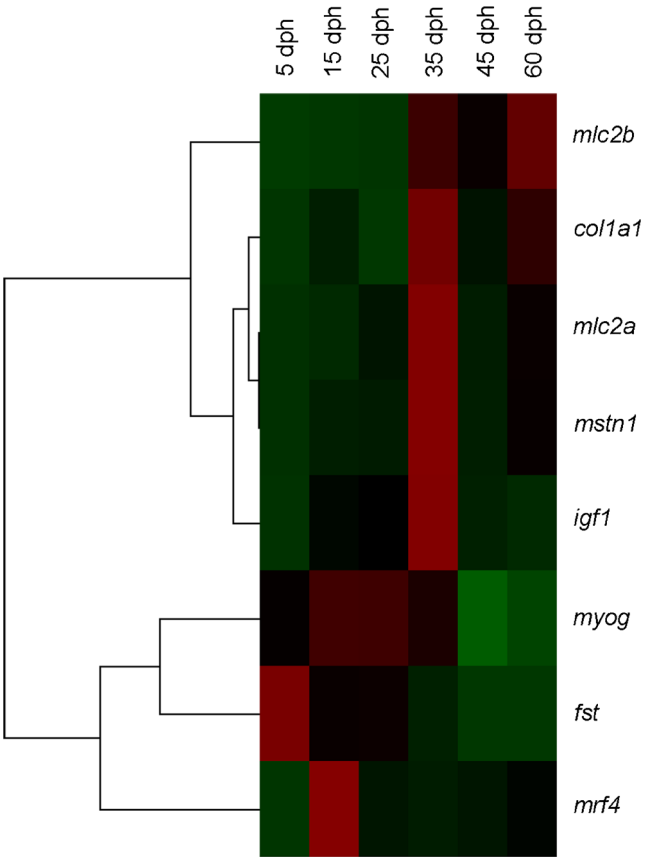

Fig. 7 Hierarchical clustering of the eight target genes during gilthead sea bream larval development. Gene clustering is based on transcript expression levels. The different ages of sea bream larvae (*dph* days post hatch) that were used in the analysis are represented in *columns*, whereas the various genes are represented in *rows*. Relative abundance of each transcript across the different ages is represented by a color scale: *red* and *green* indicate an increase and a decrease in mean gene expression level compared with an initial level of expression from 5 dph to 60 dph, respectively, whereas *black* indicates no change (*mlc2a* MLC2 isoform A, *mlc2b* MLC2 isoform B, *myog* myogenin, *mstn1* myostatin 1, *col1a1* collagen 1a1, *fst* follistatin, *igf1* insulin-like growth factor 1, *mrf4* myogenic regulatory factor 4)

Muscle cellularity reflects the collective activity of various growth mechanisms that result in the accumulation of structural muscle proteins. The total muscle fiber number has been tightly linked to the final size that fish attain and to their growth potential in the wild and under farmed conditions (Weatherley et al. 1988; Pitkänen et al. 2001; Johnston et al. 2004). The mechanisms that lead to muscle fiber formation during the early developmental stages of fish alter; the programs of embryonic muscle development are succeeded by stratified hyperplasia in larvae and mosaic hyperplasia takes over in juveniles (Rowlerson and Veggetti 2001 and references therein). The period following hatching is marked by dramatic hyperplastic growth with the formation of new fibers in apical dorsal and ventral muscle germinal zones. This pattern of development has previously been described in gilthead sea bream (Ramírez-Zarzosa et al. 1995; Rowlerson et al. 1995; Patruno et al. 1998) and has been confirmed in the present study.

Myotomal muscle consists of a superficial red muscle layer and underlying deep white muscle fibers. The cellularity of the dorsal epaxial white muscle differs significantly between early stages and hyperplasia is the predominant mechanism of muscle growth up until 25 dph (475°dph). Between 25 and 35 dph (665°dph), hypertrophic growth is initiated and marks a transition state when both hyperplasia and hypertrophy occur. A pause in fiber formation has been recorded by 45 dph (855°dph) and hypertrophy becomes the main mechanism of muscle growth. Then, at 60 dph (1140°dph), the recruitment of white muscle fibers is observed in the deep white muscle. This new phase of hyperplasia, powered by deep muscle precursor cells, was not detected in sea bream at 1000°dph by Rowlerson et al. (1995) but occurs at 1525°dph. In the latter work, no significant increase in mean fiber area was reported up until 755°dph, contrary to the present study in which high hypertrophic activity was observed from 25 dph (475°dph) onwards. Furthermore, Rowlerson et al. (1995) reported a much lower percentage of fibers with a diameter< 5 μm at 225°dph (20 %) and 405°dph (9 %), than in the present study (34 % at 285°dph and 40 % at 475°dph). The discrepancies in the chronology and intensity of hyperplasia and hypertrophy between the present study and that of Rowlerson et al. (1995) are difficult to explain but might arise from factors such as genetic background, feeding scheme, dietary composition and temperature (15-18 °C vs 19 °C constant). Temperature accounts probably for a great part of the observed differences between the studies, because the pace of both hyperplastic and hypertrophic growth and the resulting muscle cellularity are known to be highly affected by temperature during incubation and larval rearing (Alami-Durante et al. 2000, 2006; Johnston et al. 1995, 2000). The present study revealed that the total number of white muscle fibers is not directly and linearly correlated to larval axial growth in the early stages of gilthead sea bream. We found that small-diameter white fibers (<5 μm), indicative of new fiber formation (Rowlerson et al. 1995), constitute the germinal zones at the myotome edge from 15 dph to 35 dph (285-665°dph) but that new white muscle fiber formation in this zone is more pronounced between 15 and 25 dph (285-475°dph). The stage at 35 dph (665°dph) is most likely intermediate in gilthead sea bream development as muscle growth is attributable not only to hyperplasia but also to hypertrophy. This new process in muscle growth thus begins during the developmental window (475-665°dph) during which, according to Patruno et al. (1998), another major event in gilthead sea bream early development occurs, i.e., the complete flexion of the notochord. From 35 dph to 45 dph (665-855°dph), no addition of new white muscle fibers was seen to occur in the present study and by 45 dph, the stratification phase of muscle growth had come to an end.

Stratified muscle growth has been observed prior to, or at the onset of first feeding and prevails until the start or the end of metamorphosis of several marine fish species such as sea bass (*Dicentrarchus labrax*, Veggetti et al. 1990; Alami-Durante et al. 1997), Atlantic cod (*Gadus morhua*, Galloway et al. 1999a), Atlantic halibut (*Hippoglossus hippoglossus*, Galloway et al. 1999b; Campinho et al. 2007), European plaice (*Pleuronectes platessa*, Brooks and Johnston 1993), Senegalese sole (*Solea senegalensis*, Campos et al. 2013), common sole (*Solea solea*, Veggetti et al. 1999), turbot (*Scophthalmus maximus*, Calvo and Johnston 1992; Gibson and Johnston 1995) and cyprinids (*Chalcalburnus chalcoides mento*, *Rutilus frisii meidingeri*, *Rutilus rutilus*, Stoiber and Sanger 1996; *Cyprinus carpio*, Alami-Durante et al. 1997). Patruno et al. (1998) suggested that 60 dph (1140°dph in the present study; 1000°dph in the studies of Rowlerson et al. 1995) represents a true juvenile post-larval stage reached after metamorphosis, i.e., after the loss of larval features. Deep muscle progenitor cells (satellite cells) are known to be recruited in gilthead sea bream juveniles (Rowlerson et al. 1995; Patruno et al. 1998); these new small-diameter fibers arise between the mature white fibers and give it a mosaic transversal appearance. Our results suggest that this mosaic recruitment begins early during gilthead sea bream juvenile life, as soon as 60 dph (1140°dph). This recruitment of deep muscle precursor cells is a characteristic feature of species that attain a large final size (Rowlerson and Veggetti 2001). According to Weatherley et al. (1988), this fiber input proceeds until the fish achieve 40-45 % of their definite TL. However, the current experimental design did not allow for such an observation in gilthead sea bream.

Gene expression and muscle development

The larval to adult transition appears well orchestrated in white muscle of gilthead sea bream larvae at the molecular level. The results of the present study suggest that the two

isoforms of MLC2 are under differential developmental regulation with increasing expression of *mlc2a* in pre-metamorphic stages and during new fiber formation (between 15 and 25 dph). In the present study, a significant increase in *mlc2b* expression marked the onset of hypertrophy (between 25 and 35 dph) and mosaic hyperplasia (between 45 and 60 dph). In addition, another recent study in gilthead sea bream showed that *mlc2b* expression continued increasing in the post-larval stages (55-117dph), whereas *mlc2a* expression dropped to low levels (Georgiou et al. 2014). Thus, MLC2B becomes the predominant isoform in adult muscle fibers in gilthead sea bream and its expression is significantly correlated to length growth (Georgiou et al. 2014). An early preliminary study on the developmental expression of *mlc1* and *mlc2* in gilthead sea bream by *in situ* hybridization showed that *mlc2* expression marked the germinal zones of skeletal muscle and the newly formed white fibers, whereas in juveniles, its expression was restricted to myogenic cells interspersed in the fast white muscle (Moutou et al. 2005). In a following study (Sarropoulou et al. 2006), it became evident that two *mlc2* isoforms existed, namely *mlc2a* and *mlc2b*; the study by Moutou et al. (2005) referred only to *mlc2a*, the only known *mlc2* at the time. A plausible mechanism that combines the results of all studies so far is that MLC2A is the isoform expressed in the newly formed fibers and that it is replaced by MLC2B when muscle fibers reach some critical size.

The pattern of expression of *col1a1* was similar to that of *mlc2b* to support the hypertrophic growth of muscle mass. The rise in *col1a1* expression at 35 dph was consistent with earlier results in the gilthead sea bream, in which *col1a1* expression was low up to 29 dph and was significantly up-regulated between 29-37 dph (Fernández et al. 2011). A similar expression pattern was also observed during European sea bass development whereby *col1a1* expression was positively correlated with bone development (Darias et al. 2008) suggesting that *col1a1* is of huge biological importance by providing mechanical support in both the growing skeleton and the increasing muscle fiber organization.

Muscle differentiation is a complex process that requires the combined action of certain transcription factors, the myogenic regulatory factors (MRFs), the expression of which is finely regulated both temporally and spatially (Ferri et al. 2009). The transcription, subcellular localization and protein half-life of MyoD, Myf5, myogenin and MRF4 appear to be tightly regulated to ensure myogenic determination, proliferation and myotube differentiation (Zhang et al. 1995; Kassar-Duchossoy et al. 2004; Ishibashi et al. 2005; Patterson et al. 2008; Ferri et al. 2009). Myogenin and MRF4 are factors that govern the middle and terminal differentiation phases and that orchestrate the expression of the muscle-specific genes including those encoding the contractile proteins (Sánchez and Robbins 1994; Blais et al. 2005). In the present study, *myog* was expressed at highest levels during the formation of new fibers up to 35 dph and then declined to lower levels together with *mlc2a*. The results of the present study are in agreement with our previous study of developing gilthead sea bream larvae and primary muscle cell cultures and with the results from studies of other organisms that indicate that myogenin is an important regulator of muscle differentiation (Dedieu et al. 2002; Cao et al. 2006; Ferri et al. 2009; Moutou et al. 2009). Cumulative data point to its crucial role in terminal differentiation and the control of synthesis of the proteins of the contractile apparatus (Blais et al. 2005). Studies in C2C12 cells have shown that myogenin is expressed even in undifferentiated cells in which it is restricted to the cytoplasm. Only after the induction of differentiation is it translocated into the nucleus to exert its action (Ferri et al. 2009). This pattern is consistent with its high expression levels as early as 5 dph in gilthead sea bream when proliferation of small fibers prevails. The significant positive correlation between the expression of *myog* and transcripts of *mlc2a*, both of which are elevated during the phase of intensive formation of new fibers, suggests that they are good candidate markers of hyperplasia. Myogenin might drive *mlc2a* gene expression through the putative binding sites for the members of the basic helix-loop-helix (bHLH) family myogenic regulatory factors, which are present in the *mlc2a* gene promoter (Funkenstein et al. 2007). The significant rise in *mrf4* expression on 15 dph is indicative of its role in determination rather than in differentiation; this is in agreement with the contemporary hypothesis that MyoD, Myf5 and MRF4 define muscle identity and that muscle differentiation is driven by MyoD, myogenin and MRF4 (Kassar-Duchossoy et al. 2004; Ferri et al. 2009). Nonetheless, the relative expression levels of the myogenic factors MRF4 and myogenin resemble the patterns reported in differentiating C2C12 cells with myogenin being the most abundantly expressed myogenic factor (Ferri et al. 2009).

Myostatin is considered to be an inhibitor of proliferating myoblasts and a major negative regulator of fiber number (Ostbye et al. 2001). Two *mstn* paralogs have been identified in gilthead sea bream, namely *mstn1* and *mstn2* (Maccatrozzo et al. 2001a, 2001b; Nadjar-Boger and Funkenstein 2011). The gene *mstn1* is predominantly expressed in muscle and *mstn1* gene polymorphism has been associated with growth traits (Sánchez-Ramos et al. 2012). Consistent with its inhibitory role in growth, *mstn1* expression has previously been reported to be low in gilthead sea bream during the early developmental stages (1 to 44 dph; Maccatrozzo et al. 2001a). In the current study, however, *mstn1* had the same expression pattern as *mlc2b* and increased its expression significantly to peak on 35 dph (Fig. 6c), suggesting a possible role in regulating the shift from stratified hyperplasia to hypertrophy. A similar pattern has been reported in C2C12 cells with MSTN being detected in the myotubes but not in the myoblasts (Artaza et al. 2002) in which its up-regulation is initiated with nuclei accretion. Two distinct phases in *mstn1*

expression have also been detected in European sea bass (*Dicentrarchus labrax*) in which *mstn1* expression remains at low levels up to 10 dph to subsequently exhibit a rapidly increasing expression between 10 and 40 dph. Interestingly, *mstn1* expression at the pre-metamorphotic stages is localized in the apical proliferation zones, whereas post-metamorphosis, it is restricted to the proliferative zone adjacent to the horizontal septum (Patruno et al. 2008). In gilthead sea bream larvae, *mstn1* expression peaks at the same time as that of *igf1* (35 dph), suggesting that the combination of these two growth factors is associated with the end of muscle stratified hyperplasia and the switch to fiber hypertrophy. The strong correlation of *mstn1* and *igf1* (Table 2) reinforces their possible in vivo synergistic action. This is consistent with results showing that human MSTN inhibits the proliferation of cultured rainbow trout myoblasts only under IGF stimulation (Seiliez et al. 2012). It is tempting to speculate that the increase in *mstn1* and *igf1* expression between 25 dph and 35 dph in sea bream larvae acts as a signal to stop muscle stratified hyperplasia and that the decrease in *mstn1* expression between 35 dph and 45 dph permits the hypertrophic growth of muscle fibers. Stage-specific MSTN expression overlaps with strong up-regulation of the major myosin heavy chain isoform MHC-II and to a lesser degree with MHC-I in C2C12 cells (Artaza et al. 2002). A similar phenomenon was observed in the present study as based on the similarity of the expression pattern of *mstn1* clustered with structural genes (Fig. 7). Overall the results of the present study indicate that myostatin has a versatile regulatory action during sea bream ontogeny.

Myostatin action is antagonized by FST in mammals and chicks and several observations have connected FST to positive muscle growth (Matzuk et al. 1995; Lee and McPherron 2001; Amthor et al. 2004). A similar antagonistic MSTN/FST interaction has been shown in gilthead sea bream with the use of recombinant saFST over saMSTN activity in a reporter gene assay (Rebhan and Funkenstein 2008; Funkenstein et al. 2009). In adult sea bream tissues, *fst* is expressed at higher levels than *mstn1* and its expression is detectable before hatch at 15-16 h post fertilization (Funkenstein et al. 2009). The expression patterns of *fst* and *mstn1* in the present study are in agreement with the results of a previous study with larvae up to 10 dph (Funkenstein et al. 2009). The present study extended the temporal observations and revealed that *fst* expression declines slowly up to metamorphosis, and that *fst* is significantly and negatively correlated with *mstn1* expression but positively correlated with *myog* expression (Table 2). Thus, FST appears negatively to interact with MSTN-1 in vivo and it is associated more with the hyperplastic than the hypertrophic phase. FST-driven hyperplasia has also been observed in transgenic rainbow trout overexpressing FST. Transgenic trout exhibit a general increase in muscle mass accompanied by a new fiber formation and an increase in total fiber number and in the total cross-sectional area (Medeiros et al. 2009).

IGF-1 is a molecule with a pleiotropic action in gilthead sea bream and appears to mediate the effects of growth hormone in seasonal growth, to have a pivotal role in compensatory growth following starvation, to induce osteoblast and myoblast proliferation in primary cell cultures and to increase glucose and amino acid uptake in cultured myoblasts through both MAPK and PI3K-Akt signal transduction pathways (Mingarro et al. 2002; Montserrat et al. 2007; Capilla et al. 2011; Rius-Francino et al. 2011; Montserrat et al. 2012). *Igf1* transcripts have been detected in unfertilized gilthead sea bream eggs and its later expression appears to be tissue- and age-dependent, with skeletal muscle displaying high immunoreactivity (Perrot et al. 1999). In Atlantic salmon, increased *igf1* expression marks the transition from zero to fast growth and is followed by the increased expression of myosin genes including *mlc2* (Bower et al. 2008). Three alternatively spliced transcripts of *igf1* have been isolated in the gilthead sea bream (Tiago et al. 2008) and each has a distinct expression pattern during development. The primers used in the present study were designed to amplify all three *igf1* transcripts collectively. The stability in total *igf1* transcripts from 5 to 25 dph in the present study is consistent with the findings of Fernandez et al. (2011) in the same species from 2dph to 29 dph. However, whereas these authors reported that *igf1* expression increases significantly between 29 and 45 dph and between 52 and 60 dph, our findings demonstrate that *igf1* increases significantly only between 25 and 35 dph and then decreases to the level observed at the beginning of the study. A third pattern of developmental regulation was founded by Tiago et al. (2008), with expression of the two main transcripts of *igf1* in gilthead sea bream larvae (*igf1c* and *igf1a*) decreasing significantly from 48 to 67 dph. Further studies are needed to elucidate the cause of the discrepancies between the studies. However, the patterns of *igf1* expression during development vary between species; in European sea bass larvae, *igf1* expression increases continuously up to 25 dph and remains at steady levels to 80 dph (Patruno et al. 2008), whereas in shi drum (*Umbrina cirrosa*), it exhibits a transient increase at 15 dph to decrease back to low levels by 19 dph (Patruno et al. 2006). In the present study, *igf1* expression was shown to be significantly and positively correlated with the expression of the *mlc2a* and *col1a1* structural genes, with the expression of *myog* that manifests involvement in terminal differentiation and hypertrophic growth and with the expression of *mstn1* thus adding further support to the proposed dual role of *igf1* and myostatin in muscle growth.

In conclusion, *myog* accompanies a new fiber formation and *mstn1* appears to regulate the hyperplasia-to-hypertrophy transition, is probably the molecular trigger that stimulates the expression of the structural factors *mlc2a*, *mlc2b* and *col1a1* at 60 dph and is associated with a new phase

of fiber formation. In early development (15-25 dph), when hyperplasia is high, myogenin has a more prominent role in muscle differentiation and presumably acts with MRF4 with which it clusters (Fig. 7). Later in development, when new fibers appear (60 dph), up-regulation of *mlc2a*, *mlc2b* and *col1a1* occurs, all of which encode structural proteins essential for the building of muscle fibers. This fine tuning is reflected in the strong correlations (Table 2) and the gene hierarchical clustering in which structural genes form a single large cluster with *igf1* and *mstn1* (Fig. 7). As the hyperplastic process initiated at 60 dph is accompanied by the increase in expression of some of the genes analyzed, the present study provides insight into the molecular onset of mosaic hyperplasia.

Acknowledgments The authors thank M. Cluzeaud and D. Bazin for their technical support.

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