

Fish Physiol Biochem (2010) 36:1125–1132
DOI 10.1007/s10695-010-9390-y

Expression of the myosin light chains 1, 2 and 3 in the muscle of blackspot seabream (*Pagellus bogaraveo*, Brunnich), during development

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Received: 18 June 2009 / Accepted: 1 March 2010 / Published online: 18 March 2010
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Abstract Previous studies on the histochemistry and immunoreactivity of fibres in lateral muscle of blackspot seabream indicated that there is a developmental transition in the composition of myofibrillar proteins, which presumably reflects changes in contractile function as the fish grows. We hypothesize that the phenomenon underscores age and spatial differences in the expression of myosin light chains (MLC), not studied yet in this species. In this study, we examined selected stages in the post-hatching development of the muscle of blackspot seabream: hatching (0 days), mouth opening (5 days), weaning (40 days) and juveniles (70 days). The spatial expression of embryonic MLC 1 (MLC1), 2 (MLC2) and 3 (MLC3) was studied by in situ hybridization. Overall,

MLC expression patterns were overlapping and restricted to the fast muscle. At hatching and mouth opening, all MLC types were highly expressed throughout the musculature in fast muscle. The expression levels in fast muscle remained high until weaning when germinal zones appeared on the dorsal and ventral areas. The germinal zones were characterized by small-diameter fast fibres with high levels of MLC expression. This pattern persisted up to day 70, when the germinal zones disappeared and expression of MLCs was observed only in the smaller cells of the fast muscle mosaic. These results support our hypothesis and, together with previous immunohistochemistry results, allow a better understanding of the mechanism of muscle differentiation and growth in fish beyond larval stages, and form the basis for further comparative and experimental studies with this economically relevant species.

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Keywords Blackspot seabream ·
Myosin light chain · *Pagellus bogaraveo* ·
White muscle

Introduction

In most fish, the lateral musculature is the principal propulsive organ generating motion. It differs from the skeletal muscle of other vertebrates in its

segmental antero-posterior myotomal organization and by the fact that the two main types of fibres (fast and slow) are not intermingled but develop in physically distinct areas. The superficial red-slow fibres are responsible for slow swimming or cruising, whereas the bulk of deep white-fast fibres are used in fast burst locomotion (Johnston et al. 1977; Bone 1978). Another type of fibre, the so-called intermediate fibre, also occurs in most fish (Johnston et al. 1977). Also, fish muscle growth differs from that of mammals or birds as new fibres are continuously produced over much of the life cycle (Rowlerson and Veggetti 2001).

Myosin is essential for muscle contraction and comprises six polypeptides encoded by 3–5 genes for heavy (MHC) and light chain myosins (MLC). The relative content of MHC and MLC in muscle determines its contractile properties, including its ATPase activity, maximum shortening speeds and force–velocity relationship. The myosin complex is made of two heavy chains each of which consists of a structural α -helical rod portion and a globular head. The myosin head contains one essential light chain and one regulatory light chain that are encoded by different genes (reviewed by Johnston and Hall 2004). In fast skeletal muscle of carp (*Cyprinus carpio*, Linnaeus), Hirayama et al. (1997) found that the two isoforms of essential light chain are encoded by distinct genes.

In common with terrestrial vertebrates, distinct isoforms of the myofibrillar proteins, chiefly myosin, have been described in white-fast and red-slow fish muscle fibres, with great variability between taxa (Focant et al. 1976; Rowlerson et al. 1985; Karasinski and Kilarski 1989; Devoto et al. 1996). In fish, the components of the myosin complex differ between slow and fast muscle, for example different forms of MHC are found in the two muscle types (Scapolo et al. 1988; Karasinski and Kilarski 1989; Martinez et al. 1990) and there are two types of MLC in slow muscles, and three types in fast muscle (Rowlerson et al. 1985; Karasinski and Kilarski 1989; Martinez et al. 1990).

The fibre type composition of individual muscles has been shown to vary dramatically during fish ontogeny. Various biochemical, histochemical and immunohistochemical investigations have revealed that during fish muscle development there is a sequential expression of a range of myofibrillar

protein isoforms (e.g. Carpenè and Veggetti 1981; Scapolo et al. 1988; Martinez et al. 1991; Brooks and Johnston 1993; Crockford and Johnston 1993; Veggetti et al. 1993; Johnston 1994; Johnston and Horne 1994; Johnston et al. 1998; Huriaux et al. 1999, 2003; Focant et al. 2000; Moutou et al. 2001; Chauvigné et al. 2006). In the plaice *Pleuronectes platessa* (Linnaeus), larval MLC2 is gradually replaced by adult MLC2 in postmetamorphic fish, whereas changes in myosin heavy-chain composition occurs later (Brooks and Johnston 1993). In developing turbot *Scophthalmus maximus* (Linnaeus), sequential expression of different MHC, MLC2, troponin-T, and troponin-I isoforms occurs (Focant et al. 2000). More recently, molecular biology tools have been applied to follow the expression of isoforms at the mRNA level, offering more and new insights about muscle fibre composition in the course of fish development (Chauvigné et al. 2006; Nihei et al. 2006).

In a previous immunohistochemical study with blackspot seabream (*Pagellus bogaraveo*, Brunnich), a species recently adopted for aquaculture, it was shown that changes in fibre phenotype reflect developmental events in lateral muscle of this fish (Silva et al. 2008). We hypothesize that the phenomenon underscores age and spatial differences in the expression of myosin light chains. So, to further characterise the modification in myosin during the development of myotomal fibres in this species in situ hybridization was used herein to characterise the spatial and temporal expression of MLCs 1 (MLC1), 2 (MLC2) and 3 (MLC3) gene expression. This is the first study using this approach in blackspot seabream and key specific morphological and physiological development stages were chosen to investigate whether the changes in myosin are related to some precise step(s) in ontogenesis.

Materials and methods

Sample collection and preparation

Blackspot seabream were collected at 0, 5, 40, and 70 days post-hatch, fixed overnight at 4°C in 4% PFA solution (4% paraformaldehyde in phosphate buffer at pH 7.4), washed three times in buffer and stored in 70% ethanol at 4°C. Samples were decalcified before further processing by immersion in 0.5 M EDTA, pH

8.0, for at least 7 days in a dark chamber. Subsequently, samples were dehydrated and wax embedded, and the serial sections (5 μm) mounted on 3-aminopropyltriethoxysilane (APES)-coated slides.

Histology

Probe generation

Digoxigenin-labeled antisense RNA probes were synthesized using a digoxigenin RNA Labeling Kit (SP6/T7) (Roche Diagnostics). Linearised minipreps of pGEM-T Easy (Promega) plasmid vector containing inserts of the complete coding region of MLC1, MLC2 and MLC3, isolated from gilthead seabream (*Sparus aurata*, Linnaeus) were used as templates (Moutou et al. 2001). The linearised vector was purified and an in vitro transcription carried out using 20U of T7 RNA polymerase in transcription buffer (Amersham Biosciences) with 1 μl of digoxigenin-RNA labelling mix (Roche Diagnostics, Mannheim, Germany), for 1.5 h at 37°C. The reaction was stopped with 2 μl of 0.2 M EDTA. The riboprobe was purified by lithium precipitation and resuspended in 25 μl of water.

Hybridisation procedure

Sections were dewaxed in xylene and rehydrated through a graded alcohol series (100–50%), washed twice in Tris buffered saline containing 0.1% tween-20 (TBST) and pre-hybridised at 58°C for 120–240 min in hybridisation solution (50% formamide, 4 \times SSC, 0.1% torula RNA, 0.01% Heparin, 1 \times Denhart's, 0.1% Tween 20, 0.04% CHAPS). Sections were then hybridised overnight at 58°C, in hybridisation solution containing 5 $\mu\text{l ml}^{-1}$ of riboprobe. To remove nonspecifically bound probe, stringency washes were carried out at 58°C first with 2 \times SSC (2 \times 5 min) and then 5 min with 1 \times SSC. Detection of hybridised probe was carried out using anti-digoxigenin-AP Fab fragments (Roche Diagnostics) (1:500) for 120 min at room temperature, after first blocking for 120 min in a 2% solution of blocking reagent (Boehringer-Mannheim) containing 10% sheep serum. Sections were washed in two changes of TBST and colour detection was carried out at 37°C from 120 to 300 min, using the chromagens NBT (4-Nitroblue tetrazolium chloride) and BCIP

(5-Bromo-4-Chloro 3-indolylphosphate). The staining reaction was stopped by washing sections in 1 \times PBS (Phosphate Buffered Saline) and sections were further fixed for 15 min with 4% paraformaldehyde at room temperature, rinsed with 1 \times PBS and distilled water and then mounted in glycerol gelatin.

Control experiments were performed confirming a lack of signal when samples were treated with RNAase prior to hybridisation with the riboprobe and/or by omitting riboprobe from the reaction. In order to help to detect the tissue structures some sections were stained with haematoxylin-eosin. Sections were analysed using a microscope (Olympus BH2) .

Results and discussion

Previously, we used histochemistry and immunohistochemistry to characterize the muscle differentiation and growth in the blackspot seabream (Silva et al. 2008). The development of lateral muscle fibre phenotypes was then described, especially their myosin isoforms, from hatching to juveniles and young adults. In this study, we used in situ hybridization for MLC to offer new insights about muscle fibre heterogeneity and functional specialization in blackspot seabream. Thus, we were able to identify fibre types as they appeared during the post-embryonic growth. Related to this we saw a high degree of similarity between intermediate and fast fibre MLC isoforms, suggesting that intermediate fibres have the same origin as fast fibres, which differs from what was previously proposed in gilthead seabream (Mascarello et al. 1995). Accordingly, the new information we obtained is important not only to tackle our questions for this species but to highlight interspecies differences, namely because muscle development from hatching to juveniles is documented for very few aquaculture fish.

The high degree of similarity among the MLC clones isolated from the gilthead seabream (Moutou et al. 2001) with the ones previously isolated from other fish allow us to conclude that the MLC expression obtained in blackspot seabream is specific. For example, the MLC1, MLC2 and MLC3 clones showed a degree of similarity with zebrafish (*Danio rerio*, Hamilton) of 83, 92 and 91%, respectively. Overall, herein MLC expression was restricted to the

fast muscle, which agrees with data obtained in juvenile and developing gilthead seabream (Moutou et al. 2001). In common with the gilthead seabream, a notable change in the tissue expression pattern of the various forms of MLC occurs during blackspot seabream development. This may be a general feature of MLCs during fish development, as changes in myosin light chains have also been observed in an immunohistochemical study of Atlantic herring (*Clupea harengus*, Linnaeus) at different development stages (Crockford and Johnston 1993).

At hatching, MLCs were uniformly expressed in the deep fast muscle fibres of blackspot seabream. This spatial distribution of fast muscle isoform transcripts is consistent with the previous identification at hatching of two major fibre types [superficial monolayer (LSM) and fast muscle (LW)] in that species (Silva et al. 2008), as shown by immunohistochemistry using anti-myosin sera. This result suggests that the superficial monolayer fibres contain a specific myosin isoform which is in agreement with our previous immunohistochemical results (Silva et al. 2008). The MLC disposition in early developing blackspot seabream muscle, however, differs from rainbow trout (*Oncorhynchus mykiss*, Walbaum) larvae (Chauvigné et al. 2006), in which several fast isoform mRNAs, including MLC1 and MLC3, selectively accumulate in the superficial neoformed slow muscle. In blackspot seabream at 5 days of age (mouth opening, i.e. transition from endogenous to exogenous feeding), the in situ hybridization result was the same as the one obtained at hatching (Fig. 1). The small group of slow fibres adjacent to the lateral line nerve identified for the first time at this age by

immunohistochemistry (Silva et al. 2008) contains none of the three MLC transcripts studied.

At 40 days of larval life (weaning, i.e. transition from live to formula food), a strong MLC signal was detected at the periphery of the dorsal and ventral germinal zones (Fig. 2). The germinal zones were characterized by small-diameter fast fibres with high levels of MLC expression, whereas the deeper layer consisted of polygonal. This result suggests that MLC transcripts are largely restricted to myoblasts and/or young fibres that arise in those germinal zones, thus making MLC a useful marker of hyperplasia during this stratified growth phase. Previous work based on BrdU-labeling and ultrastructural observation has shown that, during larval life, differentiation of slow and fast fibre types is supported by a hyperplastic mechanism that occurs by apposition of new fibres along proliferating zones, principally under the lateral line and in the apical myomere regions, and also just under the superficial monolayer (Rowlerson et al. 1995). With the ultimate aim of examining the sites and the timing of myogenic activation in pearlfish (*Rutilus meidingeri*, Heckel) embryos and larvae, Steinbacher and co-authors (2006) studied the role of myocyte-specific enhancer factor 2 (MEF2) transcription factors, which are known to bind the promoter and enhancer regions of teleost Myogenin and MLC genes. They observed that in germinal zones, most MEF2+ cells give rise to fast fibres and that only a few such cells generate new slow fibres at the terminations of the superficial slow muscle layer, which is in agreement with our results.

Also at 40 days of life, an additional fibre type was identified by immunohistochemistry (Silva et al.

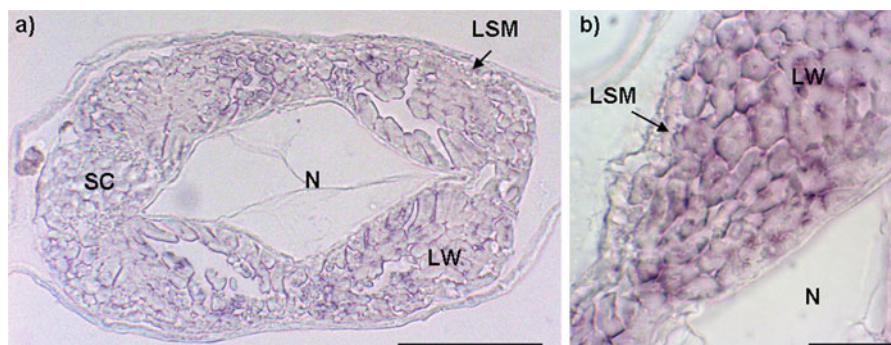


Fig. 1 Temporal and spatial expression of MLC1 in transverse sections of the posterior lateral muscle of *Pagellus bogaraveo* aged 5 days. The larvae *white-fast* muscle (LW) showed MLC1

expression. Other symbols: SC, spinal cord, N, notochord, LSM, superficial monolayer. Scale bar = 25 μ m

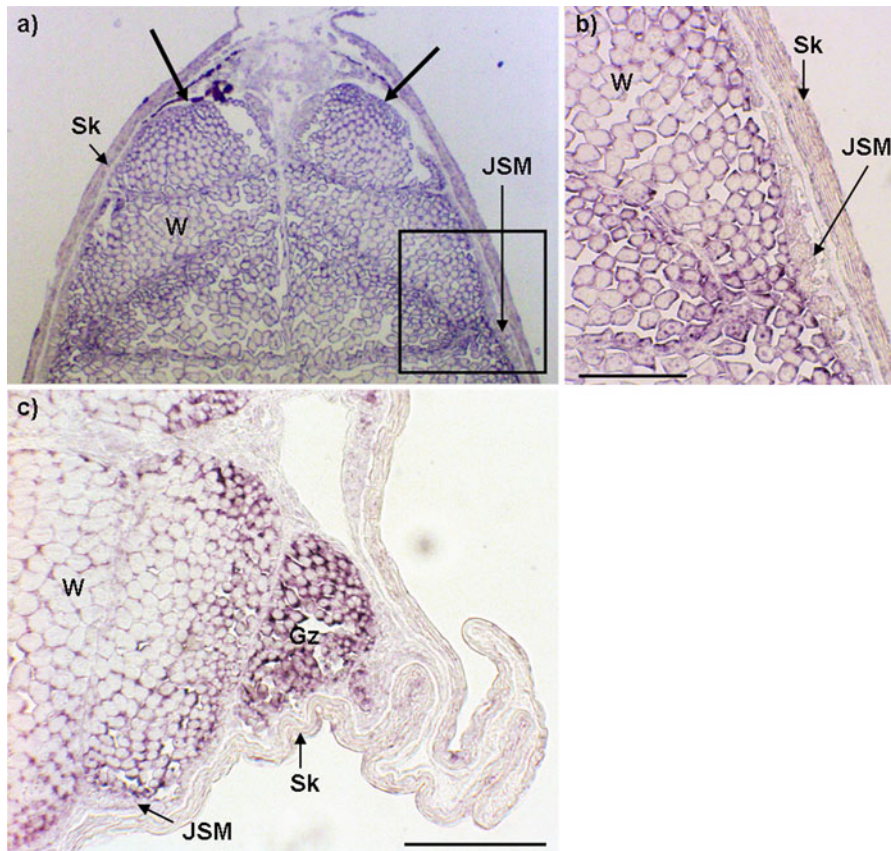


Fig. 2 In situ hybridization showing MLC3 expression in white-fast muscle of *Pagellus bogaraveo* aged 40 days. Note the lateral germinal zones—thicker arrows in (a) and Gz in (c). Boxed area in (a) is shown at higher magnification on the right

(b). The juvenile superficial monolayer (JSM) showed no MLC3 expression. Other symbols: W, adult fast white muscle; Sk, skin. Scale bar = 25 μ m

2008), forming a distinctive ‘pink’ or intermediate layer. In gilthead seabream, another sparid fish, the same germinal zone under the lateral line that gives rise to additional slow fibres from hatching to the midpoint of larval life also gives rise to intermediate (pink) fibres (Mascarello et al. 1995). In blackspot seabream, however, the new muscle cells arising at the lateral growth zones are MLC+ which suggests that the MLC gene(s) expressed by intermediate fibres diverge only slight from fast fibres in their nucleotide sequence, thus causing cross-reaction of the fast MLC probe. It remains unknown at this point whether this difference between these two sparid fish has a functional meaning. Eventually the reassessment using molecular techniques could elucidate the pink-intermediate muscle fibre origin in this important fish group.

In blackspot seabream of 70 days, several myosepta separating adjacent W-shaped myotomes are

visible in the transverse sections (Fig. 3). The expression of MLC was limited to the small diameter fast muscle fibres (W) which appeared not only near to myosepta but also dispersed in the fast muscle at 70 days post hatching, i.e. in early juvenile life, and this was reflected by a wide range of fibre sizes that gave the mosaic appearance in cross section (Fig. 3). The mosaic character of muscle has been suggested to be a result of hyperplastic processes generally only occurring in fish which grow to a large final size (Carpenè and Veggetti 1981; Rowleson and Veggetti 2001; Sanger and Stoiber 2001; Johnston 2006). The small diameter fibres in the fast muscle of blackspot seabream also differed from the large diameter fibres in their ATPase reactivity (Silva et al. 2008). It has been suggested that the de novo myogenesis process which occurs in growing muscle is similar to that observed during embryonic development (Johnston

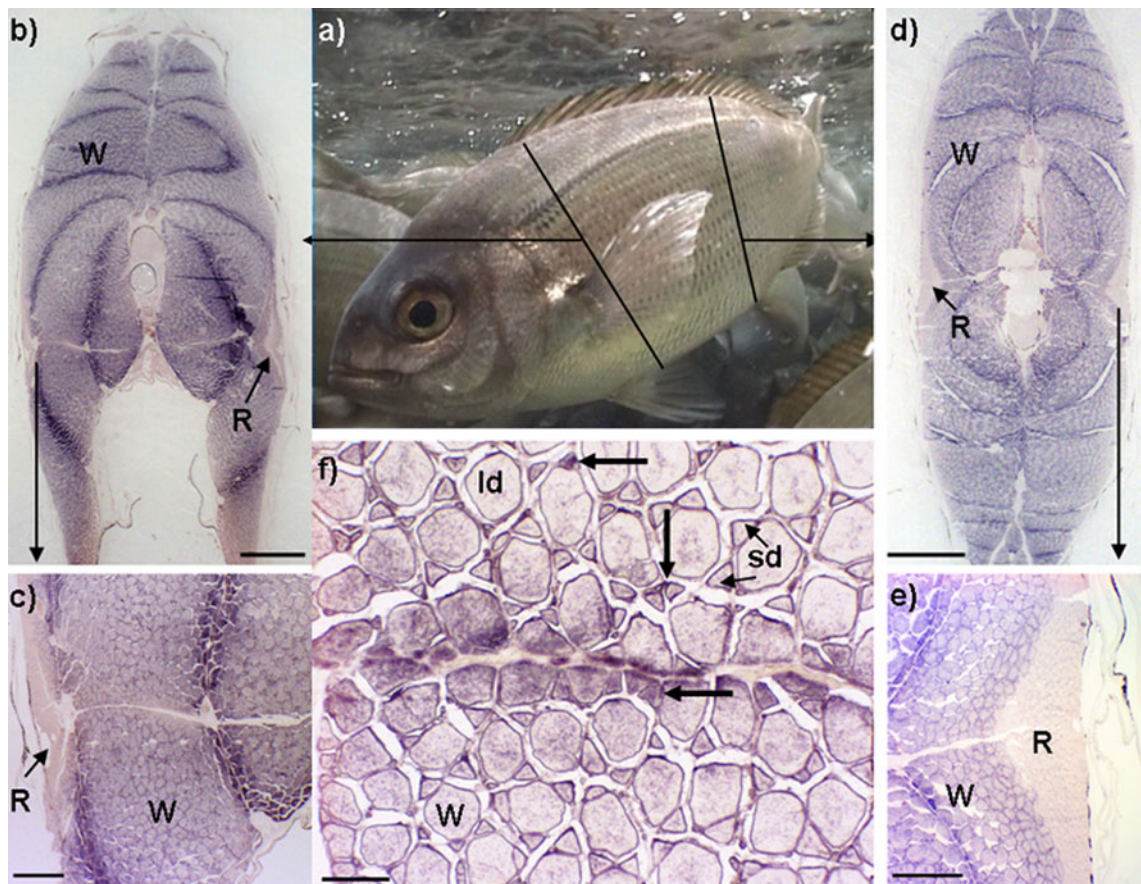


Fig. 3 Transverse section of lateral muscle of *Pagellus bogaraveo* (a) aged 70 days showing MLC1 expression at post-opercular level (b and c) and at post-anal level (d, e and f). f shows the “mosaic appearance” of the white-fast muscle. Thicker arrows indicate cells in the inter-fibre space expressing

MLC1. Symbols: *ld*, large-diameter white-fast fibres; *sd*, small-diameter white-fast fibres; *W*, adult white-fast muscle; *R*, adult red-slow fibres. Scale bar (b), (d) = 250 μ m; (c), (e) = 100 μ m and (f) = 25 μ m

1999). The persistence of early MLC isoforms in growing blackspot seabream could be related to this process. As mention above, an accumulation of MLC transcripts were noted near to myosepta which is consistent with the suggestion by Stoiber and Sanger (1996) that the mosaic precursors migrate on a longer route, probably using myoseptal gaps to reach their final position between the pre-established fibres.

In conclusion, the observations support our hypothesis that transition in the composition of myofibrillar proteins in blackspot seabream, from hatching to juveniles, is founded in age and spatial differences in the expression of myosin light chains. We saw that the temporal and spatial MLC expression patterns follow fast muscle development and mark the germinal zones and the newly formed fast

fibres, indicating their embryonic character. The changes in myofibrillar gene transcripts and probable protein composition during the early life of blackspot seabream observed in this study probably reflect a modification in the functional properties of the muscle as body size increases. The fibre type transitions described in both this and in a previous paper (Silva et al. 2008) demonstrate that gene expression of this post-mitotic cell can be altered with age. Future research will be needed to elucidate in more detail the molecular elements and mechanisms underlying fibre type determination and transformation in blackspot seabream.

Acknowledgments Work partially supported by FCT (Foundation for Science and Technology) PhD Grant SFRH-BD-14068-

2003 attributed to P. Silva. The authors are greatly indebted to the “Instituto Español de Oceanografía” (Centro Oceanográfico de Vigo, España) which provided the fish.

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