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Differentiation and growth of muscle in the fish *Sparus aurata* (L): I. Myosin expression and organization of fibre types in lateral muscle from hatching to adult

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Summary

Post-hatching development of lateral muscle in a teleost fish, *Sparus aurata* (L) was examined. At hatching only two fibre types were present; several layers of mitochondria-poor, myofibril-rich deep muscle fibres surrounded the notochord and were covered by a superficial monolayer of mitochondria-rich, myofibril-poor fibres. A third ultrastructurally distinct fibre type first appeared as one or two fibres located just under the lateral line at 6 days post-hatching. This type, which gradually increased in number during larval life, contained a slow isoform of myosin, identified by mATPase staining and immunostaining with myosin isoform-specific antibodies. Deep muscle fibres – the presumptive fast-white type – contained a fast myosin, and superficial monolayer fibres an isoform similar but not identical to that in adult pink muscle fibres. The only fibres present during larval life which showed a clear change in myosin expression were the superficial monolayer fibres, which gradually transformed into the slow type post-larvally. Pink muscle fibres first appeared near the end of larval life. Both slow and pink muscle fibres remained concentrated around the horizontal septum under the lateral line during larval life, expanding outwards towards the apices of the myotomes only after metamorphosis. Between 60 and 90 days very small diameter fibres with a distinct mATPase profile appeared scattered throughout the deep, fast-white muscle layer, giving it a 'mosaic' appearance, which persisted into adult life. A marked expansion in the slow muscle layer began at the same time, partly by transformation of superficial monolayer fibres, but mainly by addition of new fibres both on the deep surface of the superficial monolayer and close to the lateral line. The order of appearance of these fibre types, their myosin composition, and the significance of the superficial monolayer layer are discussed and compared to muscle fibre type development in higher vertebrates.

Introduction

In the skeletal muscle of higher vertebrates, the isoforms of myosin which characterise the different fibre types in adult muscle are preceeded by developmental isoforms (embryonic and foetal/neonatal/perinatal), which can be identified biochemically and immunohistochemically (Whalen *et al.*, 1981; Bandmann *et al.*, 1982; Crow & Stockdale, 1986; Weydert, 1988). Myogenesis occurs in two main stages, and fibres produced at different times show characteristic sequences of myosin isoform expression during the course of their development (Narusawa *et al.*, 1987; Stockdale & Miller, 1987; Hoh, 1991; Page *et al.*, 1992; Stockdale, 1992; Hughes *et al.*, 1993; Miller *et al.*, 1993; Russell *et al.*, 1993).

In fish, the expression of adult isoforms of myosin in slow-red, pink and fast-white muscle is preceded by 'embryonic' and 'larval' isoforms during development (van Raamsdonk *et al.*, 1978; Scapolo *et al.*, 1988; Martinez *et al.*, 1991; Focant *et al.*, 1992; Crockford & Johnston, 1993; Veggetti *et al.*, 1993; Johnston, 1994; Johnston & Horne, 1994). In higher vertebrates primary myotubes all express a slow myosin at an early stage, but in fish the myosin expression of the first fibres to be formed is uncertain. In *Brachydanio rerio* their myosin immunoreactivity is 'red-like' (van Raamsdonk *et al.*, 1978, 1982), whereas

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in *Dicentrarchus labrax* none of the fibres present at hatching react with an antibody specific for the heavy chain isoform of slow-red muscle myosin (Scapolo *et al.*, 1988), and no slow myosin light chains could be found associated with the embryonic and larval heavy chain isoforms in *Barbus barbus* (Focant *et al.*, 1992). Analysis of developmental changes in myosin expression in fish muscle is also complicated by large interspecific variations in the histochemical and immunohistochemical properties of myosin isoforms and in the timing of their developmental transitions (van Raamsdonk *et al.*, 1982; Ramanello *et al.*, 1987; Scapolo *et al.*, 1988; Focant *et al.*, 1992; Veggetti *et al.*, 1993).

In fish which reach a large adult size the embryonic and larval phase of muscle growth is followed by a second phase of myofibre formation in the fast-white muscle during post-larval life (Weatherley & Gill, 1981; Weatherley *et al.*, 1988). New fibres are formed around and in close contact with large diameter mature fibres forming during a previous developmental stage, giving the muscle a mosaic appearance in transverse section. The myosin expression in these new fibres also shows striking interspecific variations (Carpenè & Veggetti, 1981; Rowlerson *et al.*, 1985; Romanello *et al.*, 1987; Scapolo *et al.*, 1988; Rowlerson, 1994), and the cellular origin of these new fibres is currently uncertain (Koumans *et al.*, 1993; Rowlerson *et al.*, 1995).

Regulation of myogenesis and myosin isoform expression at the level of gene expression presumably employs the same mechanisms in fish as in higher vertebrates (they share some of the same myogenic transcription factors, Neville & Schmidt, 1992). However, differences in other aspects of myogenesis such as myosin isoform expression in the earliest stages and the interspecific variations are of interest because they reflect variations in biological mechanisms, and may be of practical importance in aquaculture. We have therefore studied muscle development in the gilthead sea-bream, *Sparus aurata*, a fish which is farmed throughout the Mediterranean area. Here we describe the development of lateral muscle fibres and myosin isoforms, identified by histochemical and immunohistochemical methods. In the following paper (Rowlerson *et al.*, 1995), we present a quantitative analysis of muscle growth in this fish and identify the sites of myoblast proliferation.

Materials and methods

The sea-bream, *Sparus aurata* (L) (Osteichthyes, Sparidae) is a proterandic hermaphrodite fish which can reach about 70 cm in length and a weight of about 5 kg. Sexual maturity (as males) is reached between 12 and 18 months at a weight of not less than 500 g, and towards the end of the second year of life about 80% of individuals in any given population become female, in co-incidence with an increase in body weight to greater than 1.5 kg. Males are always small (< 1.5 kg).

Embryonic life (from fertilization to hatching) lasts only 48–51 hours at 18° C and 37‰ salinity. At 2–3 h after hatching the larva, still dependent on the yolk sac for alimentation, is about 2–2.5 mm long and has 21 myotomes (final number 25). At about 50 days post-hatching the swim bladder develops and the larva completes metamorphosis to become a fry. Subsequently, at about 150 days, when a few grams in weight and about 5 cm in length, it reaches the juvenile stage which persists until sexual maturity (Lumare & Villani, 1970; Alessio & Gandolfi, 1975; Zoar *et al.*, 1984).

Larval, fry, juvenile and adult stages of *Sparus aurata* were obtained from fish hatcheries on the Adriatic coast, and killed by overdose of MS222 anaesthesia. Larval stages from hatching (day 0) to 15 days were fixed by immersion in 2.5% glutaraldehyde in cacodylate (pH 7.4) at 4° C. They were then washed in ice cold cacodylate buffer, post-fixed in OsO_4, processed through a graded series of ethanol and embedded in Epon-araldite epoxy resin. Semi-thin (~0.8 μm) sections were stained with Toluidine Blue and thin (60–80 nm) sections were viewed in a JEOL 100SX electron microscope.

For histochemistry and immunohistochemistry whole fish (larvae from 9 days post-hatching to small juveniles) or portions of lateral muscle (from larger fish) were snap frozen in isopentane at −80° C or ~ −170° C. Small individuals were combined in composite blocks with each other, with muscle from a larger fish or in liver, before freezing. Sections (10 μm) were then cut for ATPase histochemistry (methods as described in Carpenè *et al.*, 1982, Mascarello *et al.*, 1986) and immunostaining with antibodies specific for various isoforms of myosin, principally: (1) anti-SHC: for fish slow myosin heavy chain (Rowlerson *et al.*, 1985); (2) anti-FHC: for fish fast myosin heavy chain (Rowlerson *et al.*, 1985); (3) anti-FM: for mammalian adult fast myosin (reaction with lower vertebrate myosins as described in Rowlerson & Spurway, 1988 and Veggetti *et al.*, 1993). Binding of the primary antibody was visualized by indirect immunoperoxidase staining.

Most observations were made on transverse sections of the lateral muscle at the level of the anal opening, but abdominal and caudal levels were examined in some individuals, and one 0 day larva was also cut in parasaggital section.

Results

LARVAE AGED 0 DAYS (HATCHING) TO 26 DAYS

0–9 days: morphology and ultrastructure (Fig. 1a, b)

At hatching, lateral muscle already consisted of two distinct muscle layers organized around the notochord. The superficial layer was a monolayer of fibres with a rectangular profile in cross-section, and extended outwards both hypo- and epi-axially from the lateral line. Under the monolayer were rows of larger-diameter deep muscle fibres with a polygonal

profile. The deepest of these fibres lay closely adjacent to the notochord. In para-sagittal semi-thin sections, fibres of both types appeared to extend from myosept to myosept.

The number of deep muscle fibres decreased progressively in the more caudal myotomes, whereas the superficial monolayer extended relatively further, so that the most caudal (and therefore most recently formed) myotome present at hatching consisted of apparently only a complete monolayer, with no underlying deep muscle fibres.

Ultrastructurally, the deep muscle fibres contained very few mitochondria but plentiful myofibrils which were organized radially, some extending close to the sarcolemma. Some signs of immaturity, such as centrally placed nuclei and areas of cytoplasm without myofibrils, were present even in larger diameter fibres (Fig. 1a), and were more marked in small diameter fibres in the epaxial and hypaxial extremities. Superficial monolayer fibres contained many mitochondria and relatively few myofibrils (Fig. 1b). The latter were strung out across the fibre, giving rise to w- and s-shaped structures seen in transverse section. Occasional morphologically undifferentiated cells lay just deep to the superficial monolayer (Fig. 1b).

At 6 days a third fibre type appeared in some sections adjacent to the horizontal septum at the point where it meets the medial surface of the lateral line. This type consisted of one or two small diameter fibres with a round profile, filled with well organised myofibrils and few or no mitochondria (Fig. 1b). Unlike the other fibres, they were too short to span the full distance between myosepts, and so were absent from some sections in transverse series.

Larvae from 9 days: myosin immunostaining and ATPase activity

At 9 days (the earliest age examined histochemically for myosin composition), fibres of the deep muscle layer had a moderately alkali-stable mATPase activity and reacted only with anti-FHC. There was no difference between the largest (most mature) and smallest (presumed most recently formed) fibres. Occasionally, differences in pH lability of mATPase activity between the deep fibres and fast-white muscle fibres of older fish were seen, but no consistent difference could be demonstrated with either this method or by immunostaining. Deep muscle fibres are therefore already 'fast' in type, with a myosin composition very similar, if not identical, to that found in adult fast white muscle (Table 1).

At 9 days the superficial monolayer extended close to the apex of the myotomes even in relatively rostral post-anal levels. The superficial monolayer fibres had a strongly alkali-stable mATPase activity (stronger than in the deep muscle fibres, see Fig. 2b, c) and reacted very strongly with anti-FM, but not with anti-SHC (Fig. 2a, d; Table 1). Only a small group of up to four fibres present in most but not all sections, and located just under the medial border of the lateral line, reacted with anti-SHC (Fig. 2d). These fibres, clearly 'slow', also had a relatively alkali-labile mATPase activity (Fig. 1c, 2c, Table 1) and correspond to the third type first seen at EM level in 6-day larvae. Myosin ATPase and immunostaining profiles of these fibre types were the same in the most caudal myotome (no. 23 at 9 days) as at more rostral levels.

By 26 days the mATPase of superficial monolayer fibres had also become strongly acid-stable, whereas that of the slow fibres had become weakly acid-stable, as in the slow muscle of adult fish (Table 1).

The muscle wall of the abdomen (formed from hypaxial myotomes) was formed of a single continuous layer of fast fibres, with scattered very small diameter fibres of both the slow and superficial monolayer types on its external surface (Fig. 2e, f, g).

46-DAY LARVAE AND 60-DAY FRY

At 46 days very small diameter fibres lying either side of the horizontal septum just under the slow fibres were identified by their strongly alkali-stable mATPase activity (Fig. 2h, left). They increased in number and diameter over the following weeks, spreading out epi- and hypaxially to join the superficial monolayer. By 60 days their mATPase activity was strongly both alkali and acid-stable, as in the pink muscle of adult fish (Table 1; Fig. 2h, right). Immunostaining reactions in these pink muscle fibres were similar to those of superficial monolayer fibres except for their stronger reaction with anti-FHC (Fig. 3a, b).

By 60 days the slow fibres had increased in number close to the horizontal septum where they formed a layer several fibres deep (Fig. 3c) and now also appeared epi- and hypaxially on the medial border of the superficial monolayer as very small diameter fibres interspersed between the monolayer fibres (Fig. 2i–n).

Mature fast fibres maintained the same mATPase activity and immunostaining profile seen at previous stages, but in rare 60 day fry very small diameter fibres were present scattered among the mature fast fibres in the deep muscle layer, giving it a mosaic appearance.

FRY AGED 90–150 DAYS

By 90 days post-hatching the whole fast muscle layer had a mosaic appearance in all individuals examined (Fig. 3d). The small-diameter fibres did not react with anti-SHC (Fig. 3f) or anti-FM, and differed from the adjacent large diameter fibres only in their mATPase activity (Table 1). This activity was consistently more

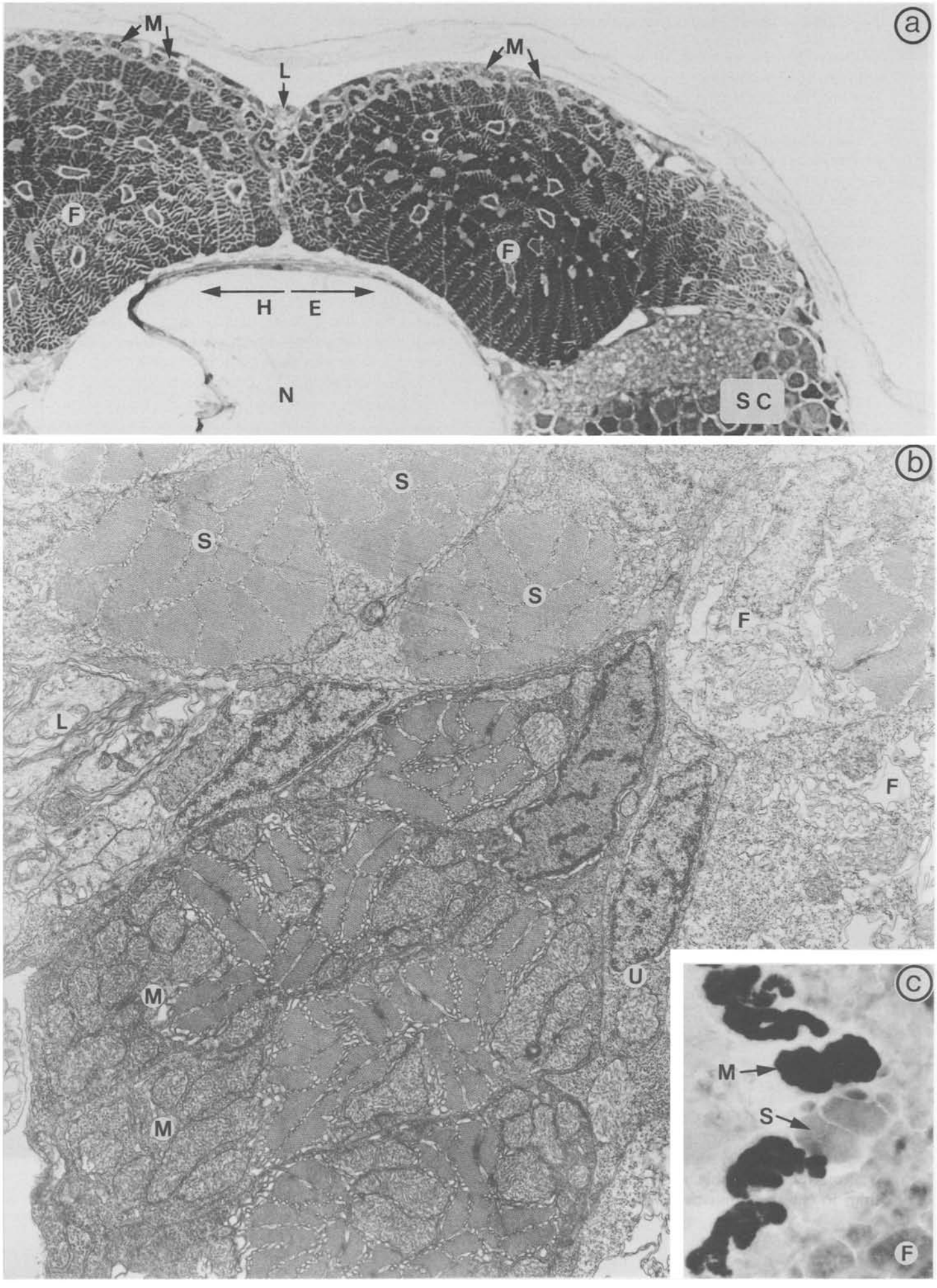
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Table 1. Myosin ATPase staining and immunohistochemical profile of adult and developmental fibre types

	Fibre type				
	*SM**	*FW*		*S*	*P*
		ld	*sd**		
mATPase:					
strong alkali[a]	+++	−	−/+	−	+++
mild alkali[b]	+++	++	++	−/+*[c]	+++
acid	++	−	+	+	+++
Immunostaining:					
anti-FHC	++	+	+	−	+++
anti-FM	++	−	−	−	++
anti-SHC	−	−	−	++	−

SM: superficial monolayer; FW: fast-white; ld: large diameter, sd: small diameter; S: slow; P: pink.
*: 'developmental' fibre types
[a]pH range 10.0–10.3
[b]pH range 9.8–10.0
[c]+ in mainly smaller diameter fibres appearing in the slow layer during juvenile life

acid-stable in the small diameter fibres (Fig. 4b), but relative alkali stability varied according to the method used (Figs 3d, 4b). Their acid and alkali stability was always less than in the pink muscle fibres, which continued to increase in number. This mosaic appearance of the fast muscle was also present at all subsequent ages.

At 120 days, very small diameter fibres with the mATPase and immunostaining profile of pink muscle appeared in a small deep area of the fast muscle either side of the dorso-ventral septum in the epaxial quadrant (Fig. 4a, b). At 150 days pink fibres appeared in the equivalent site in the hypaxial quadrant and subsequently these areas gradually increased in size, becoming identifiable macroscopically by their pink colour.

By 100 days some of the superficial monolayer fibres close to the horizontal septum had begun to react with anti-SHC (Fig. 3e, f), indicating the beginning of a gradual transformation of mATPase activity and immuno-staining properties of these fibres to the slow type.

JUVENILE AND ADULT STAGES

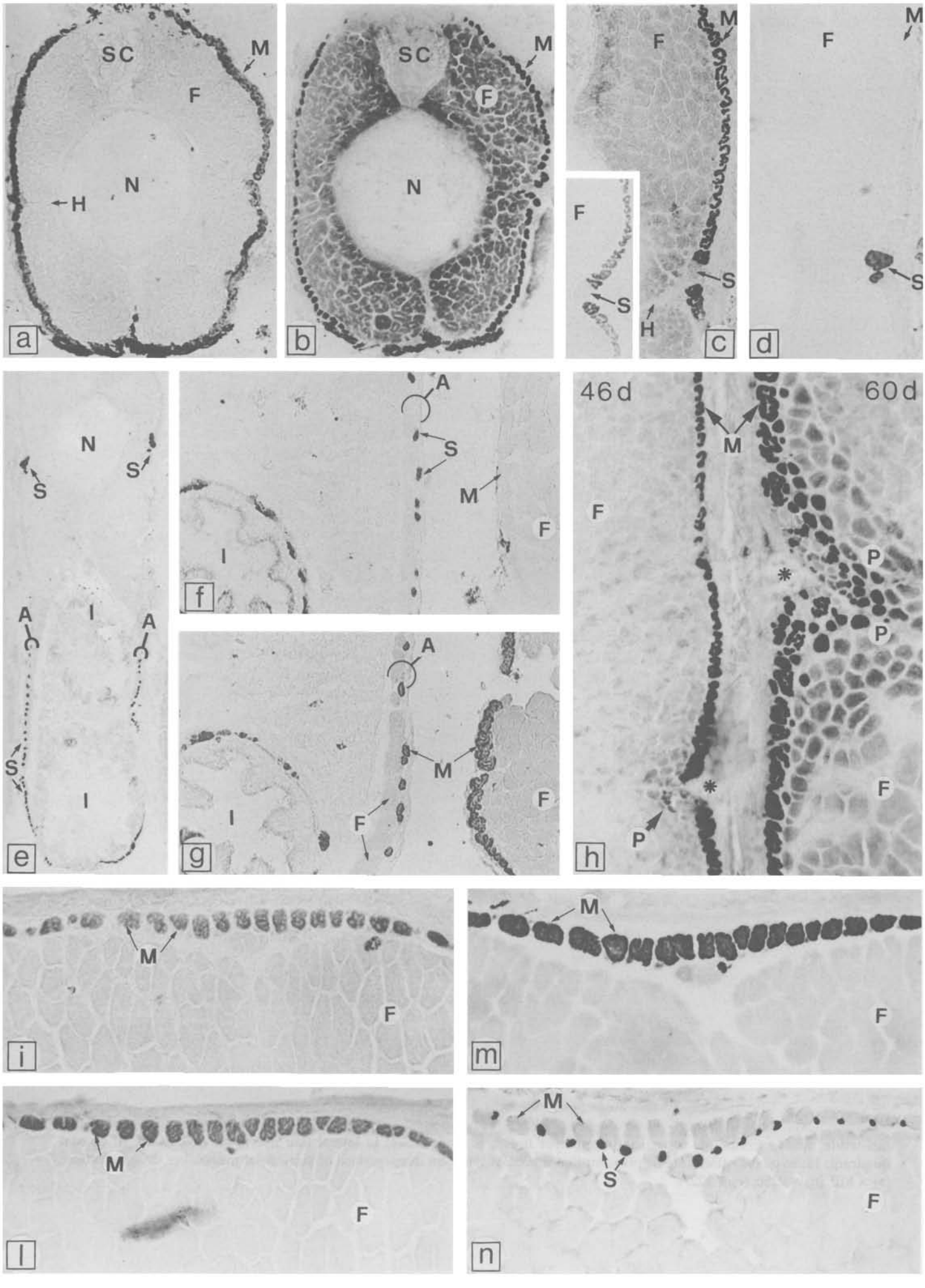
In juveniles (body weight from 2.57 to 218 g), the only significant change in fibre type properties occurred in the slow muscle layer. This increased markedly in thickness (initially close to the lateral line and later also in the hypo- and epi-axial zones), acquiring a mosaic appearance due to the addition of small diameter fibres. These small diameter fibres differed from the others in having a more alkali-stable mATPase activity (Fig. 4c). Some slow fibres also appeared in the deep pink zones of the epi- and hypaxial muscle. In the adult this zone consisted of a compact group of slow fibres encircled by pink fibres which then gradually merged with the surrounding fast fibres of the deep muscle layer.

Discussion

At hatching, lateral muscle in *Sparus aurata* consists of only two fibre types, one in the deep muscle layer which already has an mATPase activity and immunoreactivity very similar to that in adult fast white muscle, and the other in the superficial monolayer which will transform into another type at a much later stage. Thus, unlike the situation in developing higher vertebrate muscle (Draeger *et al.*, 1987; Narusawa *et al.*, 1987; Dhoot, 1988; Page *et al.*, 1992, Hughes *et al.*, 1993), neither of these two types formed during the first stage of myogenesis of *Sparus aurata* contains a slow myosin. At 6 days the first slow fibres appear, followed by the first pink muscle fibres at about 46 days. Both these types are concentrated around the lateral line and horizontal septum. Thus the main three adult fibre types have appeared by the end of larval life, before the onset of the late (post-larval) hyperplastic phase when new fibres are formed throughout the fast white muscle layer. In the sea bass *Dicentrarchus labrax* (another large, fast-growing fish) the mosaic phase of fast-white muscle also starts shortly after metamorphosis, although in this fish the large diameter fast fibres do not acquire their definitive mATPase profile until a later stage (Scapolo *et al.*, 1988).

In *Sparus aurata*, as in other fish, the lateral muscle initially develops around the notochord. According to Nag and Nursall (1972), deep muscle fibres are the first to be formed in the trout. Proctor and colleagues (1980) also examined embryonic trout, and found that the deep fibres were the first to contain well-organized myofibrils, but a recognisable superficial monolayer was also present. In *Sparus aurata* at hatching, the deep muscle layer is several fibres thick in sections taken close to the anal opening, but it

Fig. 1. Transverse sections of lateral muscle at 6 (a) and 9 (b, c) days. (a) semi-thin section showing an epaxial quadrant and its surrounding structures, Toluidine Blue stain; (b) electronmicrograph of area around the lateral line nerve; (c) frozen section of similar area to that in (b) but stained for alkali-stable mATPase activity. SC: spinal cord; F: deep, presumptive fast-white muscle fibres; M: superficial monolayer fibres; S: slow fibres; L: lateral line nerve; N: notochord; E: epaxial quadrant; H: hypaxial quadrant; U: presumptive myoblast lying on deep surface of superficial monolayer. Magnification: (a) × 810; (b) × 9250; (c) × 1227.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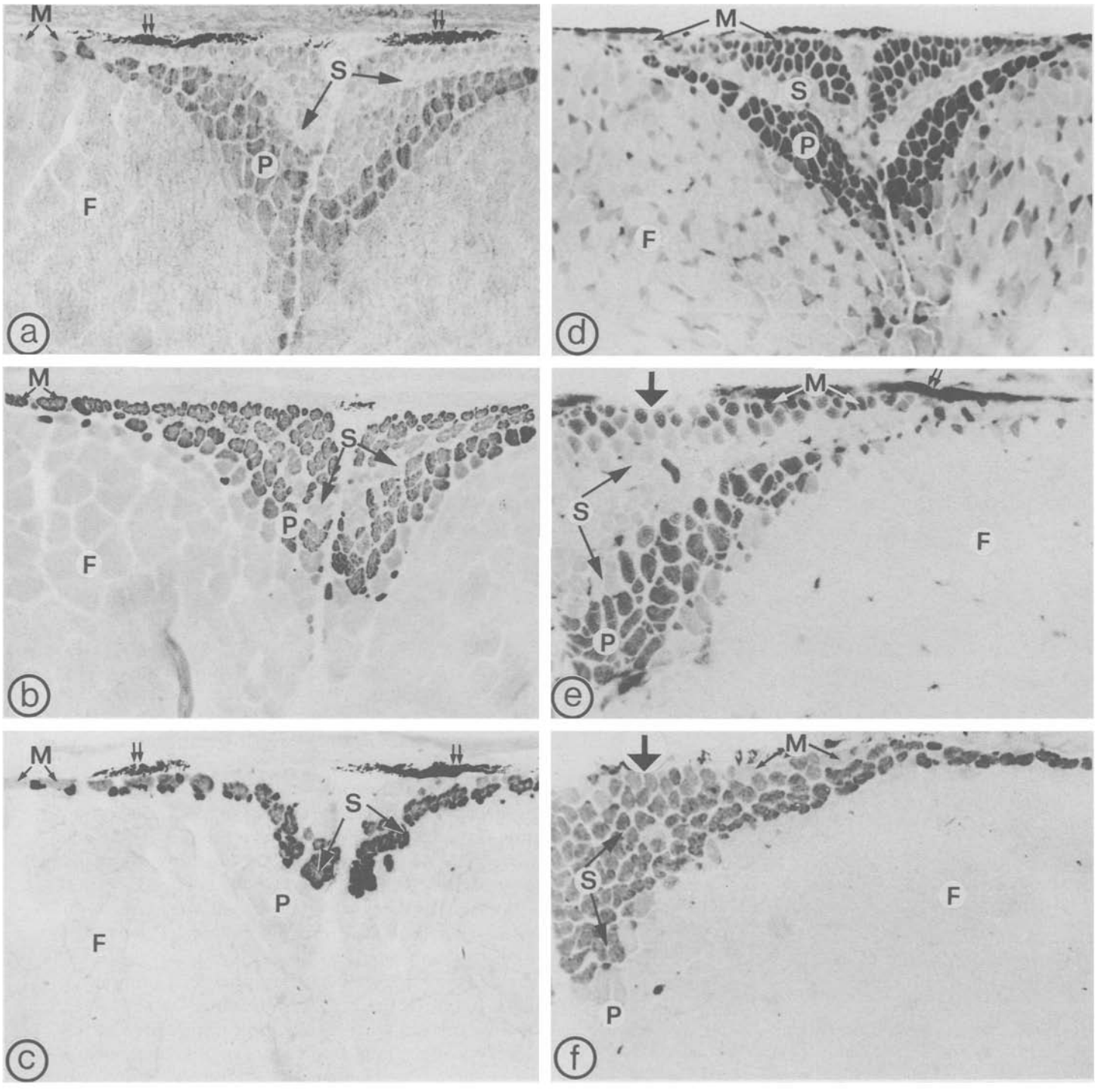

Fig. 2. Transverse sections of lateral muscle at 9 days (a, b), 14 days (c, d), 26 days (e–g), 46 (left) and 60 (right) days (composite block in h), 60 days (i, l–n). (a–d) and (h–n) are at post-anal levels, (e–g) at a more rostral level through the abdomen. In (f) and (g) the abdominal wall of one larva is on the left and a portion of post-anal muscle of another larva of the same age is on the right. (i–n) show serial sections of an epaxial area distant from the lateral line. Stains are: mATPase activity after mild alkali pH (b, c, h) or high alkali (insert in c) or acid (pH 4.8) pre-incubation (l). Immunoperoxidase with anti-FM (a, g, m) and anti-SHC (d–f, n). M = superficial monolayer; S = slow fibres; P = pink fibres; F = fast-white fibres; A = abdominal wall; I = intestine; SC = spinal cord; N = notochord; H = horizontal septum; * = lateral line. magnification: all × 260, except (e) × 80; (h) × 200.

Fig. 3. Transverse sections of lateral muscle at 60 days (a–c), 100 days (d) and 150 days (e, f). Stains are: immunoperoxidase with anti-FHC (a), anti-FM (b) and anti-SHC (c, f), and mATPase activity after mild alkali pH (d; method: Mascarello *et al.*, 1986) and high alkali (e) pre-incubation. M = superficial monolayer; S = slow fibres; P = pink fibres; F = fast-white muscle; arrow = superficial monolayer fibres which are transforming into the slow type; double arrow = pigment; magnification: (a–c) × 185; (d) × 94; (e, f) × 234.

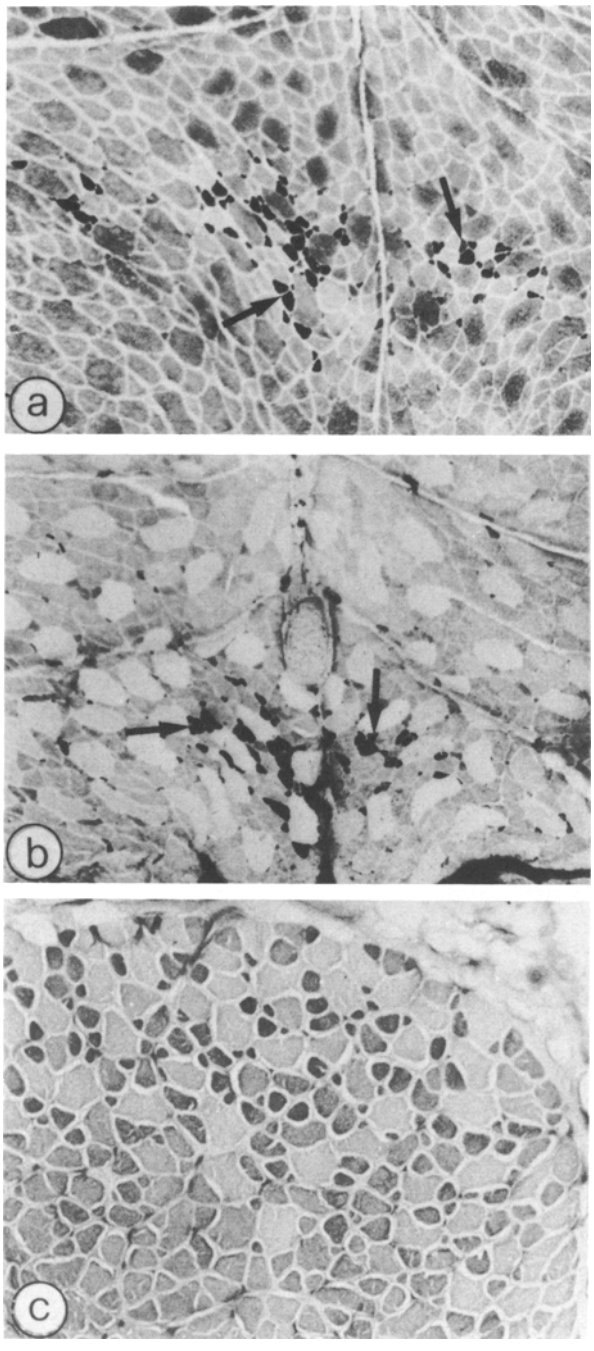

Fig. 4. Transverse section of deep lateral muscle at 150 days (a, b) and of lateral slow muscle only in a juvenile of 23.4 g body weight (c). Myosin ATPase activity after mild alkali (a, c; method: Carpené *et al.*, 1982) and acid (b) pre-incubation. Arrows = deep pink muscle fibres, magnification: (a, b) × 95; (c) × 124.

becomes progressively thinner as the number of these fibres decreases, disappearing completely in the most caudal myotome (no. 21 at this age), which appears to contain only a complete superficial monolayer. This suggests that at least in the most caudal myotomes the formation of superficial monolayer fibres can take place in the absence, and therefore independently, of deep muscle fibres. In 24-h (18 somite) embryos of *Brachydanio rerio*, van Raamsdonk and colleagues (1978) found a single layer of fibres close to the notochord; these fibres had a myosin immunostaining profile similar to that found subsequently in superficial monolayer fibres. The fibres of this primitive monolayer could perhaps correspond to the 'pioneer' fibres described by Felsenfeld and colleagues (1991) in *Brachydanio* embryos. We plan to examine myogenesis in *Sparus aurata* embryos, to see if superficial monolayer fibres are the first muscle cells to develop in more rostral myotomes.

In *Sparus aurata* the first fibres with the mATPase and immunostaining profile of slow fibres appear after hatching in two distinct sites. At all levels they are found in a tight group under the medial border of the lateral line. Their only other site of formation at this time (first half of larval life) is on the external surface of the thin layer of hypaxial muscle which forms the muscular wall of the abdomen at more rostral levels. However, only after the larval period is completed does their number increase sufficiently to form a continuous slow muscle layer extending into the apical regions of the myotomes. This is due in small measure to the transformation of superficial monolayer fibres into the slow type but mainly to the formation of new fibres both close to the lateral line and also along the deep surface of the superficial monolayer stretching out into the apices of the myotome. In *Brachydanio rerio* the transition to the definitive slow myosin composition is much more rapid (van Raamsdonk *et al.*, 1982), whereas in another small fish (*Poecilia reticulata*) the transition to slow occurs slowly near the lateral line but remains incomplete in the apical areas of the myotome (Veggetti *et al.*, 1993).

In this study of *Sparus aurata* we were unable to obtain clear evidence of developmental isoforms of myosin in most muscle fibres during larval stages. The only exception was the superficial monolayer fibres in which the original myosin is later replaced by slow myosin when these fibres transform into the slow type. Polyclonal and monoclonal antibodies to a variety of myosins were tried, but did not distinguish the fast and slow fibre myosins present in larvae from those in adults, and consistent differences in mATPase activity were not seen. By contrast, in the sea-bass *Dicentrachus labrax* and guppy *Poecilia reticulata* mATPase activity does distinguish between larval and adult forms (Scapolo *et al.*, 1988; Veggetti *et al.*, 1993).

The mosaic appearance of fast-white muscle is the result of a hyperplastic process and generally occurs only in fish which grow to a large final size (Weatherley *et al.*, 1980, 1988; Carpenè & Veggetti, 1981;

Weatherley & Gill, 1981; Stickland, 1983; Romanello *et al.*, 1987; Veggetti *et al.*, 1990; Koumans *et al.*, 1991; Rowlerson *et al.*, 1995). In *Sparus aurata* the slow muscle layer also grows by hyperplasia, especially during the juvenile period, and this too shows a distinct mosaic. The mATPase and immunoreactivity profiles of the small diameter fibres in mosaic white muscle vary widely between species. In the trout and salmon they are not different from large diameter fibres (Rowlerson *et al.*, 1985; Higgins, 1990); in mullet, as in *Sparus aurata*, only their mATPase activity differs (Carpenè & Veggetti, 1981; Rowlerson *et al.*, 1985), whereas in the carp, eel and sea bass both mATPase activity and immunostaining profiles differ (Rowlerson *et al.*, 1985; Romanello *et al.*, 1987; Scapolo *et al.*, 1988). If the presence of a developmental myosin in the small (newly-produced) fibres accounts for their distinct staining profile in some fish, it is surprising that such profiles are different or lacking in equivalent fibres of other fish species. In higher vertebrates, equivalent fibre types of different species have similar myosin immuno-reactivities, and their pattern of myosin expression reflects the myotube population from which they are derived (Harris *et al.*, 1989; Maier *et al.*, 1992; Stockdale 1992; Russel *et al.*, 1993). The reason for the much greater variability found in fish muscle is unknown and deserves further study.

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