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# MYOSIN, PARVALBUMIN AND MYOFIBRIL EXPRESSION IN BARBEL (*BARBUS BARBUS* L.) LATERAL WHITE MUSCLE DURING DEVELOPMENT

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**KEYWORDS:** Barbel, *Barbus barbus*, myosin isozymes, parvalbumin isotypes, development.

**ABSTRACT**

Histo- and immunohistochemical techniques have recently been used to study the fibre type and myosin expression in fish muscle during development. In the present work, embryonic, larval and adult myosin isozymes (heavy and light chains) and parvalbumin isotypes were analyzed, from fertization to the adult stage, by polyacrylamide gel electrophoresis of barbel (*Barbus barbus* L.) trunk muscle extracts. The examined myosins display the sequential transitions from embryonic to larval and adult forms characteristic of higher vertebrates. They are characterized by specific heavy chains but their light chains differ only by the $LC_1/LC_3$ stoichiometry with $LC_3$ exceeding $LC_1$after 10 days. Sarcoplasmic parvalbumins show considerable and unforeseen developmental transitions in their isotype distribution: the PA II isotype first appears after hatching and becomes the predominant form until the length reaches about 6 cm. One month after hatching, the amount of PA II then decreases and the synthesis of PA III and IV further increases to reach the typical adult pattern at a size of 18 cm. These observations show that the distribution of parvalbumin isotypes reflects the stage of development. It suggests a specific role for each isotype in relation to muscle activity. Microscopy illustrates the progressive development of somites, muscles cells, and myofibrils, which accelerates at hatching when movements increase.

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# Introduction

Biochemical, immunochemical and immunohistochemical investigations have shown the successive appearance and disappearance of different fibre types and myosin isozymes in skeletal muscles of higher vertebrates during their development. (Hoh 1979; Hoh and Yeoh 1979; Whalen *et al.* 1981; Lowey *et al.* 1983; Maréchal *et al.* 1984; d'Albis *et al.* 1989). Embryonic and neonatal myosins differ from adult fast myosin by several criteria such as their heavy- and light-chain complements and their ATPase activities. Their polymorphism constitutes a modulating mechanism for the speed and power of contraction of the fibre in response to the requirements of the growing animal.

In adult fish at least three fibre types can be biochemically distinguished by their myosin and parvalbumin (PA) isoforms. They have been extensively studied in various teleost species by means of electrophoretic techniques (Focant *et al.* 1976, 1981; Huriaux and Focant 1977, 1985; Johnston *et al.* 1977; Hamoir *et al.* 1980; Huriaux *et al.* 1983, 1990; Rowlerson *et al.* 1985; Scapolo and Rowlerson 1987; Ochiai *et al.* 1988; Gerday 1988; Karasinski and Kilarski 1989; Martinez *et al.* 1989, 1990a, b). Nevertheless, very poor attention has been given to the differentiation of these proteins during fish ontogeny (Van Raamsdonk *et al.* 1978, 1982; Scapolo *et al.* 1988; Martinez *et al.* 1991).

As suggested by immunohistochemical and histochemical examinations of the lateral musculature during the development of *Dicentrarchu labrax* (Scapolo *et al.* 1988), the differentiation of white and red fibres seems to occur step by step and in a non-parallel way. The authors observed developmental transitions in the myosin composition: from an early larval form (L1W and L1R respectively) to a late larval form (L2W and L2R) and then to the isozyme typical of adult white and red muscles (AW and AR). In trunk musculature, the transition from L1W to L2W happens very rapidly and early in larval life, unlike that from L IR to L2R which is more gradual. But the adult myosin types, distinguished by their histochemically well-characterized myosin ATPase activities, appear very late (by 20 months) in the fast white fibres and by about 80 days in the slow red fibres. Recently, embryonic myosin isoforms, characterized by a specific heavy chain complement and an additional fastest-migrating $LC_1$ light chain, were also found during development of the fast white muscles of the Arctic charr *Salvelinus alpinus* (L.) (Martinez *et al.* 1991).

As for the parvalbumins, their appearance has been monitored during ontogeny of the frog (Schwartz and Kay 1988), chicken (Le Peuch *et al.* 1979) and rabbit (Leberer and Pette 1986). In these animals, their synthesis is switched on immediately after birth and progressively increases. They appear tardily in myogenesis, together with the sarcoplasmic reticulum, in correlation with the onset of high-frequency neural activity and the differentiation of fast fibres. Until now, the concentration of the different isotypes of parvalbumins during the ontogeny of fish muscles has not been investigated.

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In this work, we have monitored the growth of several barbel (*Barbus barbus* L.) batches from an experimental hatchery, focusing on electrophoretic analysis of the polymorphism of trunk white muscle myosins (heavy and light chains) and parvalbumins in relation to development. We have investigated from fertilization onward the embryonic, eleutheroembryonic, larval, juvenile, and adult stages.

# Materials and methods

## FISH

*Barbus barbus* eggs were obtained from the experimental hatchery (Philippart 1982; Philippart *et al.* 1989) of the University of Liege (CERER, Tihange, Belgium). Embryos, eleutheroembryos, larvae and juveniles (Krupka 1988) were reared at 20°C until the age of 64 days (3 batches) (**Fig. 1**). Older specimens were captured in the hatchery fishponds. All fishes were first anaesthetized with tricaine methanesulfonate (MS 222, Sandoz) and killed by decapitation. First stages (2 batches) were handled under a magnifying lens in a cold solution of 0.01M Tris, 0.05M KCI, 0.001M DTT, 0.005% $NaN_3$, pH 7.5. The yolk sac of each embryos, present until day 8 post-fertilization, was removed; as early as day 23, the caudal fin and internal organs were also discarded and from day 64 (at a standard length around 2.4 cm), trunk dorso-lateral white muscle was dissected. Per sample we pooled 10 embryos, 5 larvae, and 3 juveniles. The material used for biochemical analyses was minced and suspended in 10 vol of a solution containing 0.01M Tris, 0.05M KCL, 0.01M DTT, 0.005% $NaN_3$, 50% glycerol, pH 7.5 solution. Samples were kept at 4°C for 24 h, mixed, and stored until use at −18°C. A third batch of developing barbels was preserved in 70% alcohol for the morphological study or processed for electron microscopy examination (see below).

## CRUDE PARVALBUMIN AND ACTOMYOSIN EXTRACTS

Two to 44 day samples in glycerol conservative solution were centrifuged for 10 min at 8,500 × g (Beckman Microfuge) at 4°C and the supernatant retained for parvalbumin analysis. The myofibrillar pellet was directly dissolved in the urea or SDS incubation solution (see below). The amount of actomyosin was too small to allow the usual high-ionic-strength extraction and precipitation by the dilution method. This latter method (Huriaux and Focant 1977) was routinely employed with muscle samples from 64-day and older specimens, after prior removal of sarcoplasmic proteins by centrifugation (30 min at 18,000 × g) for the parvalbumin analysis.

Sarcoplasmic protein concentrations were measured according to Bradford (1976), using bovine serum albumin as standard.

## INCUBATION OF PROTEINS

Actomyosin pellets were dissociated in 3 vol of 8M urea, 0.02M Tris, 0.12M glycine, 3% betamercaptoethanol, pH 8.6 or in 3 vol of a solution containing 0.69M sodium dodecyl sulfate

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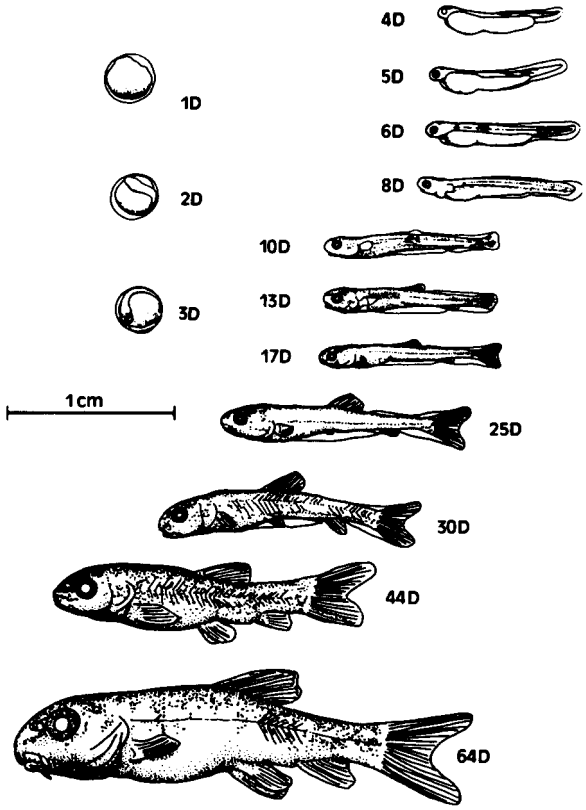
(SDS), 0.0625M Tris, 10% glycerol (v/v), 5% betamercaptoethanol, pH 6.8, and heated for 2 min at 100°C. Parvalbumins were incubated by mixing sarcoplasmic protein fractions with 2 vol of a solution containing 0.02M Tris, 0.12M glycine, 3% betamercaptoethanol, 10% glycerol, pH 8.6.

***Fig. 1.*** *Morphological aspect of the developing barbel (age in days). 0-3 D: embryos, 4-8 D: eleutheroembryos, 10-30 D: larvae and 44-64 D: juveniles.*




## POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

PAGE was performed under 4 sets of conditions: (1) myosin light chains were separated in 8M urea, 10%-acrylamide gel at pH 8.6 (Perrie and Perry 1970) or (2) in a 20%-acrylamide discontinuous gel system in the presence of SDS, at pH 8.4 (Laemmli 1970); (3) myosin heavy chains were discerned in a similar 6%-acrylamide discontinuous gel system in the presence of SDS (Danieli Betto *et al.* 1986) but with further inclusion of 30% glycerol (w/v); (4) parvalbumin isotypes migrated in a Tris-glycine buffer at pH 8.6 as in (1), but with urea replaced by 10% glycerol (w/v) (Focant *et al.* 1981). A same amount of sarcoplasmic proteins was loaded in each sample well.

Conditions for staining and destaining have been previously described (Huriaux and Focant 1977). Densitometry was performed with a HELENA Quick-Scan apparatus (Beaumont, Texas).

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### PEPTIDE MAPPING OF MYOSIN HEAVY CHAINS

Myosin heavy chains were isolated from myofibrillar samples on a 10%-acrylamide discontinuous SDS gel. Peptide maps were obtained by digestion of heavy chains with 20ml (0.02mg/ml) of *Staphylococcus aureus V8* protease and separation of resulting peptides on a 15%-acrylamide discontinuous SDS gel at pH 8.8 (Cleveland *et al.* 1977).

### ELECTRON MICROSCOPY

Samples taken 42 h post-fertilization were immersed for 90 min in fixative solution (2% glutaraldehyde LADD, 0.1M $Na_2HPO_4$, 0.1M $NaH_2PO_4$, pH 7.2), changed 3 times at 4°C. They were rinsed, dehydrated and embedded in an EPON mixture. Semithin sections (1$\mu$m) were stained with toluidine blue for light microscopy. Ultrathin sections (60 to 80nm) were cut with a diamond knife on a REICHERT OMU3 ultramicrotome, stained with uranyl acetate and lead citrate and viewed with a JEOL CX100II transmission electron microscope.

## Results

### MORPHOLOGICAL ANALYSIS

**Figure 1** illustrates the developmental stages in barbel from fertilization till two months. Short, quick quivering movements are perceptible just after hatching, which occurs 4 days after fertilization. Eleutheroembryos are essentially benthic until major resorption of the yolk sac on the eighth day post-fertilization. They then become more active. These morphological observations obviously demonstrate the difficulties encountered in taking trunk muscle samples with minimal contamination by other tissues such as skin, neural tube and chord, until the age of 44 days when the larvae present the first adult morphological characters (juveniles).

On semithin sections, the first well-shaped somites are observed in 42-h embryos. These sections make it possible to accurately orientate the fibres in the muscle samples dedicated to the electronmicroscope examination. This technique was used to monitor, on ultrathin sections, the development of contractile fibres (**Fig. 2**). At 42 h, somites are composed of uninucleate cells regularly distributed between intercellular spaces (**Fig. 2a**). At a higher magnification, cells appear devoid of any myofilaments (**Fig. 2b, c**). The next stage (65 h) shows polynuclear cells containing a few myofilaments organized into primitive myofibrils (**Fig. 2d, e, f**) with visible Z-lines and ordered thin and thick filaments in transverse sections. At hatching and during the following stages (**Fig. 2g, h, i**) myofibrils increase in number and size and display the typical longitudinal cylinder shape of striated muscle.

***Fig. 2.*** *Transverse- (c, f, h, i) and longitudinal (a, b, d, e, g) sections of barbel trunk muscle fibres at the stage 42h (a, b, c), 65h (d, e, f), 90h (g, h) and 115h (i). Arrows indicate myofibrils. Bar = 2$\mu$m (a, d, g, h, i) and 0.3$\mu$m (b, c, e, f).*

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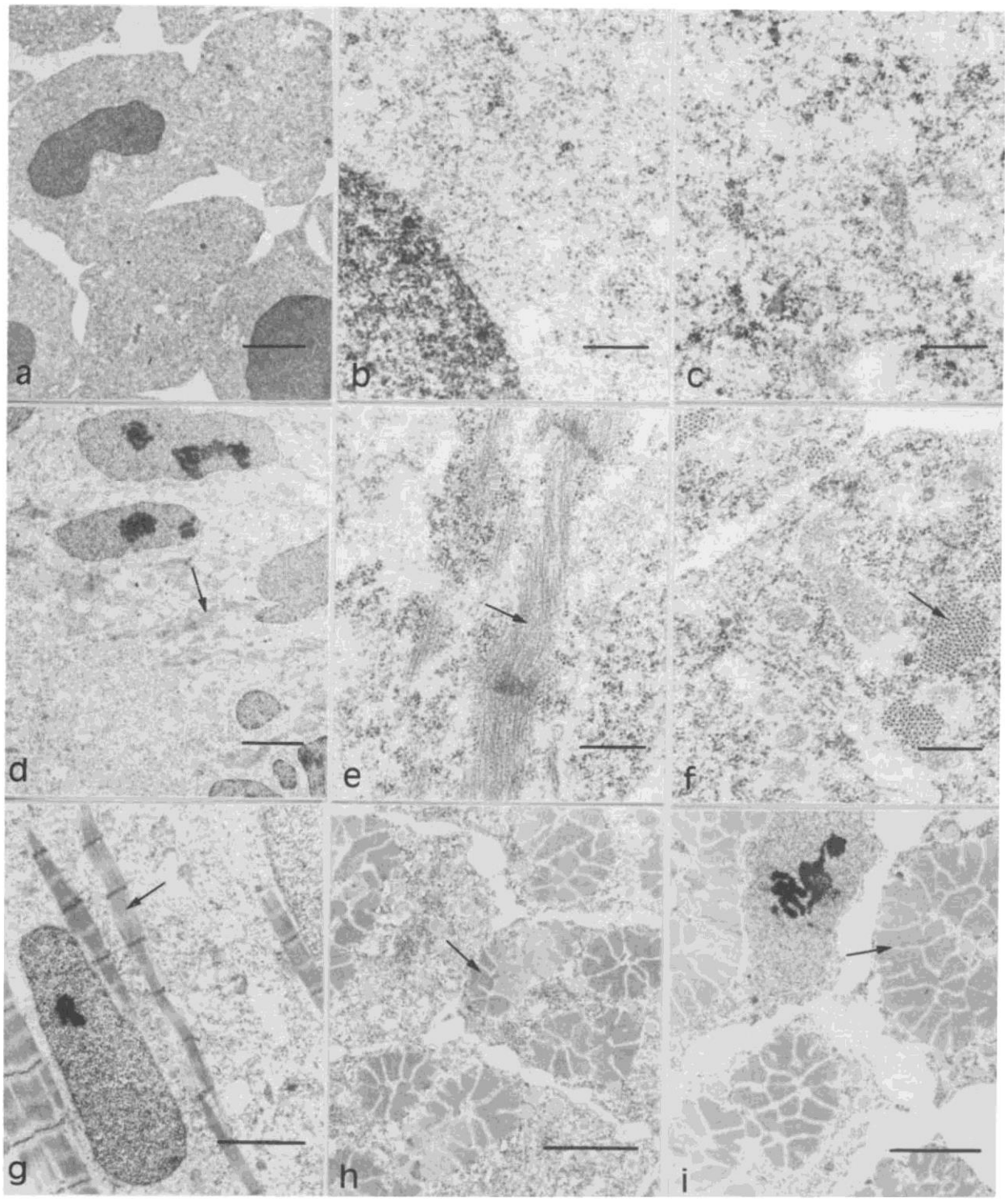


## DEVELOPMENTAL TRANSITIONS IN MYOSIN COMPOSITION

The three myosin light chains from adult barbel white muscles have recently been identified on urea and SDS gels (Huriaux *et al.* 1990) (**Fig. 3a, b**). They exhibit the typical fish characteristics with five times as much $LC_3$ as $LC_1$. However, to discriminate between $LC_2$ (17400 D) and $LC_3$ (16200 D) requires unusual SDS-PAGE conditions (separating gel 20%-acrylamide, pH 8.4).

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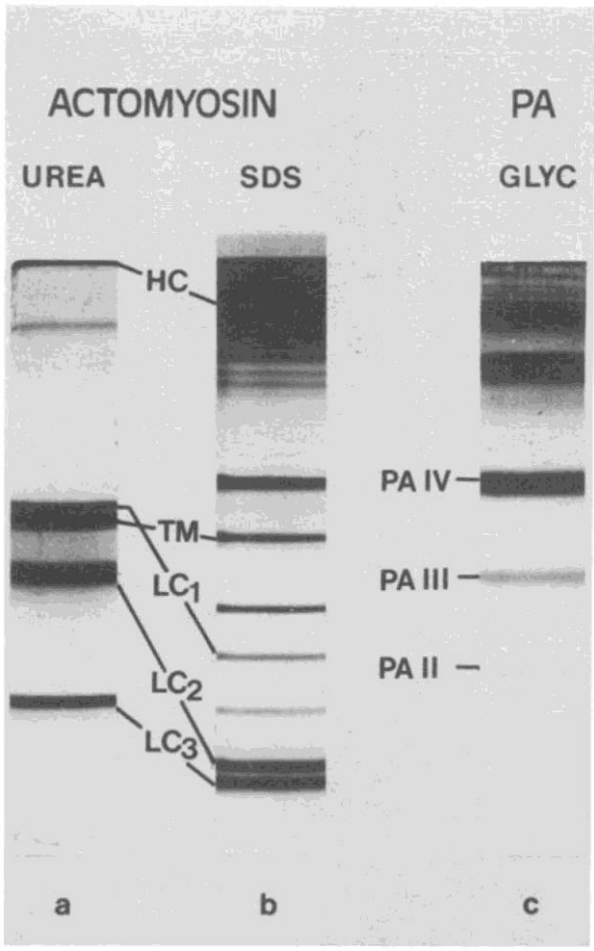
***Fig. 3.*** *Electrophoretic pattern of actomyosin components (a: urea-gel, b: SDS-gel) and of sarcoplasmic parvalbumin isotypes (c: glycerol-gel) from adult barbel trunk white muscle.*




The myosin light chains from the trunk muscle were examined in the course of development from the 3rd-day until the 64th-day stage. Proteinic bands presumed to be the myosin light chains were unequivocally identified by their isolation from urea gels, incubation with SDS, and comigration with adult and larval actomyosins on SDS gels. On urea gel (**Fig. 4a**), the initial actomyosin loads being roughly equal, younger stages show a lower total amount of myosin, with predominantly $LC_2$ and $LC_1$ (comigrating with tropomyosin); $LC_3$ appears later but increases proportionally faster until the age of 8 days. The three light-chain bands from older specimens are qualitatively and quantitatively identical to adult ones. A similar comparison on discontinuous SDS-PAGE (**Fig. 4b**) reveals all the myofibrillar components. Only the relative proportions of myosin light chains slightly change during the early stages of development: compared to the $LC_2$ content, $LC_1$ increases steadily from the beginning whereas $LC_3$ becomes visible at 4-5 days. At 10 days, the stoichiometry of LCI and $LC_3$ reverses and the percentage of $LC_3$ looks higher, as usual in adult fish myosins. Numerous additional unidentified bands are also observed in the embryo actomyosin. Yolk proteins isolated from eggs were screened under the same conditions on urea and SDS gels but no proteinic band comigrates with the myosin

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components. Myosin heavy chains from developing barbel muscles were analysed by high-porosity SDS-PAGE and compared to the white and red adult myosin heavy chains. Two heavychain isoforms, differing from both adult myosin heavy chains, are visible in the early stages (**Fig. 5**). The fast-migrating minor isoform decreases regularly and disappears at 8-10 days. The slow-migrating main isoform which exhibits an electrophoretic mobility intermediate between the two adult heavy chains (Ad.W and Ad.R), is the only form present in the early larval stages. It is then progressively replaced by the adult white isoform. The percentages of adult white heavy chains versus larval heavy chains are roughly 30°%7/70% at 64 days (2.4 cm), 50%/50% at 84 days (3.0 cm), 60%/40% at 127 days (3.9 cm) and 80%/20% at 149 days (4.8 cm). Peptide maps displayed on SDS-PAGE after digestion of heavy chains by the *Staphylococcus areus V8* protease show an evolution in the composition of isoforms: three peptides of very high molecular weight disappear after hatching; on the other hand, a set of five slightly faster-migrating peptides progressively diminish, vanishing at the juvenile stage.

***Fig. 4.*** *Electrophoretic separation of myofibrillar proteins from 3 to 64 days old barbel trunk muscle in the presence of urea (a) and SDS (b). Myosin light chains only are labelled.*

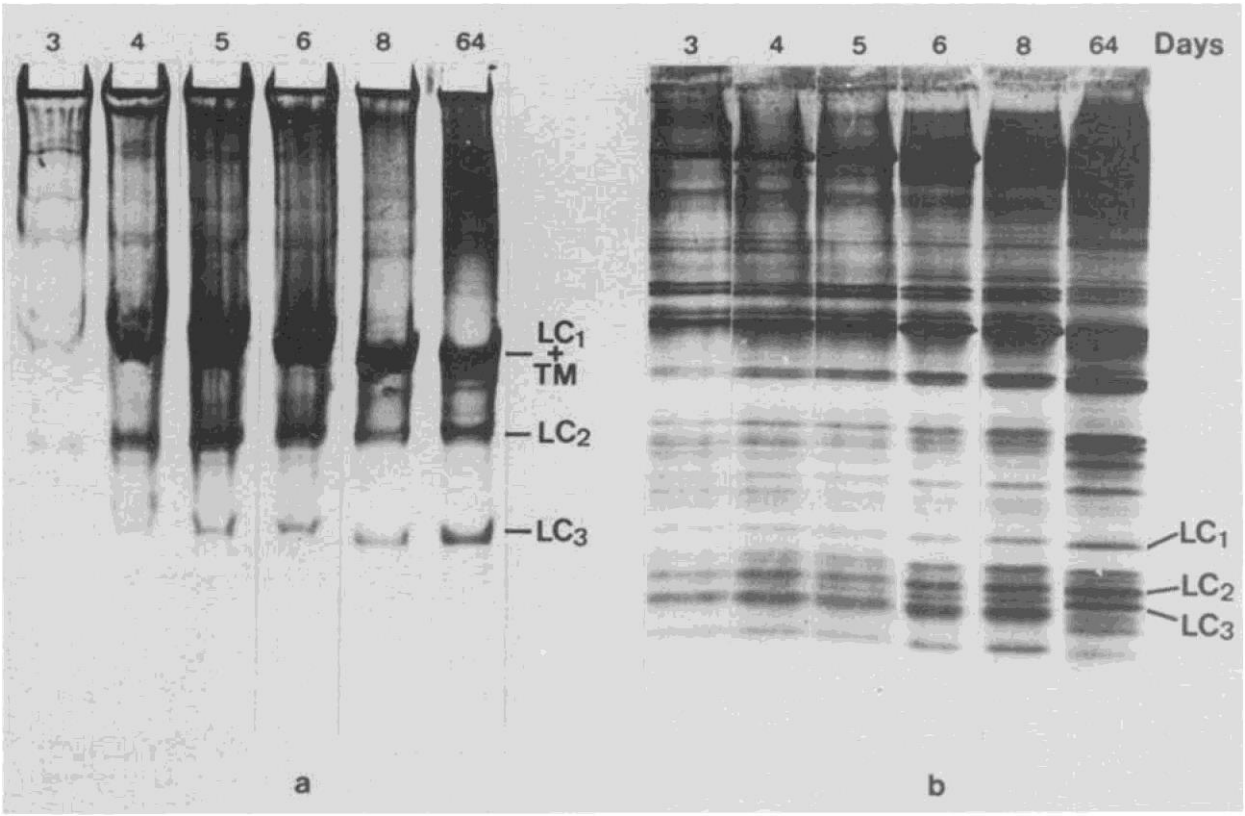


***Fig. 5.*** *Densitometer traces of the electrophoretic separation of myosin heavy chain isoforms from 4 to 64 days old barbel trunk muscle. Myosin heavy chains from adult white (Ad.W) and red (Ad.R) muscles are used as references. The vertical dotted line indicates the main larval isoform.*

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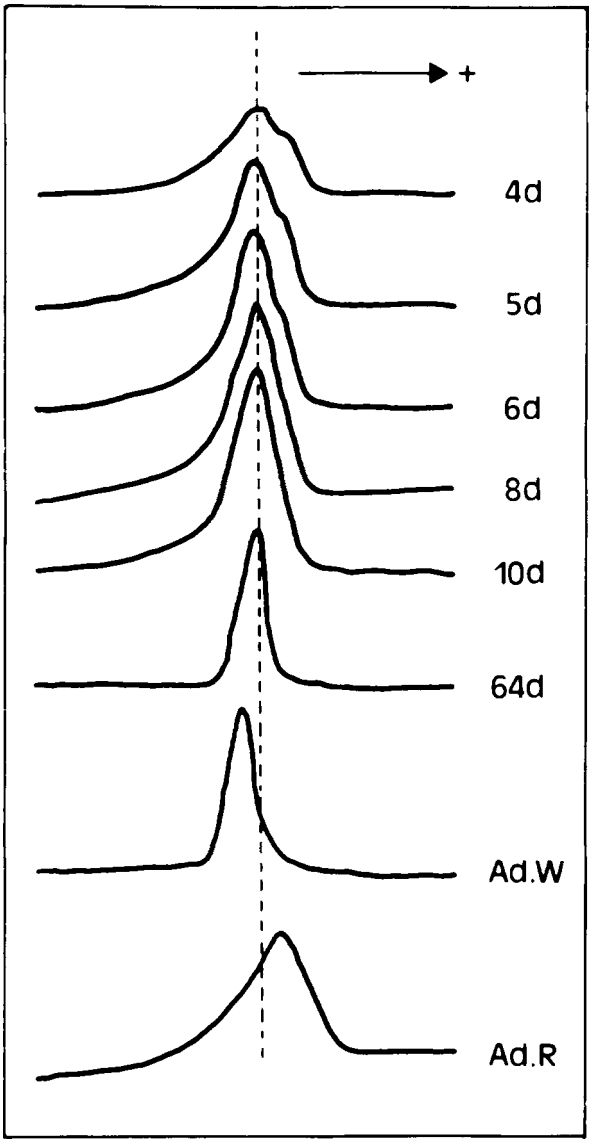


## DEVELOPMENTAL TRANSITIONS IN PA COMPOSITION

First described on starch gel by Pironi and Gosselin-Rey (1974), barbel parvalbumins separate well on glycerol-PAGE at pH 8.6 (Huriaux *et al.* 1990). In trunk white muscle from adult fish they are composed of three different isotypes of decreasing negative electric charge at pH 8.6: PA 11 (10%), PA III (20%) and PA IV (70%) (**Fig. 3c**).

By computing of densitometer traces of parvalbumin electrophoretograms, it is possible to see the evolution of isotype proportions in the trunk muscle from the egg to the adult barbel stage (**Fig. 6**). During early development (until 64 days), the major feature is the non-parallel appearance of the three isotypes found in adult muscles (**Fig. 6a**). The first isotype synthetised is PA II, which appears in measurable amount at the day 4-day 5 stage; it steadily augments in the larval sarcoplasm and then diminishes although it remains the predominant form. PA IV appears at 5-10 days and increases slowly. PA III is still near the method's limit of detection at 64 days.

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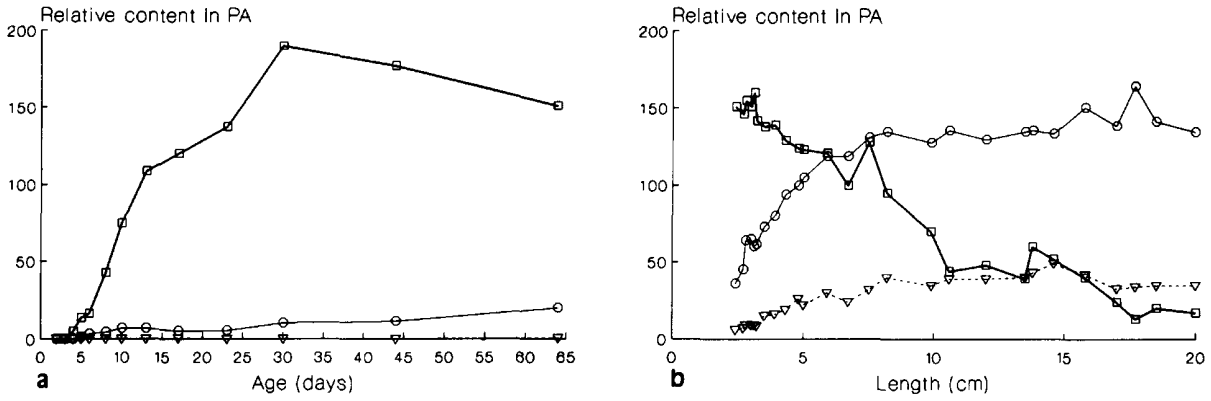
***Fig. 6.*** *Evolution of the parvalbumin isotype distribution in barbel trunk muscle during the development, a: from the fertilization until 64 days (standard length of 2.4 cm), b: from 2.4 to 20 cm.* □, *PA II;* ▽, *PA III;* ○, *PA IV.*




During ulterior barbel growth, the size of specimens of the same age becomes more variable. As previous findings shown the distribution of parvalbumin isotypes to depend on barbel size and not on the growth rate (Huriaux *et al.* 1990), the variation of this distribution was followed in older specimens according to their standard length, from 2.4 cm (64 days old) up to 20 cm (adult). **Figure 6b** shows the progressive establishment of the adult parvalbumin pattern characterized by a large predominance of PA IV. The reduction of PA II synthesis, already observed after 30 days (*cfr.,* **Fig. 6a**), regularly follows its course whereas the PA III and PA IV contents increase up to a length of 8-10 cm. The stoichiometry of the three isotypes appears constant after the adult stage, 18 cm long.

# Discussion

In the early development of barbel trunk muscle, well-formed somites appear 42 h after fertilization, primitive myofibrils after 65 h, and typical striated muscle at hatching. At this stage, myofibrils are well-organized in a ribbon-like pattern and the muscles are active. This differentiation scheme apparently parallels that of developing zebrafish, *Brachydanio rerio,* though it is slightly slower (Van Raamsdonck *et al.* 1974). As in the trout, *Salmo trutta fario,* not until hatching do white muscle cells exhibit considerable hypertrophy (Josse *et al.* 1986). Post-hatching development is characterized by an increased myofibrils density in the muscle cells.

Previous histochemical and immunohistochemical studies have revealed the presence, in developing teleost, of different myosins (Van Raamsdonk *et al.* 1978, 1982; Scapolo *et al.* 1988; Martinez *et al.* 1991).

This biochemical study is a first advance in clearing up the question of how their heavy- and light-chain compositions continuously vary from fertilization to embryo, eleutheroembryo, larva and juvenile.

The presence of clearly identified adult white myosin light chains from the outset of barbel development (3 days after fertilization) is consistent with the observation of Van Raamsdonk *et*

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*al.* (1978) that the bulk of the zebrafish musculature consists of white fibres in the period around hatching. The excess of LC, versus $LC_3$ at that stage evokes the lower concentration of $LC_3$ in neonatal mammals (Dabrowska *et al.* 1977; Syrovy 1979). The synthesis of $LC_3$ starts around birth whereas $LC_1$ is already present (Roy *et al.* 1979). The gradual increase of $LC_3$ linked with higher ATPase activity, could explain the increased contraction speed during post-hatching development. No red myosin light chains can be observed (Huriaux *et al.* 1990) pointing out the absence of red fibres. The additional bands detected in actomyosin extracts from embryos and young larvae originate from tissues such as the skeleton, neural tube or digestive tract, owing to the very low amount of muscle fibres present. It cannot be totally excluded, however, that some of these low-molecular-weight proteins may proceed from specific embryonic or larval light-chain isoforms.

It is obvious that barbels, from hatching to at least the age of two months, are characterized by a major specific myosin heavy chain isoform distinct from adult white and red ones. The other specific early and transient myosin-heavy chain isoform might be what remains of the embryonic red-cell precursor population, as found in zebrafish embryo muscles (Van Raamsdonk *et al.* 1982). The peptide maps confirm these changes in heavy chain composition. The successive appearance of three myosin heavy-chain isoforms related to different stages of myotomal development is also in agreement with the findings of Scapolo *et al.* (1988) and Martinez *et al.* (1991). Concerning *Dicentrarchus labrax* white muscle myosin composition, these authors describe a fast transition from an early larval from (L1W) to a late larval form (L2W) followed by a slow transition to the isoform typical of adult myosin. In the barbel, the transition from L2W to the adult form seems to be more rapid. As in higher vertebrates, the early stages consitute a "hinge step" in the postnatal development of fishes. As in the other lower vertebrates examined (d'Albis *et al.* 1985), larval and adult barbel myosins apparently possess the same light chains, but distinct heavy chains.

The abundance of parvalbumins in fish white muscle makes it possible to study them by means of the useful PAGE technique, which separates the various parvalbumin components and allows their quantification. Undetectable in white trunk muscles during the egg stage, parvalbumins appear only after hatching when, according to microscopical examinations, the myofibrils are well-structured and the fish begins to respond to outer stimuli. The same occurrence of parvalbumins with the start of activity was observed in frog development (Schwartz and Kay 1988). Le Peuch *et al.* (1979) detected no parvalbumins in the chicken muscles before hatching, whereas most of the contractile proteins are present. The delayed appearance of parvalbumins could be a mechanism restricting muscle contractibility in the egg. Proteins such as parvalbumins, which modulate muscle contraction, probably don't become essential until the muscle is on duty. Our results show that synthesis of the three isotypes is asynchronous during barbel development, PA II being the principal larval form and PA IV the essential adult form. As parvalbumin expression is controlled by motor unit activity, the observed distribution could reflect the innervation status at the various stages of development (Kullberg *et al.* 1977). The temporal variability of parvalbumin distribution raises the question of the exact physiological role of each isotype. Is there a specialization according to the developmental stage? As these calcium-binding proteins may be involved in the relaxation process in cold-blooded vertebrates (Gillis and Gerday 1977), it is plausible that a particular isotype could be related to a type of muscle or a contraction speed: for example, the growth-linked increase in contraction speed

could require a specially adapted isotype such as PA IV, predominant in adult fast-muscle sarcoplasm. The relationship between developmental stages and parvalbumin isotype expression should be confirmed by the study of other fish species.

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